DISSERTATION

ISOLATION AND CHARACTERIZATION OF A NOVEL BACTERIOPHAGE, ASC10, THAT LYSES *FRANCISELLA TULARENSIS*

Submitted by

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF A NOVEL BACTERIOPHAGE, ASC10, THAT LYSES *FRANCISELLA TULARENSIS*

Francisella tularensis is an extremely infectious intracellular bacterium and the etiological agent of tularemia. Inhalation of *Francisella tularensis* can cause pulmonary tularemia, which has a mortality rate of 35% in the absence of treatment. Studies investigating the biology and molecular pathogenesis of *Francisella tularensis* have increased in the last few years especially after the U.S. Centers for Disease Control and Prevention (CDC) classified *Francisella tularensis* as a Category A Select Agent.

In this dissertation, the identification and characterization of a novel temperate bacteriophage specific for *Francisella* species is described. Initial experiments focused on developing a media that would allow optimum growth of *Francisella* and recovery of bacteriophages. The preferred growth media for *Francisella* researchers is Mueller Hinton broth supplemented with IsoVitaleX enrichment mix. The research presented in this dissertation included development of a simple and low-cost brain heart infusion and Mueller Hinton base media, designated (BMFC), for enhancing the growth rate of all *Francisella* strains. BMFC media was compared with brain heart infusion media supplemented with cysteine (BHIc) for growth of *Francisella tularensis* subspecies *novicida* U112, *Francisella tularensis* subspecies *holarctica* LVS, and *Francisella tularensis* subspecies *tularensis* NR-50. Results from these experiments demonstrated that *Francisella* strains grow more rapidly when inoculated into BMFC media than when grown in BHIc media.

A bacteriophage, designated ASC10, was discovered to be active against the majority of *Francisella* strains including *F. tularensis Schu S4*. This is the first bacteriophage reported to infect and lyse several *Francisella* species including *F. tularensis* subspecies *holarctica* strain LVS, *F. tularensis* subspecies *tularensis* strain *Schu S4*, *F. philomiragia* and *F. t. novicida.* Bacteriophage ASC10 was found to possess an icosahedral head of approximately 114 nm in diameter and a non-contractile tail of 92 nm in length. These measurements place ASC10 into the family of *Siphoviridae.*

Bacteriophage ASC10 was isolated by mitomycin C induction of *F. t. novicida* NR-575 and plating of the phage lysate on *F. t. novicida* NR-584. Since these two strains are both mutants of *F. t. novicida* U112, it was hypothesized that they had genomic alterations that allowed *F. t. novicida* NR-584 to serve as a host for phage production. NextGen sequencing of the *F. t. novicida* NR-575 and NR-584 genomes and comparison by alignment revealed that *F. t. novicida* NR-584 had a 11.3 kb deletion encoding an abortive phage infection protein and a restriction-modification system.

The location of the ASC10 prophage was identified within the *Francisella novicida* U112 genome by searching for homologous phage proteins using BLASTP. Based on significant similarity of clusters of phage-like proteins, it was determined that the ASC10 prophage is located between nucleotides 369,143 and 779,775. This location was chosen since it contained the integrase gene and other proteins associated with phage and was flanked by tRNA-serine loci.

In summary, this dissertation describes the optimization of growth media for *Francisella* and the discovery of a unique phage that lyses *Francisella* species. The availability of a *Francisella*-specific bacteriophage is essential to develop rapid, field-deployable diagnostic

assays, and would provide another tool in the arsenal for genetic manipulation of the *Francisella* genome.

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1.1 Introduction

Tularemia is a severe and sometimes fatal disease in humans caused by *Francisella tularensis*, a gram-negative coccobacillus. This disease was reported in the United State as early as 1911 and was described in all states except Hawaii. Tularemia was named based on an outbreak of a plague-like disease in rabbits In Tulare County in the state of California. A small gram-negative bacterium was isolated from infected rabbits and named *Bacterium tularensis.* In 1921, Edward Francis identified this bacterium and named it *Francisella tularensis.* Variable names were used to describe this disease in different parts of the world for example, it was known as deer-fly fever in Utah, rabbit fever in the central U.S. water rat trapper's disease in Russia, and Ohara's disease in Japan (Mörner, 1992). In 1939, human tularemia peaked to 2,291 cases and has decreased has since to 100-200 cases annually (Dennis et al., 2001).

In the United States from 1981 to 1987, approximately 60% of the tularemia cases were reported in Arkansas, Missouri, Oklahoma and Texas (Taylor et al., 1981). The highest incidence of tularemia reported from 1990 to 2000 were from localized outbreaks in Massachusetts, (i.e. Martha's Vineyard), and in the central states of Arkansas, South Dakota, Missouri and Oklahoma.

1.2 Vectors and Transmission

Many blood-feeding arthropods serve as vectors for *Francisella tularensis*, the bacteria that causes tularemia. In the United States, *Dermacentor variabilis*, also known as American Dog tick, *D. andersoni* (Rocky Mountain Wood tick) and *Amblyomma americanum* (the Lone Star tick) are considered important vectors for *F. tularensis* (Farlow et al., 2005). Tularemia infections have been reported in several species of birds, reptiles, mammals, and fish. *Sylvilagus* spp, the cottontail rabbit, and *Lupus* ssp. hares are important hosts in North America (Jellison, 1974). Most cases of human tularemia result from direct interaction with infected wild rabbits or from bites of blood-sucking insects. Transmission of these diseases occurs mostly in the spring and summer and decreases during winter months (Jacobs et al., 1985).

1.3 Diseases

Human tularemia can take different forms depending on the path of infection or the entry of the bacterium into the human body. Ulceroglandular tularemia is the most prevalent form of this disease. This type is transmitted by a bite from an arthropod that has fed on an infected animal. Several occurrence of tularemia have been reported in hunters as a result of their handling infected animals. Three to six days is the incubation period of this disease, and after that, the patient will have symptoms including fever, headache, chills and ulcer which generally forms at the site of infection, and remains for several months (Evans et al., 1985).

Glandular tularemia has similar symptoms to ulceroglandular but without the formation of an ulcer. In this case, *F. tularensis* is disseminated from the site of infection through the

human lymphatic system to the nearest lymph nodes causing inflammation and swelling. Ulceroglandular and glandular tularemia are seldom fatal even without treatment. The mortality rate from these diseases is less than 3%, although the recovery is sometimes extensive in length.

Typhoidal tularemia has a mortality rate of 30 to 60% and is characterized as septicemia without the appearance of ulcers. In this case, *F. tularensis* enter the bloodstream through mucous membranes or through breaks in the skin and may affect the reticuloendothelial organs as well as the lungs. A patient with this disease experiences symptoms similar to ulceroglandular disease and may develop shock and delirium. Oculoglandular tularemia is usually occurring as a result of infecting the conjunctiva with *F. tularensis* (Steinemann et al., 1999). This disease is identified by the formation of nodules and ulcers on the conjunctiva.

Orpharyngeal or gastrointestinal tularemia can be a result of ingesting or drinking infected materials or water. The symptoms of orpharyngeal tularemia include sore throat, mouth ulcers, and enlargement of the tonsils, diarrhea and swollen cervical lymph nodes.

Pneumonic tularemia is the most serious type of tularemia and is associated with the inhalation of as low as 10 cells of *F. tularensis.* Signs and symptoms of this disease may vary, which make the diagnosis difficult but overall symptoms include cough, chest pain and difficult breathing.

These human diseases are primarily associated with two *Francisella tularensis* subspecies: *tularensis* (type A) and subspecies *palaearctica* (type B or subspecies *holarctica*). Subspecies *holarctica* is now the most common terminology used instead of *palaearctica* (Olsufiev.N.1970). Strains of *F. tularensis* subsp. *tularensis* are extremely virulent in humans and are found in North America in addition to different parts of Europe (Guryčová, 1998). Strains of *F. tularensis* subspecies *holarctica* are moderately virulent with the mortality rate of

untreated diseases in human of less than 0.5% (Ellis et al., 2002). *F. tularensis* subsp. *mediaasiatica* is found mostly in central Asian republics of the Former Soviet Union.

According to Bergey's Manual of Systematic Bacteriology, *F. tularensis* and *F. novicida* are considered two species of the genus *Francisella*, due to the inability of *F. novicida* to generate acid from sucrose and its lack of virulence for human and rabbits. On the other hand, the 16S ribosomal DNA sequences of *F. novicida* and *F. tularensis* show 99.6% of sequences similarity (Forsman et al., 1994). Furthermore, *F. novicida* is not differentiated from *F. tularensis* based on DNA hybridization studies (Hollis et al., 1989). Based on these studies, *F. novicida* is proposed to be a subspecies of *F. tularensis*, (Table 1.1). Recently, F. *novicida* has been reclassified as subspecies of *F. tularensis* (Huber et al., 2010). *F. philomiragia* and *F. tularensis* are two separate species in the *Francisella* genus in the *Francisellaceae* family. *F. philomiragia* can be distinguished from *F. tularensis* because it is easily cultured and can cause diseases only in immunocompromised individuals.

Table 1.1: Taxonomy and Characteristics of *F. tularensis* subspecies. NR, not reported (Ellis et al., 2002).

Current terminology	Previous names	16s rRNA genotype	Geographic location	Citrulline ureidase activity	Glycerol fermentation	Glucose fermentation	Sensitivity to erythromycin
Francisella tularensis subsp. *tularensis*	*Francisella tularensis* A; *Francisella tularensis*	A	Primarily N. America	+	+	+	+
Francisella tularensis subsp. *holarctica*	*Francisella tularensis* B*;* *Francisella tularensis* subsp. *palaearctica*	B	Primarily Europe, Siberia, Far East, Kazakhstan, and N. America	–	–	+	+
Francisella tularensis subsp. *mediaasiatica*	*Francisella tularensis* subsp. *mediaasiatica*	B A A	Primarily Eurasia Japan Primarily central Asia and some parts of the former USSR	– – +	– + +	– – +	– + +
Francisella novicida	*Francisella tularensis* subsp. *novicida*	NR	Primarily N. America	NR	NR	+	NR

1.4 Treatment

In vitro studies of *F. tularensis* strains are isolated from humans and animals found that all of these isolates were resistant to azithromycin and beta-lactams. Tetracycline and chloramphenicol have good bactericidal activity against *F. tularensis* and have been prescribed to treat tularemia. However, treatment failures have been reported for these antibiotics (Dennis et al., 2001). The antibiotic ciprofloxacin had the lowest level of treatment failure reported in the epidemic outbreak in Spain(Pérez et al., 2001).

Streptomycin and gentamicin are the antibiotics of choice for treating tularemia, but relapses are associated with gentamicin treatment.

Telithromycin is known to be effective against *Legionella* spp. and *Chlamydia* and has been proven to be bactericidal against *F. tularensis* in cell culture systems (Maurin et al., 2000). Since there is no proven way to contain this organism in the natural environment, public awareness of the potential for human infection should be maintained. Handling of dead animals should be strongly avoided specially in tularemia endemic areas.

Studies investigating the biology and molecular pathogenesis of *Francisella tularensis* have increased in the last few years especially after the U.S. Centers for Disease Control and Prevention (CDC) classified *Francisella tularensis* as a Category A Select Agent with potential for biological terrorism. A wide range of genetic tools and techniques have been developed to understand the molecular basis of pathogenicity and to identify virulence factors of this organism. These include random transposon mutagenesis systems using EZ-*Tn5*, Himar 1 and *Tn5* (Buchan et al., 2008), *E. coli-Francisella* shuttle vectors (Maier et al., 2004), methods for allelic exchange (LoVullo et al., 2006), as well as a single copy integration system based on *Tn7* transposon (LoVullo et al., 2009). However, the use of these tools is limited due to the relatively low transformation frequency of *Francisella sp*. (Anthony et al., 1991). In addition, few of these tools have been evaluated on Select Agent strains of *Francisella* due to the need for BSL3 laboratories and the restricted use of antibiotic resistance markers on these strains. One method that has not been attempted is the integration of genetic elements into the *F. tularensis* chromosome via temperate bacteriophage. Recombineering, i.e. genetic engineering using recombination proteins, is mutagenesis system in *E. coli* based on λ Red system. Similar systems have been developed in *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* using

recombination proteins produced by their bacteriophages (Hoang et al., 2000). Using bacteriophages specific for *Francisella* will allow the development of a recombineering system in this pathogen.

1.5 Bacteriophage Biology:

Bacteriophages are the most abundant living entities on the planet and play an important role in bacterial evolution and microbial balancing in the ecosystem. Bacterial viruses or bacteriophages were first discovered in 1915 and 1917 by Frederick William Twort in England and then in 1917 by Felix d'Herelle who named it bacteriophage or phage, short for the Greek word "phagein", which means "to eat". Luria, Delbruk and other virologists in the 1930s utilized these phages as model systems to study virus genetics, structure and replication.

Bacteriophages are extremely diverse, can carry single- or double-stranded DNA or RNA (Casjens, 2003), and follow different replication cycles. After bacterial infection, phage can follow two different propagation pathways - either the lytic or lysogenic pathway. If the lytic route is followed, the phage uses the bacterial machinery to replicate its DNA, expresses essential genes, assembles viron particles, and lyses the bacterial cell. In the lysogenic pathway, the phage DNA integrates into the bacterial chromosome. The prophage genome replicates as part of the bacterial chromosome and establishes a stable long-term relationship with their host bacteria. These bacterial cells are called lysogens due to their potential to lyse the bacterium. Prophage DNA makes the cell resistant to further phage infection (Figure 1.1).

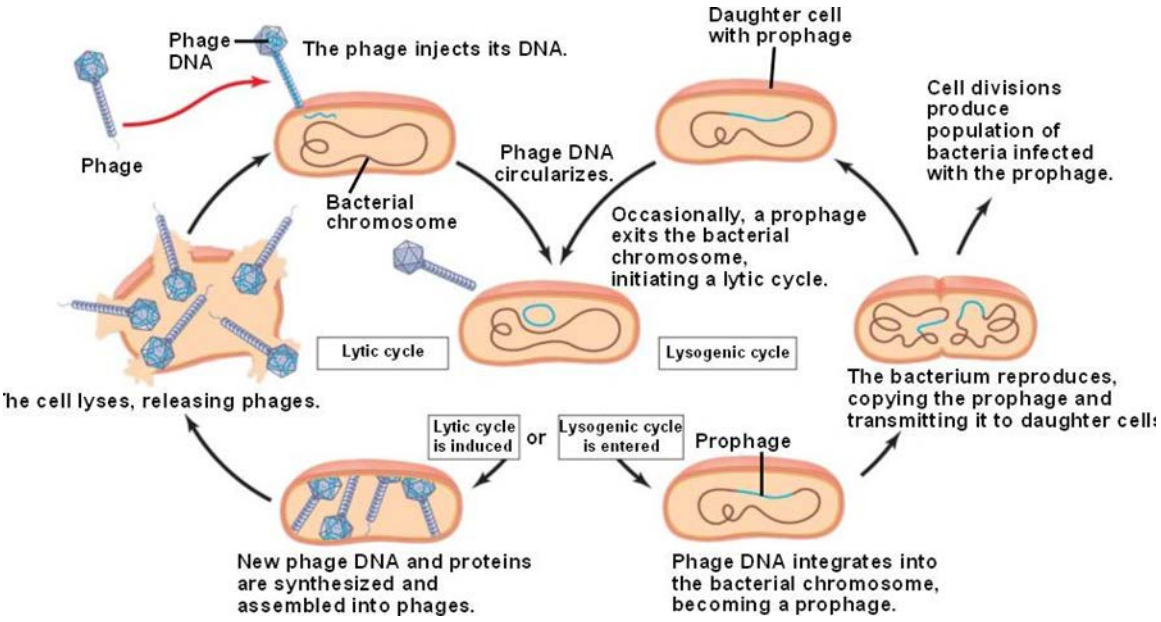


Figure1.1: Prokaryotic viruses life cycle (lytic and lysogenic)

<http://henspasitaspasii.blogspot.com/2013/04/virus-paper.html>

Temperate phages can propagate lytically under some circumstances. Induction is the process whereby prophage excise from the bacterial chromosome, replicate, and lyse the bacteria to release phage virions. During this process the genes that repress phage replication are damaged and required genes for lytic growth are turned on. Induction can occur spontaneously in the environment or in vitro using UV radiation or genotoxic agent such as mitomycin C (Casjens, 2003).

From the evolutionary perspective, prophage integration and phage transduction are major mechanisms for lateral DNA transfer in bacteria. There is a general assumption that the prophage decreases the fitness of its lysogenic host because the bacterial cell has to replicate extra DNA and can lyse the cell after induction. On the other hand, prophage gives the lysogenic

cell immunity and protects it from other phage infections and prophage acquisition of certain genes that could help the lysogenic cell to survive under certain conditions. For example, phage genes *bro* and *lom* confer serum resistance and better survival of *E.coli* in macrophages.

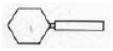
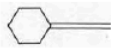
1.6 Bacteriophage Classification and Taxonomy

Phages are extremely diverse and can carry single or double stranded DNA or RNA. When d'Herell first discovered bacteriophage in the 1917, he thought that there was only one bacteriophage species with many races and he named that *Bacteriophagum intestinal*. In 1943, Ruska proposed a classification of viruses based on electron microscopy and that reveal the viral nature of bacteriophage. Lowff, Horne and Tournier in 1962 published a classification system of viruses based on morphology and nucleic acid type. In 1967, Bradley recognized six different phage-types filamentous, tailed and icosahedral phage that contains single-stranded DNA or RNA. Later on in 1971, the International Committee on Taxonomy of Viruses (ICTV) classified phage also based on the two major criteria of phage morphology and nucleic acid type. ICTV classified phage into six general types corresponding to five of Bradley's basic phage types (Table 1.2).

The main purpose of classification is generalization and simplification. Classification is essential for phylogenetic studies because it allows comparisons, which assist in understanding the relationships between phage groups. It is required as well for the identification of novel phages.

The ICTV taxnomic system requires the use of electron microscopy to visualize phage particles. However, many investigators do not perform this procedure, which explains the fact that GenBank has the complete sequence of many phages that have not been classified by the ICTV system. In 2002, Forest Rohwer and Rob Edwards proposed the phage proteomic tree, which is based on metagenomics data, that is, the cultured-independent identification of the phage using sequence data. They proposed this new method of classification to resolve the discrepancies between the available genomic data and traditional taxonomic methods. Metagenomics provides gene order of the phage and direction of transcription, as well as identification of genes coding for restriction modification enzymes and capsid proteins. Furthermore, metagenomics emphasizes horizontal gene transfer and provides important insights about the evolutionary history of phage. On the other hand, electron microscopy illustrates phage structure with measurement details of the capsid size and the tail length. Metagenomics data does not indicate to which family a tailed phage belongs because there are no specific sequences to *Sipho-*, *Myo-*, or *Podoviridae*. However, in the filamentous or polyhedral phages, metagenomics data allows separation into *Levio-*, *Micro-*, and *Inoviridae* (Ackermann, 2011). Metagenomics cannot replace electron microscopy because there is no proof that the detected sequences belong to complete active and functional phage. Metagenomics and electron microscopy are not mutually exclusive, but are complementary because each method answers different questions and both are essential for phage classification.

Table 1.2: Basic classification of phage families (Ackermann, 2011).

Shape	Order or family	Nucleic acid, particulars, size	Member	Number[a]
	Caudovirales	dsDNA (L), no envelope		
	*Myoviridae*	Tail contractile	T4	1312
	*Siphoviridae*	Tail long, noncontractile	λ	3262
	*Podoviridae*	Tail short	T7	771
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174	38
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2	3?
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1	19
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2	38
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6	3
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd	66
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2	5

1.7 The Lambdoid λ Phage System

Bacteria and their temperate phage, for example, *E.coli* and wild-type λ phage, can establish a dormant lysogenic or active lytic state. In the lysogenic cycle, the λ double-stranded DNA inserts into the *E.coli* chromosome and remains there for several generations. Phage λ can also enter the *E.coli* cell, multiply inside, and lyse the cell.

The gene organization of λ phages is what led to the formation of the "modular genome hypothesis". This hypothesis was proposed in 1980 by Botstein and it states that phage evolve by interchanging genetic elements (modules) that each can be considered as functional units. Thus,

for example, λ phage genes can be functionally separated into early, delayed early, and late genes, based on the timing of gene expression and transcription (Botstein, 1980).

In a prophage λ, the repression of the promoters *pR* and *pL* often prevents the expression of the lytic genes (Figure 1.2). This repression is associated with the CI protein, which binds to *oL* and *oR* operators and inactivates *pR* and *pL* promoters, and that provide the maintenance of lysogenic state (Oppenheim et al., 2005).

During induction of lambdoid phages, the SOS response inactivates CI, which induces the latent co-protease activity of RecA. RecA binds to the *cI* repressor and activates its by proteolysis. The inactivation of CI allows the transcription of the *pR* lytic promoter, which is the first step in initiation of the lytic cycle. Successful induction results in excision of the prophage, replication, and lysis of the bacteria.

1.7 The Lytic Cycle Pathway

The lytic mode of phage growth occurs in three stages, and involves the transcriptional cascade of early, delayed early, and late genes. *pR* and *pL* promoters transcribe genes for the two regulators, N and Cro, respectively. These regulators are attenuated by the *tL1* and *tR1* terminators that act as weak repressors for both *pR* and *pL* promoters (Oppenheim et al., 2005).

The N protein is considered an antiterminator that up-regulates the assembly of a transcription complex (i.e., an N-modified RNA polymerase). This complex can overcome the *tL1* and *tR1* terminators leading to the transcription of the delayed early genes. These genes encode the DNA replication proteins, O and P, the lysogenic regulators CII and CIII, and the late gene regulator Q (Figure 1.2).

When Q protein accumulates at a sufficient level, it modifies RNA polymerase and makes it resistant to the effect of the terminators downstream to *pR'*. Leading to the transcription of the late genes. These late genes encode for proteins involve in phage morphology, assembly, and host cell lysis (Yang et al., 1987).

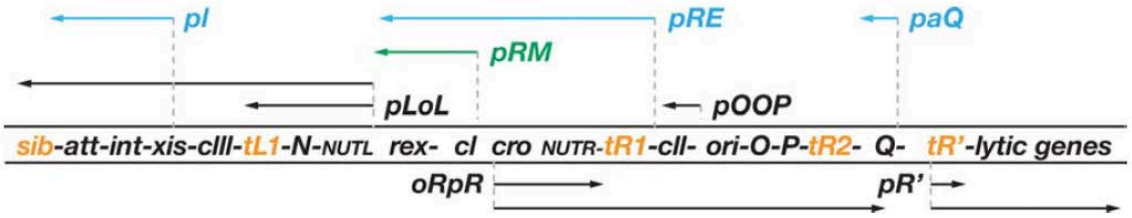


Figure 1.2: Genetic map and transcriptional modules of phage λ regulatory region (Oppenheim et al., 2005).

1.8 Switching to the Lysogenic Pathway

The transcription of the delayed early genes leads to the initiation of the lysogeny process. Under certain conditions, CII lysogenic regulator accumulation leads to the inactivation of the Q protein (late gene regulator) so that lytic pathway is blocked and switches to the lysogenic state. CII protein accumulation is essential for the transition to the lysogenic state. It catalyze the synthesis of the Int protein that stimulate phage λ DNA insertion into the bacterial chromosome, as well as the synthesis of the CI repressor which binds to the *oR* and *oL* promoters. Once the lytic pathway is switched off and CI represses *pL* and *pR*, the prophage is maintained in the bacterial chromosome and it is extremely stable.

1.9 Bacteriophages and their Implication on Biotechnology

In the 20th century, following the discovery of bacteriophage and their potential to lyse bacteria, extensive research was performed to utilize phages as therapeutic agents. However, after the discovery of antibiotics, this idea became less attractive and phages were used mainly as genetic tools in the molecular biology field.

Nowadays, due to increased bacterial resistance, and the advantages of modern biotechnology, bacteriophages are being studied because of their potential to be used as an alternative treatment against antibiotic resistance bacterial strains. They are also being considered as bio-control agents in the petroleum and agriculture industry. Moreover, phages can be used as diagnostic tools and as a display system for proteins and antibodies.

1.9.1 Phage Therapy

Phage therapy has many advantages over antibiotics for therapy, but there are also concerns. This approach has been used in animal, plants, and human beings with varied degrees of success. One of the advantages of using phage is their specificity to the target bacteria and lack of interference with the host normal flora. After phage administration, they spread quickly through the human body reaching every organ. However, the immune response generates antibodies that clear the systemic phage and that is one of the major concerns about using phage therapy. One way to circumvent this is to use the phage's lytic enzymes such as endolysins and holins for therapy instead of using the whole viron particle (Haq et al., 2012).

1.9.2 Phage Display

In the phage display technique, DNA, which encodes the desired polypeptide, is fused within the coat protein genes for the phage. Then the desired protein is produced and expressed on the surface of the phage (Smith, 1985). Phage display can be used to generate antibody fragments libraries using the filamentous phage such as M13 phage. These display libraries have been involved in many applications such as in the treatment of cocaine addiction. First, the phage are administrated nasally until it reaches the central nervous system where the displayed antibody binds to the molecules of cocaine and prevent its effect on the brain (Dickerson et al., 2005).

1.9.3 Phage Typing

The use of phages as a diagnostic tool or for phage typing depends upon the sensitivity patterns of bacteria to certain phages. There are various methods that can be used to detect pathogenic bacteria. For example, phages can be used to deliver reporter genes, which can be detected post infection. Phage adsorption can be detected by using phage that have fluorescent dye attached to their coats (Goodridge et al., 1999). Detection of released bacterial proteins following bacterial lysis due to phage infection, such as adenylate kinase, can be detected by using antibodies produced by phage display that will bind specifically to these complexes (Petrenko, 2003). The phage amplification assay is the most technique that have been used to detect bacteria such as *Pseudomonas, E.coli, Mycobacterium tuberculosis, Salmonella, Campylobacter* and *Listeria* species (Barry et al., 1996).

1.9.4 Phages Bioprocessing and Biocontrol

Bacteriophages are used in bioprocessing to decrease the bacterial load in foods. Especially foods that are minimally processed to avoid cooking associated texture or flavor (Garcia et al., 2010). Phage bioprocessing has been employed to reduce the growth of many food pathogens such as *Salmonella enteritidis* in cheese, *Campylobacter* and *Salmonella* on chicken skin, and *Listeria monocytogenes* on meat. In addition, this approach can be used to extend the shelf life of animal products (Dykes et al., 2002).

Phages can be used as well as predators of bacteria that associated with fungi, plants or their products. Phage biocontrol of plants pathogens has been a successfully used against *Xanthomonas pruni* on peaches, peppers, and cabbage plants. These methods also worked against *Ralstonia solanacearum* on tobacco and *Xanthomona campestris* on tomatoes.

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Chapter 2

Modification of media for optimized *in vitro* growth of *Francisella* strains

2.1 Introduction

Francisella tularensis subspecies *tularensis* and *holarctica* are known for their fastidious growth requirements *in vitro.* However, *Francisella tularensis* subspecies *novicida* has less fastidious requirements than the first two (Gray et al., 2002). The difficulties associated with cultivation *F. tularensis in vitro* is a consequence of a paucity of information about the factors needed for extracellular growth. Chamberlain's defined medium (CDM) and Mueller Hinton broth (MHII) are the media most commonly used to cultivate *F. tularensis* (Chamberlain, 1965). In addition, cysteine heart agar (CHA) enriched with hemoglobin, tryptic soy agar (TSA) with 0.1% cysteine, Mueller Hinton broth (MHII) with IsoVitaleX and, more recently, brain heart infusion with cysteine (BHIc) has been utilized to cultivate *F. tularensis* (Mc Gann et al., 2010; Ellis et al., 2002). The IsoVitaleX supplement is costly and the Chamberlain's defined medium is time consuming to prepare. In addition, a clear agar medium is needed for visualizing opaque phage plaques, and media containing blood or hemin are not transparent. Therefore, for these reasons, the goal of this research was to develop an inexpensive, easy to prepare, clear agar media for screening for phage plaques.

In this report we described the development of a low-cost media consisting of a combination of BHI and MHII base media supplemented with ferric pyrophosphate and L-cysteine designated BMFC. This study was undertaken to measure the growth of multiple *Francisella* strains on BMFC media and to compare it with the recently reported BHIc media (Gann et al., 2010).

2.2 Materials and Methods

2.2.1 Comparison of the growth rate of *Francisella tularensis LVS* in five different media

Francisella tularensis LVS strain was provided by BEI (Manassas, VA, USA). The bacteria were initially grown from a freezer stock on chocolate agar and incubated at 37°C for 24 h. Five different types of media were prepared: 1) 1X Mueller Hinton II broth (Becton Dickinson, MD, USA) with 1% IsoVitaleX (Becton Dickinson, France), 2) 1X Mueller Hinton II broth with 1% rabbit blood, 3) 1X Mueller Hinton II broth with 0.2% hemoglobin (MP Biomedicals, Santa Ana, CA, USA), 4) 1X Mueller Hinton II broth with 1% rabbit blood and 1% IsoVitaleX, and 5) 1X Mueller Hinton II broth with 0.2% hemoglobin and 1% IsoVitaleX. The five media were inoculated with either a single colony of *F. tularensis LVS* from the chocolate agar plate or with 100 µl of the frozen stock culture from BEI Resources. The inoculated media was incubated at 37°C on a platform shaker (Innova 4000, Incubator Shaker, New Brunswick Scientific, Enfield, CT, USA) at 225 rpm for 24 h. The optical density (OD_{600}) of each culture was measured every 6 h, using 5 Genesys spectrophotometer. *Francisella tularensis* LVS growth curves were constructed by plotting the OD_{600} versus the time using Xcel software.

2.2.2 Comparison of *F. tularensis LVS* growth rate on BHI agar media containing different iron sources and amino acid supplements

Francisella tularensis LVS was plated onto four different agar media: 1) Brain heart infusion (BHI, Becton Dickinson) agar with 1% IsoVitaleX and 0.5% ferric pyrophosphate (Sigma-Aldrich), 2) BHI agar with 1% IsoVitaleX and 0.2% hemoglobin, 3) BHI agar with 1% L-cystine with 0.5% ferric pyrophosphate, and 4) BHI agar with 1% L-cystine and 0.2% hemoglobin. All bacterial plates were incubated at 37°C for 48 h and observed for growth. Growth was scored as 'good growth', 'light growth', and 'no growth'

2.2.3 Comparing the effect of IsoVitaleX and L-cysteine on the growth rate of *Francisella tularensis* LVS in BHI-MHII (BM) media

Francisella tularensis LVS strains 1145 and 1281were each grown on two media: 1) 1XBHI and 1XMHII agar (designated BM) supplemented with 0.1% L-cystine, 0.25% ferric pyrophosphate and 1% IsoVitaleX, and 2) 1XBHI and 1XMHII agar with 0.1% L-cystine (ThermoFisher scientific, Waltham, MA USA), 0.25% ferric pyrophosphate, and 25μg/ml (0.0025%) L-cysteine. Plates were incubated at 37°C for 48 h. Next, single colony was inoculated from each agar media type into their respective broths and incubated at 37°C on a platform shaker for 16 h. Optical densities (λ=600 nm) were measured every 4 h using a 5 Genesys spectrophotometer blanked with the media.

2.2.4 Comparison of the growth rates of *Francisella tularensis LVS* and *Francisella philomiragia* strains grown in BM media with either L-cysteine and glucose or IsoVitaleX

Francisella tularensis LVS and *Francisella philomiragia* strains 25016, 25017, and 25018 were grown on two different media: 1) BM agar containing 0.25% ferric pyrophosphate and 1% IsoVitaleX and 2) BM agar with 0.25% ferric pyrophosphate, 250 μg/ml L-cysteine (0.025%) and 1% glucose. Plates were incubated at 37°C for 48 h. oneat single colony of each strain was inoculated into the same broth and incubated at 37°C on a platform shaker at 250 rpm for 16 h. The optical density of each culture was read every 4 h using a 5 Genesys spectrophotometer (Genesys, Daly City, CA).

2.2.5 The effect of varying concentrations of L-cysteine on the growth of *Francisella tularensis* LVS

In this experiment, the base media was composed of BM agar containing 0.25% ferric pyrophosphate, 0.1% L-cystine, 0.1% glucose. Four different concentrations of L-cysteine: 1) 0, 2) 0.0125%, 3) 0.025%, and 4) 0.05% were added to the base media broth and *Francisella tularensis* LVS strain 1145 was plated onto each of these media and incubated at 37°C for 48 h. A single colony was inoculated into its respective broth media and grown overnight at 37°C on a shaker incubator at 225 rpm (Innova 4000, Incubator Shaker, New Brunswick Scientific, Enfield, CT, USA). An aliquot (100 microliters) of the overnight culture was added to 10 ml of the same media and incubated at 37°C with shaking (225 rpm) for 30 h. OD_{600} values were recorded at 0, 5, 10, 20, and 30 h time points.

2.2.6 The effect of L-cystine and glucose on growth of *Francisella tularensis LVS* in BHI and MHII base.

In this experiment, four media were prepared: 1) 1XBHI, 1XMHII, 0.25% ferric pyrophosphate, 0.025% L-cysteine, 0.1% glucose, 0.1% L-cystine 2) 1XBHI, 1XMHII, 0.25% ferric pyrophosphate, 0.025% L-cysteine, 0.1% glucose, no L-cystine, 3) 1XBHI, 1XMHII, 0.25% ferric pyrophosphate, 0.025% L-cysteine, 0.1% L-cystine, and 0.1% glucose, 4) 1XBHI, 1XMHII, 0.25% ferric pyrophosphate, 0.025% L-cysteine, 0.1% L-cystine, and no glucose. *Francisella tularensis* LVS strain 1145 was grown on agar plates of each of these media and incubated at 37°C for 48 h. Single colonies were inoculated into the same broth media and grown overnight at 37°C with shaking (225 rpm). The overnight culture (100 µl) was added to 10 ml of the appropriate broth and incubated at 37°C with shaking (225 rpm) for 30 h. OD_{600} values were recorded at 0, 5, 10, 20, and 30 h time points, and growth curves were generated by plotting time versus OD_{600} using Xcel software. Duplicate tubes were used for each time point.

2.2.7 Determination of the essential supplements for growth of *Francisella tularensis* LVS

Three different media were prepared in this experiment as described in Table 2.1 and hereafter are designated BM, BMFC, and BMFCGC. In order to illustrate the impact of the broth formulation on *Francisella tularensis* LVS growth, the bacterium was initially cultured from a −80°C, 40% glycerol stock culture on BM, BMFC, and BMFCGC solid media for 48 h at 37°C. Single colonies were inoculated into the corresponding broths and incubated overnight at 37°C with shaking (225 rpm). After that, a 1:100 dilution of the overnight culture was prepared in 10 ml of a fresh broth and incubated at 37°C, in a shaker incubator at 225 rpm for 30 h. At 0, 5, 10,

20, and 30 h time points, the OD_{600} values were recorded using a 5 Genesys spectrophotometer. Duplicate tubes were used for measuring the optical densities of the bacterial cultures.

Table 2.1: Composition and preparation of BM, BMFC, and BMFCGC.

Designation	Constituent	Amount/liter
BM	**Brain heart infusion**	**37 g**
	Mueller Hinton	**22 g**
	Dissolve in 941ml of distilled water and sterilize by autoclaving	
BMFC	**Brain heart infusion**	**37 g**
	Mueller Hinton	**22 g**
	Dissolve in 928.5 ml of distilled water and sterilize by autoclaving allow to cool down then add the following	
	Ferric pyrophosphate	**10 ml**
	Dissolve 2.5 g in 97.5ml of distilled water and sterilize by autoclaving to make 25 mg/ml stock concentration	
	L-cysteine	**2.5 ml**
	Dissolve 0.5 g in 9.5ml of distilled water and filter sterilize (0.2µm syringe filter) to make 50 mg/ml stock concentration	
BMFCGC	**Brain heart infusion**	**37 g**
	Mueller Hinton	**22 g**
	Dissolve in 891 ml of distilled water and sterilize by autoclaving allow to cool down then add the following	
	Ferric pyrophosphate	**25 ml**
	Dissolve 10 g in 90 ml of distilled water and sterilize by autoclaving to make 100 mg/ml stock concentration	
	L-cysteine	**5 ml**
	Dissolve 0.5 g in 9.5ml of distilled water and filter sterilize (0.2µm syringe filter) to make 50 mg/ml stock concentration	
	Glucose	**10 ml**
	Dissolve 10 g in 100 ml of distilled water and filter sterilize (0.2µm syringe filter) to make 10% stock concentration	
	L-cystine	
	Dissolve 10 g in 100 ml of distilled water and filter sterilize (0.2µm syringe filter) to make 10% stock concentration	**10 ml**

2.2.8 Growth of *Francisella philomiragia, Francisella tularensis* subsp. *tularensis, holarctica,* and *novicida* strains in BMFC media

A total of 18 different *Francisella* strains (see Table 2.2) were used in this experiment. For growth on agar media, all bacterial strains were cultured from −80°C, 40% glycerol stocks

on BMFC media (as described in table 2.1) for 48 h at 37°C.

Single colonies from each strain were inoculated into BMFC broth and incubated overnight at 37°C with shaking (300 rpm, Innova 4000, Incubator Shaker, New Brunswick Scientific, Enfield, CT, USA). Duplicate tubes of fresh BMFC broth (10 ml) were inoculated with overnight culture (100 µl) and incubated at 37°C, for 20 h at 300 rpm on a platform shaker. OD_{600} values were recorded at 0, 5, 10, and 20 h time points. A growth curve was constructed using Xcel software and optical densities were plotted against time points.

Table2.2: *Francisella* strains

***Francisella* Strains (freezer#)**	**BEI strains #**
1239 *F. t. novicida*	NR-13 *F. t. novicida*
1241 *F. t. holarctica*	NR-14 *F. t. holarctica*
1243 *F. t. tularensis*	NR-50 *F. t. tularensis*
1245 *F. t. novicida*	NR-573 *F. t. novicida*
1247 *F. t. novicida*	NR-574 *F. t. novicida*
1249 *F. t. novicida*	NR-575 *F. t. novicida*
1251 *F. t. novicida*	NR-576 *F. t. novicida*
1253 *F. t. novicida*	NR-577 *F. t. novicida*
1255 *F. t. novicida*	NR-578 *F. t. novicida*
1257 *F. t. novicida*	NR-579 *F. t. novicida*
1259 *F. t. novicida*	NR-580 *F. t. novicida*
1261 *F. t. novicida*	NR-581 *F. t. novicida*
1263 *F. t. novicida*	NR-582 *F. t. novicida*
1265 *F. t. novicida*	NR-583 *F. t. novicida*
1267 *F. t. novicida*	NR-584 *F. t. novicida*
1269 *F. t. holarctica*	NR-585 *F. t. holarctica*
1271 *F. t. holarctica*	NR-597 *F. t. holarctica*
1273 *F. t. holarctica*	NR-646 *F. t. holarctica*

2.2.9 Comparison of growth rates of selected *Francisella* strains in BMFC and BHIc media- impact of changing the iron source from heme-histidine to ferric pyrophosphate and vice versa

The modified brain heart infusion (BHIc) media was prepared as described by Mc Gann et al in 2010 (see table 2.4). BMFC media was prepared as described in table 2.1**.** Chamberlain's defined media was prepared as described previously (Chamberlain, 1965) and the components are detailed in table 2.3. *F. tularensis* LVS, *novicida* U112, and *tularensis* NR-50 were selected to perform the comparison in this experiment. For growth on solid media, all strains were cultured on chocolate and BMFC agar media and incubated at 37°C for 48 h. Single colonies from each strain were inoculated from chocolate agar into BHIc broth and BMFC broth and the growth curves in the graph were designated as BHIc and BHIc-BMFC (to indicate that the bacteria were first grown on chocolate agar and then inoculated into BHIc or BMFC broth). In addition, a single colony of each strain was transferred from BMFC agar to both BHIc and to BMFC broth (BMFC-BHIc and BMFC-BMFC). All broth cultures were inoculated in triplicate and were incubated at 37°C overnight with agitation at 200 rpm. Each culture was subsequently diluted with the corresponding broth to an OD_{600} of 0.2, and the diluted culture (700 μl) was added to 11.3 ml of the same broth formula to ensure that the starting number was comparable among all strains. All tubes were incubated at 37°C with shaking at 200 rpm for 34 h. Growth curves were generated for each growth condition by plotting the OD_{600} obtained every two hours (for 34 h) versus the time post-inoculation.

Table2.3: Chamberlain's defined media

Ingredient	Stock Concentration mg/ml	Final concentration mg/ml	#of ml from the stocks to the final media in 1000 mL	# of g in 100 ml stock solution
L-Arginine	11.0 mg/ml	0.4 mg/ml	36.3 mL	1.1 g
L-Aspartic Acid +HCl	10.0 mg/ml	0.4	40 mL	1 g
L-Cysteine HCl	11.0 mg/ml	0.2	18.18 mL	1.1 g
L-Histidine	11.0 mg/ml	0.2	18.18 mL	1.1 g
DL-Isoleucine	10 mg/ml	0.4	40 mL	1 g
L-Leucine (Methionine free)	10 mg/ml	0.4	40 mL	1 g
L-Lysine (mono HCl)	44.0 mg/ml	0.4	9 mL	4.4 g
DL-Methionine	10 mg/ml	0.4	40 mL	1 g
L-Proline (hydroxyl-L-proline)	15 mg/ml	2	133.3 mL	1.5 g
DL-Serine	10.0 mg/ml	0.4	40 mL	1 g
DL-Threonine (allo-free)	40 mg/ml	2	50 mL	4 g
L-Tyrosine+HCl	2.0 (0.001 N NaOH)	0.4	200 mL	0.2 g 2 bottles each has 100 mL
DL-Valine	20 mg/ml	0.4	20 mL	2 g
Spermine Diphosphate	10 mg/ml	0.04	40 mL	1 g
Thiamine HCl	10 mg/ml	0.004	0.4 mL	1 g
DL-Calcium Pantothenate	10 mg/ml	0.002	0.2 mL	1 g

Table 2.4: Composition of BHIc media

Constituent	Amount (per liter)
Brain heart infusion	37g
Cysteine	1g
B-Nicotinamide Adenine Dinucleotide (1mg/ml)	10 ml
Heme-histidine (1mg/ml)	10 ml
Glucose (20%)	25 l

2.3 Results

Francisella tularensis LVS and *novicida* U112 are considered the most common strains used in *Francisella* research. Studies on these strains can be done in BSL2 facilities making them ideal models for research. The primary goal of this aim was to develop agar and liquid media that is inexpensive and efficient for *Francisella* growth, phage production and plaque visualization. BMFC media was developed in our laboratory, was low cost, and resulted in rapid growth for most *Francisella* strains. Our goal was to develop a media with the ability to (a) allow high-density and rapid growth and (b) a low-cost media that promotes efficient growth from a small inoculum. In addition, we found that BMFC was useful for growth of *Francisella philomiragia, holarctica, tularensis,* and *novicida,* and it could be used to grow *Francisella* strains that require BSL3 facilities, including *F. tularensis* subsp. *tularensis* Schu S4 and WY96-3418 as well as *F. tularensis* subsp. *holarctica*, Strain OR96-0246, MA00-2987 and K499-3387. Individual colonies grew on agar and very dense growth was observed in broth within 12 h of inoculation. Our data indicate that BMFC exhibits superior growth for *Francisella* strains with reduced cost

and preparation time over currently available media formulations.

2.3.1 Hemoglobin supports better growth of *F. tularensis LVS* as compared with rabbit blood

As *F. tularensis* LVS is considered the most common strain used in the study of *Francisella* this experiment focused on identifying a suitable agar and liquid media for the cultivation of this strain. The growth curves for this experiment can be seen in figures 2.1, 2.2, 2.3, 2.4, and 2.5. *F. tularensis* LVS reached a maximum OD_{600} of 1.27 when cultured in MHII media with 1% IsoVitaleX and 0.2% hemoglobin (Figure 2.3). A significant delay in growth was recorded in media that was inoculated with bacteria (100 µl) obtained directly from thawed frozen stock cultures. However, *F. tularensis* LVS reached an OD_{600} of 1.08 when grown on MHII and IsoVitaleX only (Figure 2.2). This strain grew better in media that contained MHII, IsoVitaleX and hemoglobin than in media with MHII, IsoVitaleX and rabbit blood (Figures 2.3 and 2.5). These results indicate that *F. tularensis* LVS grew better on media containing IsoVitaleX as an amino acid source and hemoglobin as an iron source.

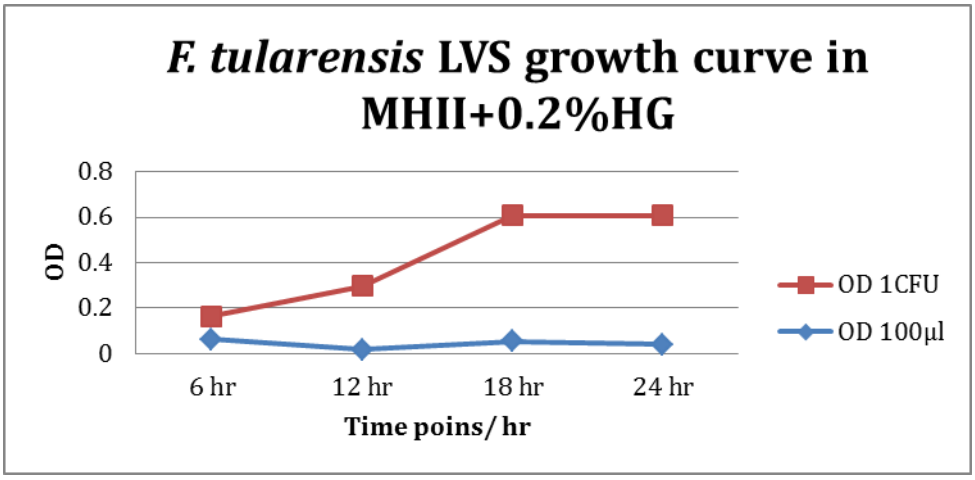

Figure 2.1: *F. tularensis LVS* growth curve in MHII and 0.2% hemoglobin

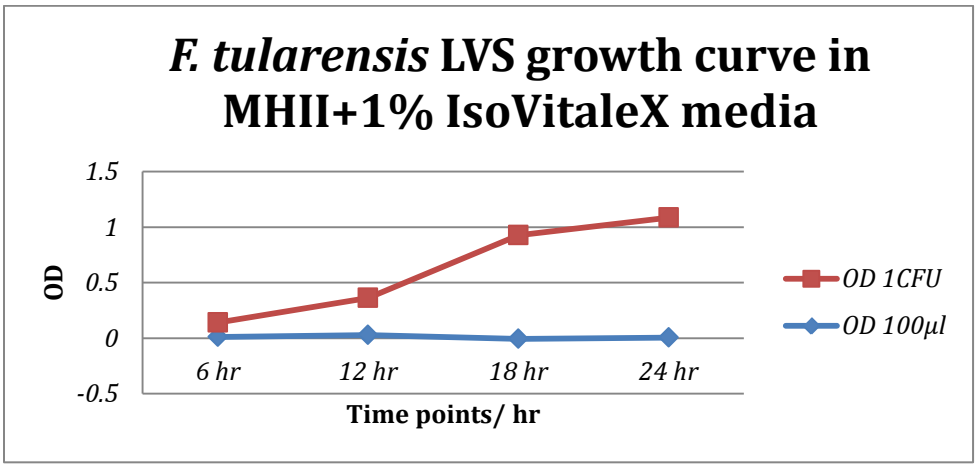

Figure2.2: *F. tularensis LVS* growth curve in MHII and 1% IsoVitaleX

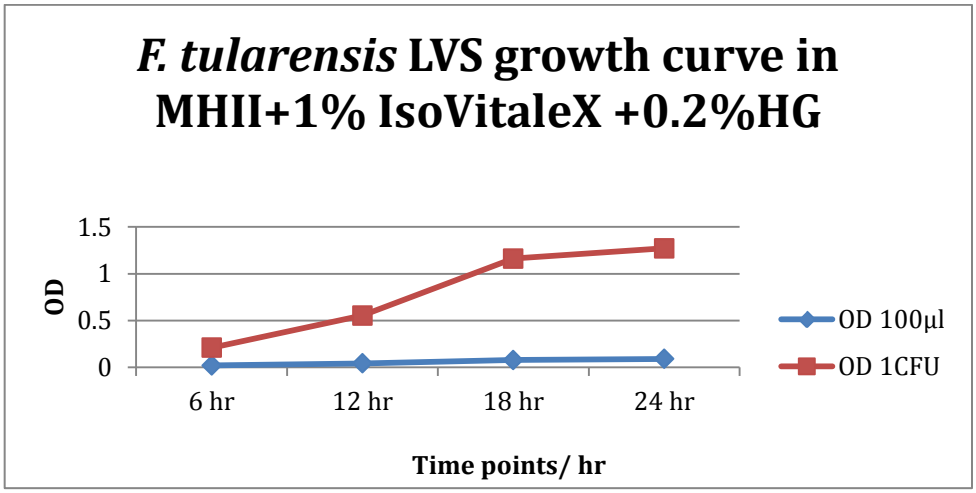

Figure2.3: *F. tularensis* LVS growth curve in MHII, 1% IsoVitaleX and 0.2% hemoglobin

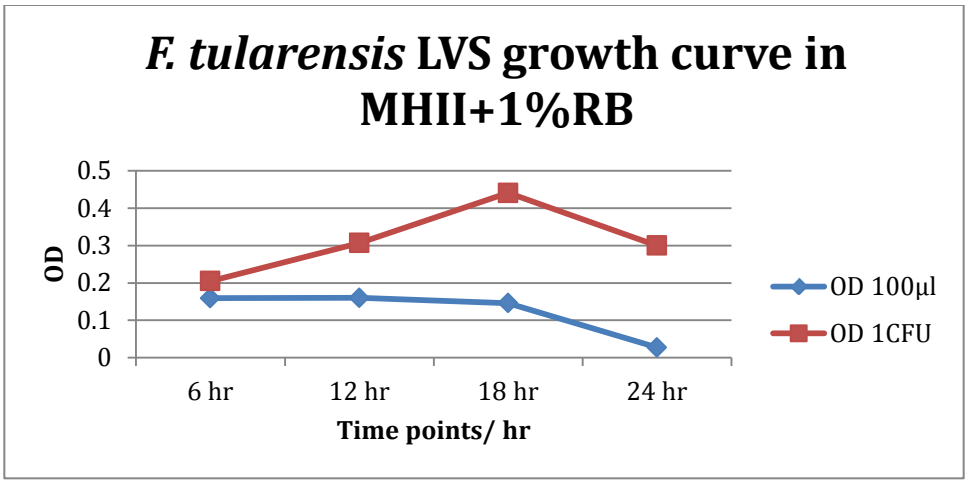

Figure 2.4: *F. tularensis* LVS growth curve in MHII and 1% Rabbit blood

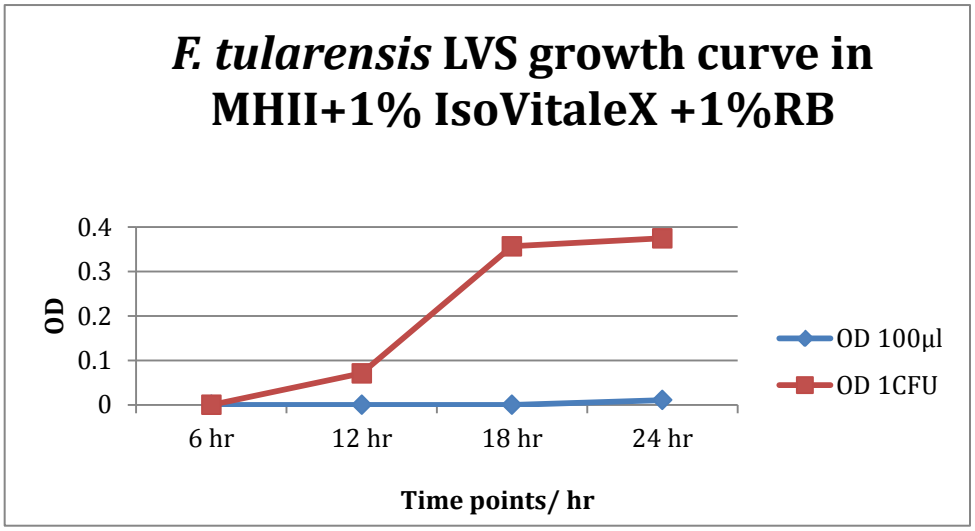

Figure 2.5: *F.tularensis* LVS growth curve in MHII, 1% IsoVitaleX and 1% Rabbit blood

2.3.2 Ferric pyrophosphate and IsoVitalex allow better growth of *F.tularensis LVS* than hemoglobin and L-cystine, respectively

F. tularensis LVS is a fastidious organism and requires the presence of specific amino acids and an iron source for enhanced growth. This experiment evaluated whether *F. tularensis* LVS grew better on media containing hemoglobin versus ferric pyrophosphate, and L-cystine versus IsoVitaleX. The performance of each agar formulation was evaluated based on the overall bacterial growth and the morphology of the colonies. The ability of *F. tularensis LVS* to grow on these four different agar media was assessed: (a) 1XBHI, 1% IsoVitaleX and 0.5% Ferric pyrophosphate, (b) 1XBHI, 1% IsoVitaleX and 0.2% hemoglobin, (c) 1XBHI, 1% L-cystine and 0.5% ferric pyrophosphate, (d) 1XBHI, 1% L-cystine and 0.2% hemoglobin. It took 48 h at 37^{o}C for visible colonies to appear on all media types. *F. tularensis* LVS growth was best on the media supplemented with IsoVitaleX and ferric pyrophosphate. On this media, the individual colonies were larger and growth was denser.

2.3.3 A mixture of brain heart infusion (BHI) and Mueller Hinton II media provides better growth characteristics for *F. tularensis LVS* than either media alone; IsoVitaleX is better at supporting growth as compared with L-cysteine when L-cysteine is at suboptimal concentration in the media

The results of these experiments highlighted the suitability of a mixture of BHI and MHII media for the cultivation of *F. tularensis* LVS and compared its growth in BHI and MHII media with L-cysteine versus IsoVitaleX. After 24 h incubation, all plates for each media type showed

visible growth and individual colonies were observed. This media produced faster and superior bacterial growth than all media previously tested, which contained either BHI or MHII alone. *F.tularensis LVS* reached an OD_{600} of 1.7 when cultured in media containing 0.5XBHI and 1XMHII, 0.1 % L-cystine and 1% IsoVitaleX after 18 h of incubation (Figure 2.6). Another media with the same ingredients except that it contained 25µg/ml L-cysteine instead of IsoVitalex also exhibited good growth with an OD_{600} reading of 1.4 after 18 h (figure 2.7). Mixing brain heart infusion with Mueller Hinton media provides more essential nutrients that support rapid growth of *F. tularensis* LVS. IsoVitaleX appeared to support *F. tularensis* LVS growth more than L-cysteine due to a combination of low concentration of L-cysteine (25µg/ml) used in this experiment and the presence of glucose in IsoVitaleX. Therefore, we decided to increase the concentration of L-cysteine to 250µg/ml and add glucose in the next experiment.

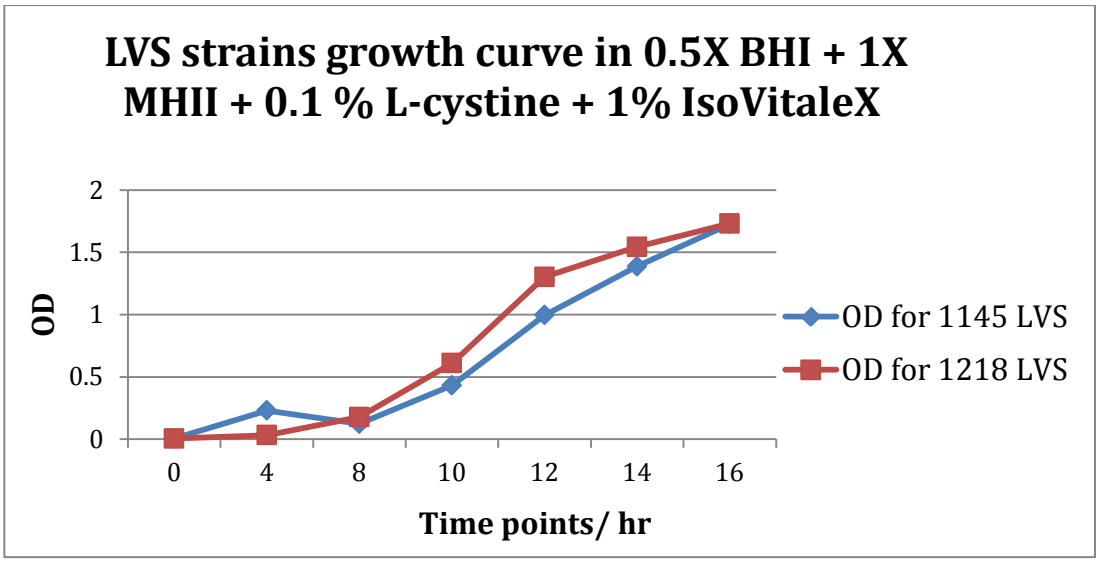

Figure 2.6: *Francisella tularensis* LVS strains growth curve in 0.5X BHI + 1X MHII + 0.1 % L-cystine + 1% IsoVitaleX

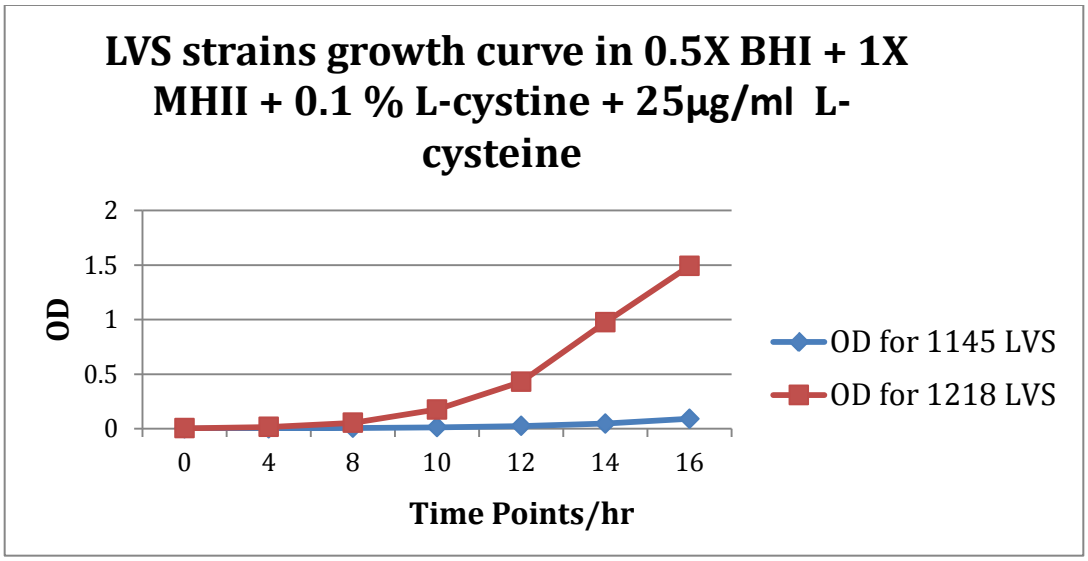

Figure 2.7: *Francisella tularensis* LVS strains growth curve in 0.5X BHI + 1X MHII + 0.1 % L-cystine + 25µg/ml L-cysteine

2.3.4 L-cysteine and glucose can replace IsoVitaleX in growth media for *F.tularensis LVS* and *F. philomiragia* strains

L-cysteine and glucose are the major components in the IsoVitaleX enrichment mix. This experiment illustrates the difference between using IsoVitaleX versus its major components (L-cysteine and glucose) on the growth of *F. tularensis* LVS and *Francisella philomiragia* strains. Both *F. tularensis* LVS and *Francisella philomiragia* displayed very similar growth characteristics in media containing IsoVitalex or L-cysteine and glucose as supplements (Figure 2.8 and 2.9). Therefore, we substituted IsoVitaleX with L-cysteine and glucose in our media since these components are much cheaper.

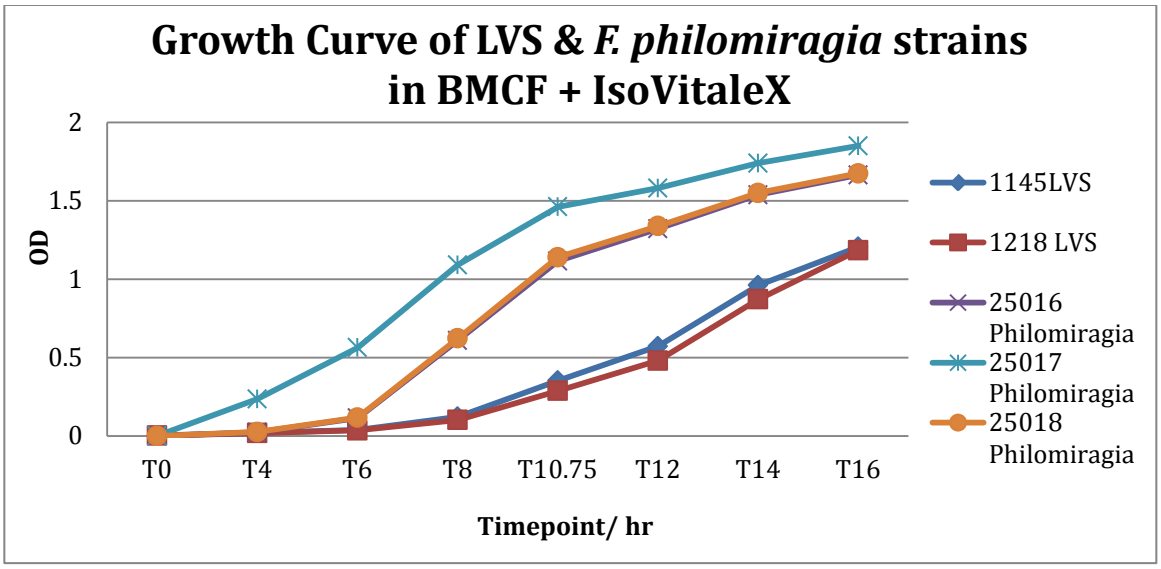

Figure 2.8: Growth curve of *F. tularensis* LVS & *F. philomiragia* strains in BMCF + IsoVitalex

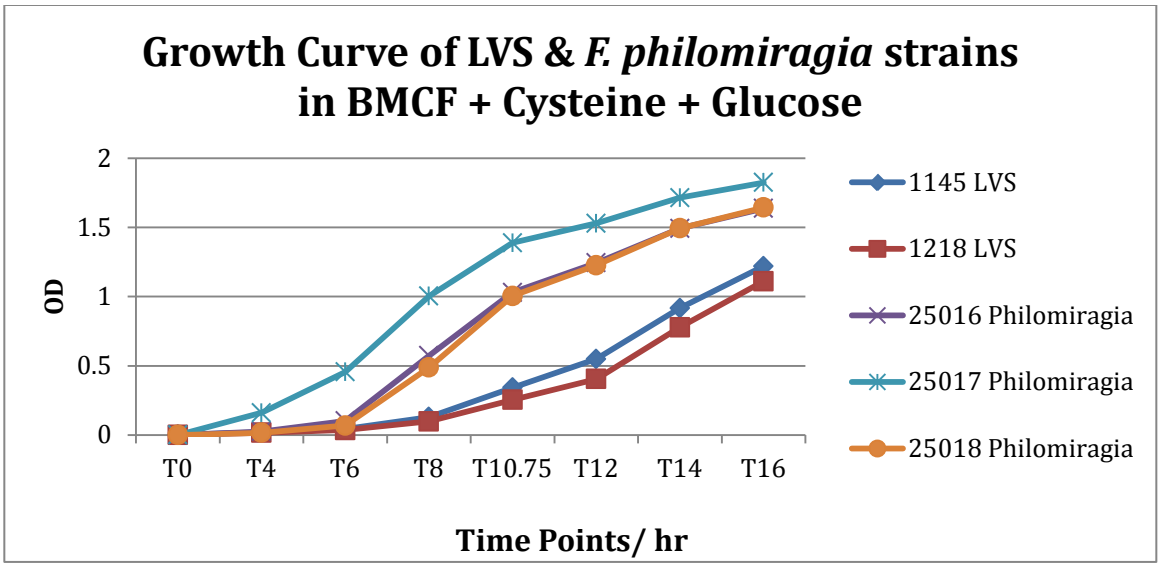

Figure 2.9: Growth curve of *F. tularensis* LVS & *F. philomiragia* strains in BMCF + Cysteine + Glucose

2.3.5. Either L-cystine or L-cysteine is sufficient for growth of *F. tularensis* LVS

L-cystine is an amino acid formed from two L-cysteine molecules bound together by a disulfide bond. This experiment illustrated which of these amino acids is essential for *F. tularensis* LVS growth by measuring the growth rate in media supplemented with 0.1% L-cystine and different concentrations of L-cysteine (0, 0.0125, 0.025, and 0.05%). There was no significant difference between the growth rates of *F. tularensis* LVS in each of these media (Figure 2.10), indicating that the presence of either one of these amino acids is sufficient for *Francisella* growth.

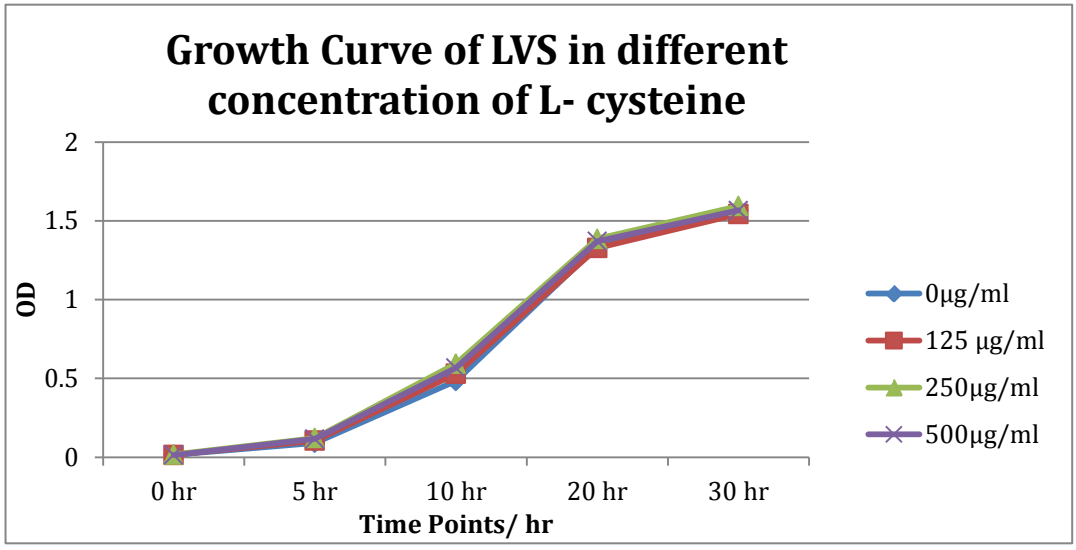

Figure 2.10: Growth curve of *F. tularensis* LVS in different concentration of L- cysteine

2.3.6 L-cystine and glucose are dispensable in BHI-MHII growth media containing L-cysteine and ferric pyrophosphate

In order to reduce cost, time, and effort preparing standard media to grow *Francisella*, we needed to eliminate unnecessary supplements. This experiment emphasized the impact of L-cystine and glucose on *F. tularensis* LVS growth. *F. tularensis* LVS showed very similar growth rates in all four media formulations with and without L-cystine and glucose (Figures 2.11, 2.12). BHI (1X) and MHII (1X) media are the basis of this media and BHI contains glucose as an ingredient. These data confirmed the results of the previous experiment, which indicated that the presence of either L-cystine or L-cysteine is sufficient for *Francisella* growth.

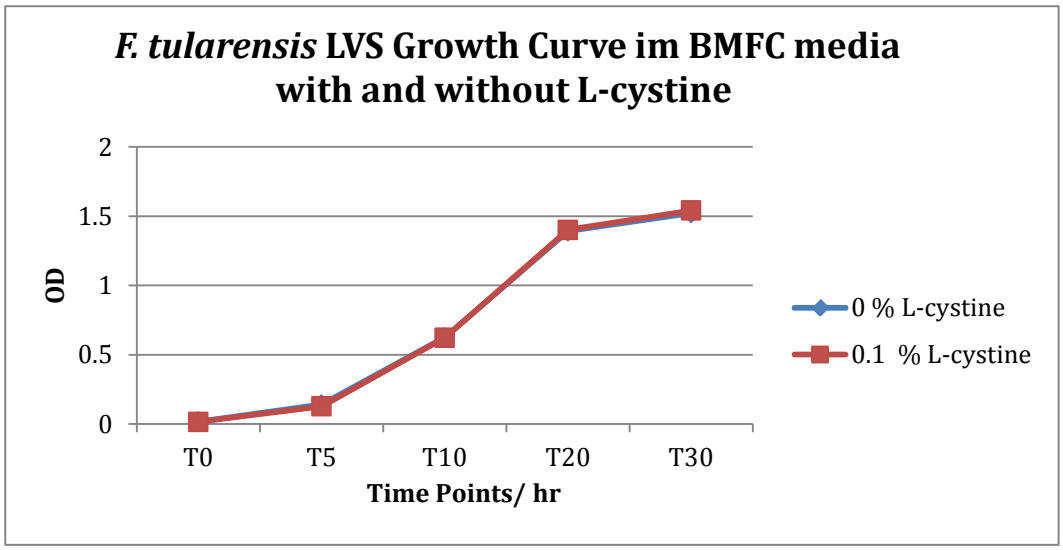

Figure 2.11: *F. tularensis* LVS growth curve im BMFC media with and without L-cystine

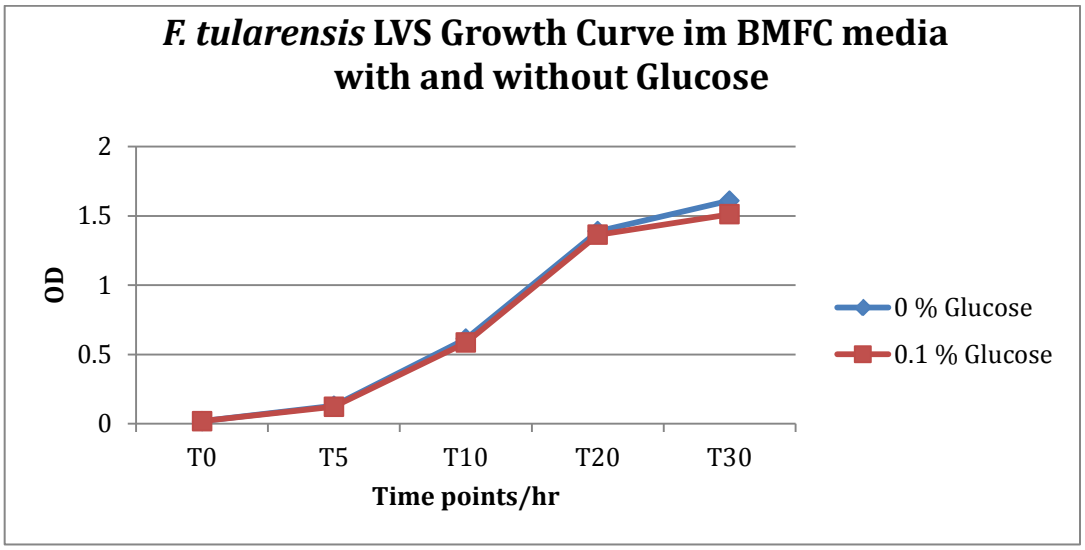

Figure2.12: *F. tularensis* LVS growth curve im BMFC media with and without Glucose

2.3.7 BMFC media provides necessary nutrients for *F. tularensis LVS*

This experiment finalized the formula of the *Francisella* standard growth media, which was used through this project. *F.tularensis LVS* growth curve was recorded for BM, BMFC, and

BMFCGC media (Table 2.1). The growth curves displayed very similar patterns for *F. tularensis* LVS in BMFC and BMFCGC media (Figure 2.13). This confirmed the previous results. However, the growth rate of *F. tularensis* LVS was significantly lower when grown on BHI-MHII media (without supplements), which indicated that L-cysteine and ferric pyrophosphate are absolute requirements for *Francisella* growth

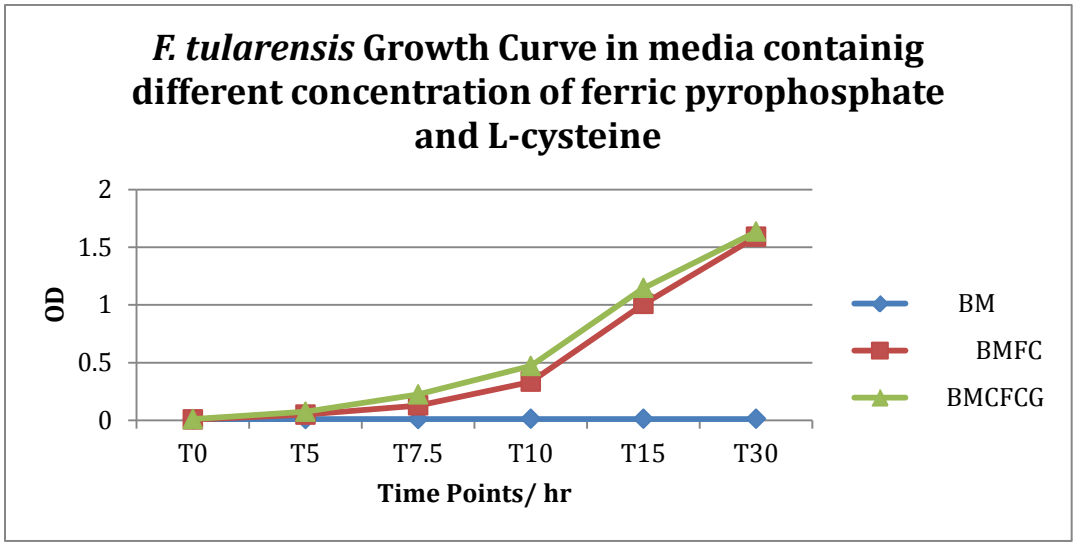

Figure 2.13: *F. tularensis* growth curve in media containing different concentration of ferric pyrophosphate and L-cysteine

2.3.8 BMFC promotes efficient growth of *Francisella philomiragia and Francisella tularensis* subsp. *tularensis, holarctica,* and *novicida*

In order to test the suitability of the BMFC media for the cultivation of *Francisella* strains, an additional 18 strains were obtained from BEI Resources (Manassas, VA, USA), and used in this experiment. A total of 22 strains were evaluated in BMFC media, including thirteen *Francisella novicida* strains, four *F. t. holarctica* strains, two *F. t. tularensis* strains, and three *F. philomiragia* strains (Figures 2.14, 2.15, 2.16, and 2.17). These strains represent a broad cross-

section of *Francisella* subspecies. All of the bacterial cultures were sufficiently turbid within 12 h of inoculation and incubation at 37^{o}C. BMFC media, with the final formulation of 1X BHI, 1X MHII, 0.25 mg/ml ferric pyrophosphate, and 0.125 mg/ml L-cysteine appears to be an excellent media for growth of all *Francisella* strains tested.

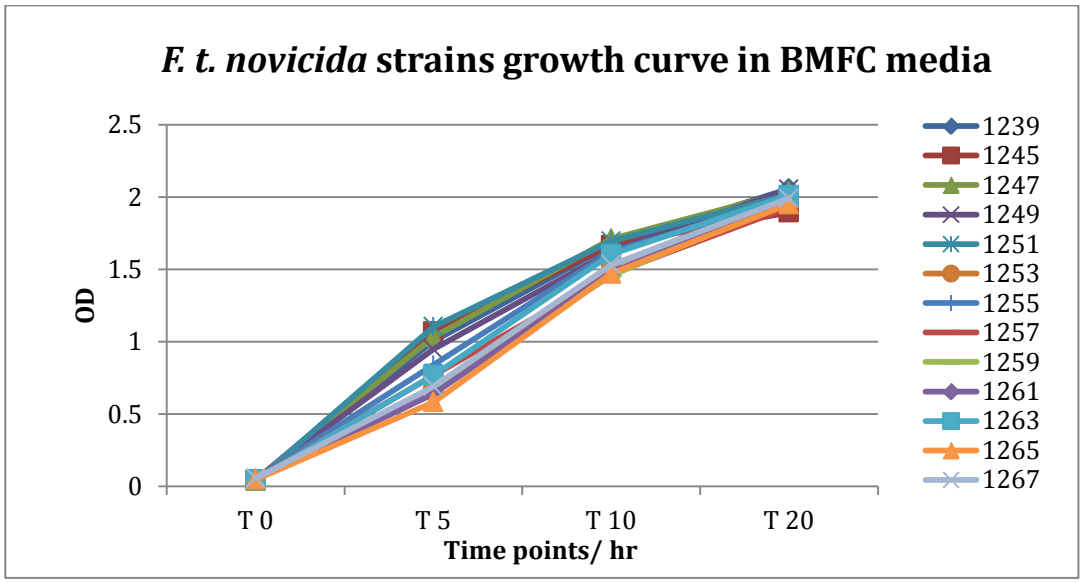

Figure2.14: *F. t. novicida* strains growth curve in BMFC media

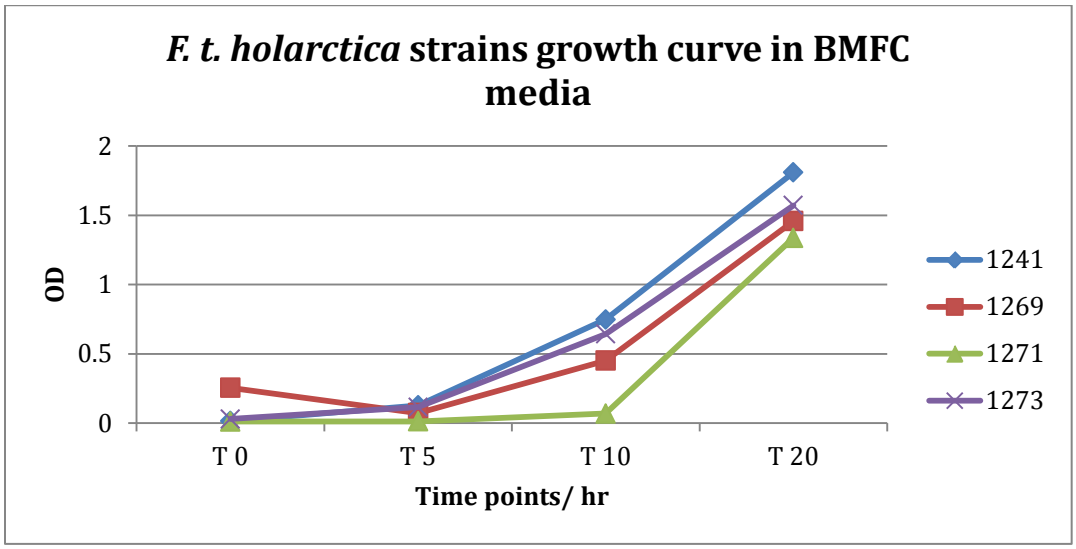

Figure2.15: *F.t. holarctica* strains growth curve in BMFC media

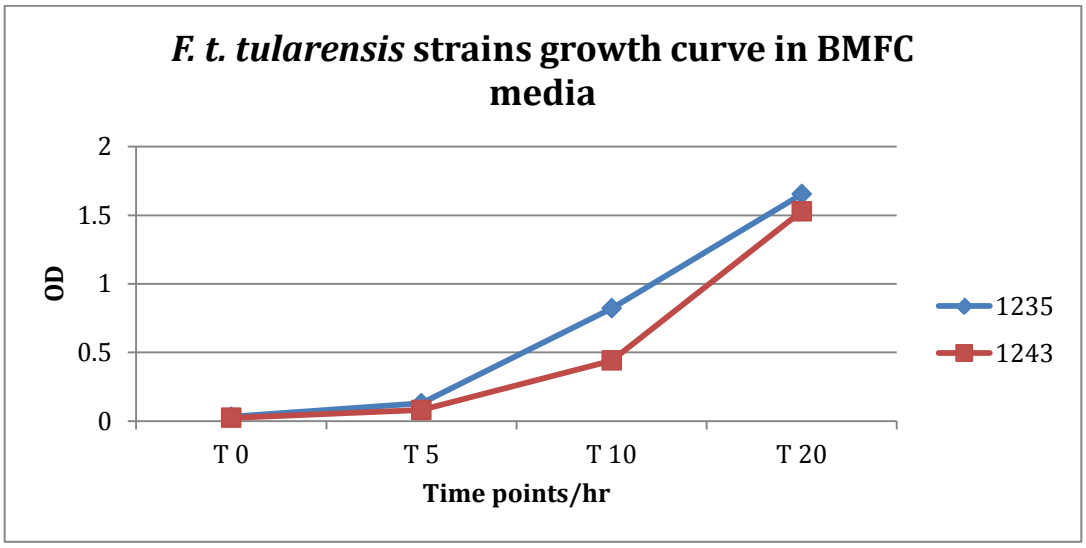

Figure 2.16: *F.t. tularensis* strains growth curve in BMFC media

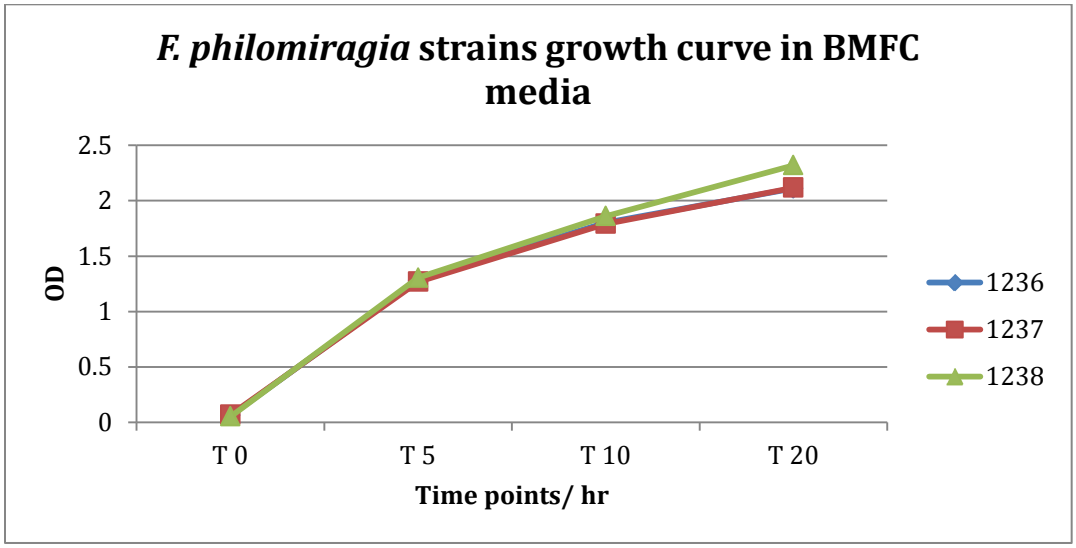

Figure 2.17: *F. philomiragia* strains growth curve in BMFC media

2.3.9 BMFC is a better growth media than BHIc for three selected *Francisella* strains

Francisella tularensis LVS, *F. t. novicida* U112, and *F. t. tularensis* NR-50 were used in this experiment as model organisms for *Francisella* cultivation. The growth rates of these

organisms were compared using BMFC media and BHIc (BHI supplemented with cysteine-see table 2.4) that was described by Mc Gann et al in 2010. Chamberlain's defined media was eliminated due to the growth delay that occurred in the overnight cultures. *F.t. novicida* U112 reached an OD_{600} of 1.5 after 30 h of incubation in BMFC broth but it reached an OD_{600} of 1.3 at the same time point in BHIc broth (Figure 2.18). *Francisella tularensis LVS* reached the highest OD_{600} of 1.3 after 32 h of incubation in BMFC while it reached 0.5 in BHIc media at the same time (Figure 2.19). *F, tularensis* NR-50 is the most fastidious strain of the three organisms. This organism reached an OD_{600} of 1.2 within 30 h of growth and it failed to grow in the BHIc media (Figure 2.20).

In addition, in this experiment, bacteria were grown on agar media and transferred to broth that contained a different source of iron. Switching the iron source from ferric pyrophosphate to heme-histidine (BMFC agar to BHIc broth) or vice versa did not have a major impact on *F.t. novicida* U112 growth and overall, *F. t. novicida* U112 grew better in BMFC broth as compared to BHIc broth. The same growth pattern was observed with *F. tularensis* LVS under these conditions. Unlike *F. tularensis* LVS and *F.t. novicida* U112, *F. t. tularensis* NR-50 grew well when the iron source was switched from heme-histidine to ferric pyrophosphate (BHIc agar to BMFC broth) and did not grow vice versa (BMFC agar to BHIc broth). All three *Francisella* strains displayed superior growth rates when grown in BMFC broth, probably because of the additional nutrients supplied by MHII media and the ferric pyrophosphate.

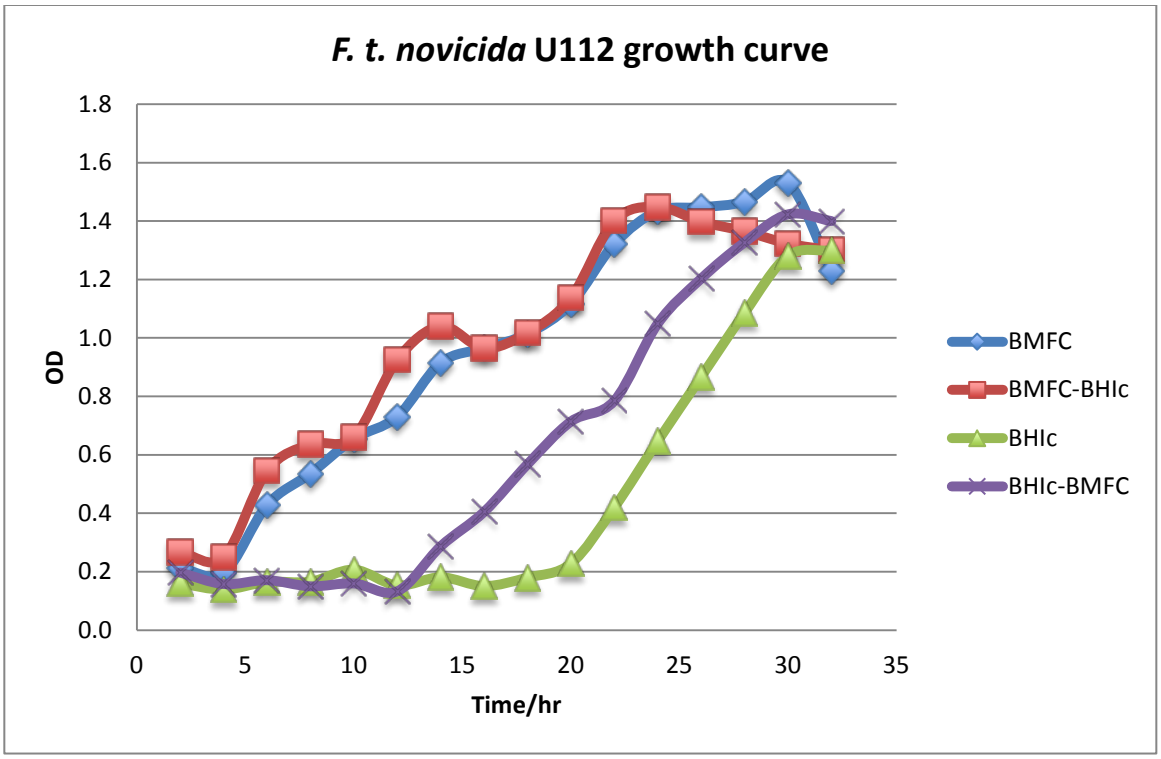

Figure2.18: *F. novicida* U112 growth curves grown in four different combinations of media. BMFC agar to BMFC broth (BMFC), BMFC agar to BHIc broth (BMFC-BHIc), BHIc agar to BHIc broth (BHIc), BHIc agar to BMFC broth (BHIc-BMFC).

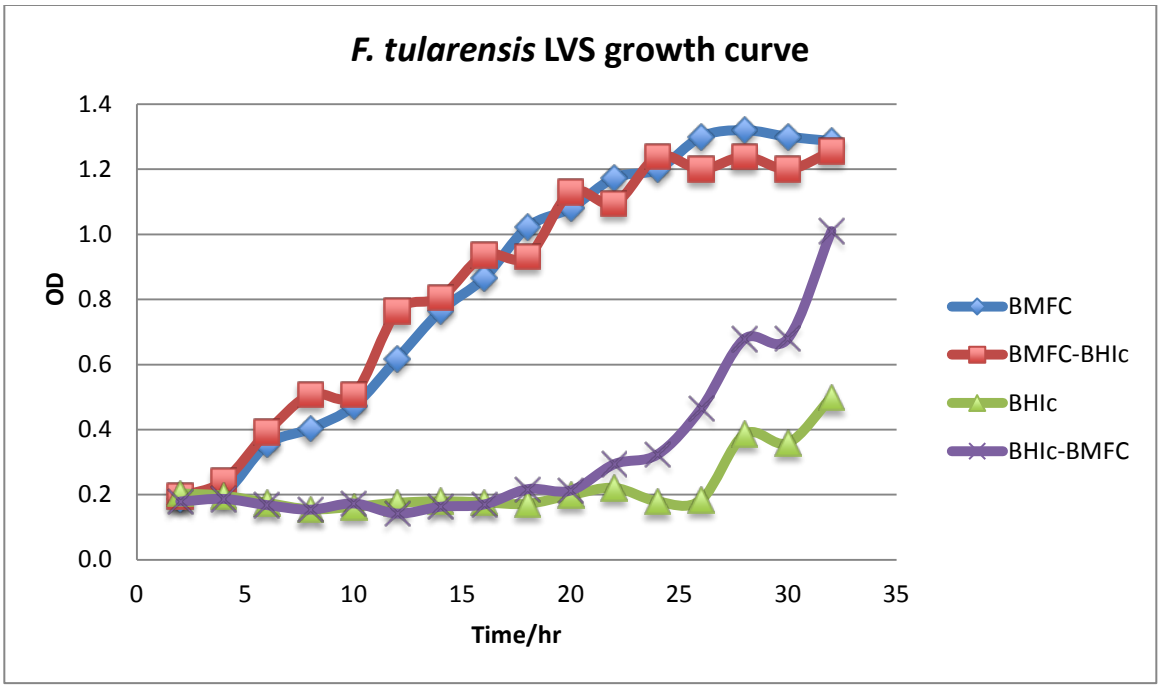

Figure2.19: *F. tularensis* LVS growth curve growth curve in four different media BMFC agar and broth(BMFC), BMFC agar to BHIc broth (BMFC-BHIc), BHIc agar and broth (BHIc), BHIc agar to BMFC broth(BHIc-BMFC).

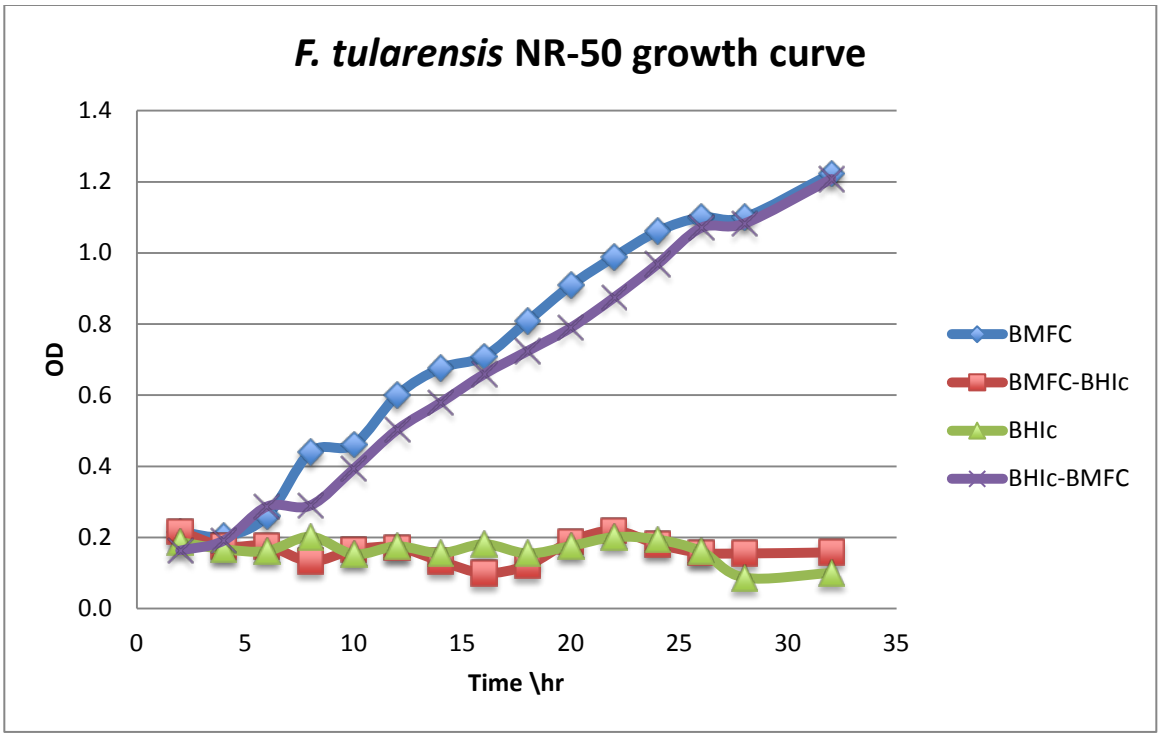

Figure2.20: *F.tularensis* N-50 growth curves grown in four different combinations of media. BMFC agar to BMFC broth (BMFC), BMFC agar to BHIc broth (BMFC-BHIc), BHIc agar to BHIc broth (BHIc), BHIc agar to BMFC broth (BHIc-BMFC).

2.4 Discussion

When dealing with fastidious organisms like *Francisella*, the absence of specific nutrients can lead to the loss of the bacterial culture. Therefore, in our laboratory we devoted time and effort to improve a standard formulation to enhance *Francisella* growth and reduce costs and cultivation time. In this chapter we introduced a novel Mueller Hinton and brain heart infusion base media that provides good growth for *Francisella* strains. Since *Francisella tularensis* LVS and *F. t. novicida* U112 can be cultured and manipulated in BSL2 facilities, they represent the most common strains used in *Francisella* studies.

A literature review showed that Mueller Hinton broth supplemented with IsoVitaleX is the preferred media for *Francisella* cultivation. Using this approach, we began with this formula and added bovine hemoglobin or rabbit blood, since many intracellular pathogens require iron in their metabolism. Deng and co-workers demonstrated that iron limitation directly affects expression of virulence genes in *F. tularensis* (Deng et al., 2006). In their study, they used ferric pyrophosphate as the form of iron in their media. Based on that, we tested *Francisella tularensis* LVS growth on media containing ferric pyrophosphate side by side with media containing bovine hemoglobin. *Francisella tularensis* LVS *growth* was better on media containing ferric pyrophosphate because it is less complex than hemoglobin thus readily available for bacterial metabolism.

Hazlett et al showed in their study that growth of *F.tularensis* in brain heart infusion media mimics gene expression that occurs in *F. tularensis* grown in macrophages. Based on these observations, we decided to use a mixture of brain heart infusion and Mueller Hinton media as our base media. *Francisella* strains tended to grow faster and reached log-phase in a shorter incubation time than with other formulation containing only BHI or MHII. L-cysteine and glucose are the major components of IsoVitaleX enrichment mix. *Francisella tularensis* LVS growth rate was not affected by the absence of glucose since it is available in brain heart infusion media.

Based on a review by Peterson et al, L-cysteine is an absolute requirement for *Francisella* growth. A series of experiments were performed in our laboratory to determine whether L-cystine or L-cysteine was more suitable for *Francisella* growth and the concentration required. L-cystine is basically two molecules of L-cysteine bound by a disulfide bond. In our experiments, either one of these amino acids is acceptable for *Francisella* growth.

The addition of ferric pyrophosphate and L-cysteine to the BHI and MHII media, resulted in a final formulation with a pH of 7, unlike the formulation developed by Hazlett and co-workers where the pH needed to be adjusted to support good growth. The finalized formula of BMFC media is 1X BHI, 1X MHII with a final concentration of 0.25 mg/ml ferric pyrophosphate and 0.125 mg/ml L-cysteine. This media produces superior growth for all *Francisella* strains used in our laboratory. *Francisella* growth on solid media usually takes from 48 to 72 h in media that has been routinely employed (Ellis et al., 2002). It took only 24 h for visible colonies to appear on BMFC agar and 12 h is enough time to reach turbidity in broth.

Different strains of *Ftrancisella* were grown on BMFC, highlighting the suitability of this media for use across the spectrum of *Francisella* research. To evaluate this possibility, three model organisms including *Francisella tularensis LVS, F.t. novicida U112,* and F. t. *tularensis* NR-50 were selected to compare BMFC and BHIc (the latter media was recently developed by Mc Gann et al). To provide a more comprehensive evaluation, the source of iron was switched from heme-histidine to ferric pyrophosphate (by initial growth on agar and inoculation into broth containing an alternate iron source) was tested as well to see if that would delay the bacterial growth. These strains grew more rapidly on BMFC as opposed to BHIc media, probably because BMFC contains MHII with all its essential nutrients, in addition to BHI. While switching the iron source revealed some interesting results, both *F. tularensis* LVS and *F. t. novicida* U112 grew better when cultivated first on ferric pyrophosphate-supplemented solid media and then transferred to heme-histidine supplemented broth (BMFC agar to BHIc broth) and not vice versa. Similar results were found when *F. tularensis* LVS grown on BMFC agar was switched to BMFC broth (BMFC agar to BMFC broth). Interestingly, regardless of the source of iron in the broth, *F. t. tularensis* NR-50 revealed the same growth pattern and reached a final optical density

of 1.3 at 32 hours post-inoculation. However, the growth of *F. t. tularensis* NR-50 was significantly affected by the iron source in the initial agar media. These data emphasize the significant differences among these strains that reflect differences in broth formulation and subsp. type. Both *F. tularensis* LVS and *F. t. novicida* U112 revealed comparable growth rates when grown on BMFC agar-BMFC broth or BMFC agar-BHIc broth, as well as when grown on BHIc agar-BHIc broth or BHIc agar-BMFC broth. On the other hand, *F. t. tularensis* NR-50 displayed comparable growth when grown on BMFC agar-BMFC broth and BHIc agar-BMFC broth. *F. tularensis* LVS and *F. t. novicida* U112 and *F. t. tularensis* NR-50 may undergo bacterial metabolic adaptation that needs to be studied further.

The use of standard growth media such as BMFC for the cultivation of various *Francisella* species would benefit researchers since it is affordable, easy to prepare, and produces more rapid growth as compared to existing media. Our data clearly shows that BMFC is the optimal media for *Francisella* cultivation.

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Chapter 3

Isolation and characterization of bacteriophage ASC10, a novel temperate bacteriophage active against *Francisella tularensis* strains

3.1 Introduction

F. tularensis ssp. *tularensis* (type A) and *F. tularensis* ssp. *holarctica* (type B) are the most virulent strains associated with human tularemia. These pathogens have a wide host range and can be transmitted by direct contact with diseased animals, by inhalation or ingestion of contaminated food or water, and by tick bite. These organisms can persist for a long time in moist environments like mud, water, and animal carcasses.

Francisella tularensis strains are generally beta-lactam antibiotic resistant. Due to its potential use for bioterrorism, antibiotic resistance remains a big concern. Studies on phages as a therapeutic alternative to control bacterial infection have increased recently because of the emergence of bacterial resistance to antimicrobial agents.

Despite the proven virulence of *F. tularensis*, there is limited research on bacteriophages active against this organism. A review of the literature done by Canchaya et al in 2003 showed that 70% of sequenced bacterial genomes contain prophage elements within the bacterial chromosome. Based on this information, our hypothesis was that one or more of the *Francisella* strains may contain prophage within its chromosome.

In this chapter, we describe the isolation and characterization of a novel temperate bacteriophage active against *Francisella tularensis* strains. The bacteriophage, designated ASC10, was isolated from *F. tularensis* ssp. *novicida* lysogen by mitomycin C induction. The bacteriophage was named ASC10 based on the first names of the three lab members that

discovered this phage (Abeer Alharby, Sheridan Potter, and Claudia Gentry-Weeks) and the year of discovery (2010). Phage ASC10 is a member of the *Siphoviridae* family and forms plaques on different *Francisella* strains including *F. tularensis* subsp. *tularensis* strain MA00-2987, *F. tularensis* subsp. *holarctica*, strain K499-3387 and *F. tularensis* subsp. *tularensis* Schu S4 as well as the less virulent *Francisella* strains, *F. tularensis* subspecies *holarctica* strain LVS, *F. t. novicida* U112 and derivatives, and *F. philomiragia.*

In addition to the isolation and characterization of ASC10 in this chapter, we investigated the influence of physicochemical parameters on phage production and stability.

3.2 Materials and Methods

3.2.1 Isolation of phage ASC10 from lysogenic Francisella strains and identification of indicator strains

BMFC media was prepared as described previously in table 2.1 in broth and agar form. For phage and plaque formation, BM base media containing 0.8% agar was used for the upper layer of the overlay and supplemented with ferric pyrophosphate and L-cysteine to reach final concentration of 0.25 mg/ml and 0.125mg/ml, respectively. The supplements were added just before pouring the top layer in the overlay process. *Francisella* strains used in these experiments have been listed previously in table 3.2. Each strain was grown in BMFC at 37°C and 200 rpm until they reached late logarithmic phase (OD_{600} of 0.4). Mitomycin C was added to a final concentration of 0.5 μg/ml in the bacterial culture, followed by incubation for an additional 20 h (i.e. 24 h post inoculation). To prepare the phage lysate, bacterial cultures were passed through a 0.22 μm syringe filter (MILLEX-GP, Millipore Express PES membranes, Millipore Ireland Ltd.,

Cork, IRL). To identify indicator bacteria, the strains were grown overnight at 37°C with agitation (200 rpm) and then tested as indicators in the overlay process. Plaque assays were performed as described in Erauso et al., with slight modifications. For the top layer, 4 ml of BM media was prepared with 0.8% agar, autoclaved, and subsequently molten and dispensed into tubes in 50^{o}C heat-blocks. The supplements (50 µl of a mixture of 1ml of L-cysteine and 4 ml of ferric pyrophosphate) was added to 4 ml soft agar, 250 µl of the indicator cells, and 500 µl of phage filtrate and the mixture was poured over 20 ml of supporting BMFC agar. The plates were incubated at 37°C for 12 hours and observed for phage plaques. This entire experiment was performed three times for confirmation.

3.2.2 Host range

In order to determine the specificity of this phage, more indicator strains need to be tested, especially those bacteria closely related to *Francisella tularensis*. *F.t.novicida* strain NR-575 was selected as the lysogen for induction since it consistently produced phage upon induction. A list of bacterial strains used as indicators in this experiment is presented in table 3.2. The plaque assay was performed as described in the previous experiment except that lysogenic bacterial cells were removed from the lysate by centrifugation at 10,000 x g at 4°C for 15 min instead of by filtration. *Francisella* strains were tested for their phage sensitivity. For experiments with *F. tularensis* subsp. *tularensis* Schu S4, *F. tularensis* subsp. *tularensis* strain WY96-3418, *F. tularensis* subsp. *holarctica*, strain OR96-0246, *F. tularensis* subsp. *tularensis* strain MA00-2987, and *F. tularensis* subsp. *holarctica*, strain K499-3387, all work was performed in the CSU BSL3 facility using approved standard operating procedures. Select gram-

negative and gram-positive bacteria as well as *Mycobacteria* strains were tested for phage sensitivity.

3.2.3 Improved phage production and plaque formation

3.2.3.1 Determination of the optimum time for collecting phage lysate after mitomycin C induction

Based on the previous results (Table 3.1), *F.t. novicida* NR-575 and NR-584 were selected for use as the lysogen and indicator strains, respectively, in our experiments due to their relative consistency in phage production and plaque formation. A literature review showed that bacteria were collected a short time after induction with Mitomycin C to avoid continuous exposure to the mutagen. To test if continuous exposure to Mitomycin C affected bacterial growth and phage production, *F.t. novicida* NR-575 were grown in BMFC broth at 37°C with agitation (225 rpm) until the culture reached an OD_{600} of 0.3. Mitomycin C was then added to a final concentration of 0.5 µg/ml. Aliquots were collected every two hours for the first twelve hours and then at 16, 24, and 48 h post induction. Chloroform (1%) was added to each aliquot to lyse the bacterial cells, followed by centrifugation at 10,000 rpm for 10 min. Phage lysates were then added to the soft agar using *F.t. novicida* NR-584 as the indicator in the overlay. Plates were incubated at 37°C for 24 h and observed for plaques.

In another experiment, bacterial cells were centrifuged after one hour of mitomycin C induction, and the cell pellet was suspended with fresh BMFC broth, and the incubation continued overnight. The phage lysate and the plaque assay were performed as described previously.

3.2.3.2. Examination of mitomycin C stability when suspended with milliQ-purified water and stored at 4°C

Mitomycin C is an antitumor agent used to inhibit DNA synthesis, eukaryotic nuclear division, and induce prophage from bacterial lysogens. Mitomycin C (MMC) was obtained from Thermo Fisher Scientific and was packaged in small amber glass vials containing 2 mg of lyophilized powder. Sterile milli-Q water (1ml) was used to suspend the mitomycin C. It barely dissolved in water and precipitated over time. Plaques were obtained when a fresh suspension of mitomycin C was used, but no plaques were detected after the mitomycin C had been stored for greater than one month at 4°C. Therefore, four vials of mitomycin C that had been suspended in milli-Q water on different dates were tested for their ability to induce λ phage from *E. coli* K-12 lysogens, using *E. coli* LE392 as the indicator strain. Vials were numbered from 1 to 4, corresponding from the earliest date to the latest date of suspension. Four cultures of *E.coli K-12* were grown in LB broth at 37°C at 225 rpm until they reached log phase. Mitomycin C (0.5 μg/ml final concentration) was added to the cultures and they were incubated for 2 h. One ml aliquots from each culture suspension were filtered through a 0.22 μm filter. Eight-fold serial dilutions were made from each filtrate and 10 μl was spotted on soft agar overlays containing *E. coli* LE392, incubated overnight at 37°C, and observed for plaques.

3.2.3.3 Induction of phage ASC10 using phleomycin and hydrogen peroxide

In a research article by Heinemann and Howard, different antitumor agents were used to induce λ from *E-coli* K-12 and they correlated the induction efficiency with the antitumor activity of these agents. Therefore, phleomycin and hydrogen peroxide (H_2O_2) were tested in this experiment to see if they would increase phage production. To do this, 13 *F. t. novicida* strains were grown overnight in BMFC media at 37°C, 225 rpm. 50 µl of each of the 13 strains were added simultaneously to three flasks of 25 ml BMFC and incubated at 37°C, 225 rpm for 4 h. The first flask was treated with 0.5 µg/ml Mitomycin C, the second flask with 0.2 µg/ml phleomycin and the third one with 0.001% H_2O_2 and incubation was continued for 20 h. Bacterial cells were then removed by filtration through 0.22 µm pore-size filters. Phage lysate from each treatment was used in a plaque assay using *F.t. novicida* NR-584 as an indicator strain. Overlay plates were incubated at 37°C for 24 h for plaque count.

3.2.3.3. Improved phage production by limiting mutation rate for lysogenic cells

Mutations occur naturally during cell division. For example the mutation rate of *E. coli* is estimated to be 5.4×10^{-10} per nucleotide per replication. However, there is no research that has investigated the mutation rate for *Francisella* strains. Since we are dealing with prophage with its DNA integrated into the chromosome, any mutation that occurs in the bacterial chromosome could have an effect on the prophage biology and production. To test whether the *Francisella* mutation rate affects phage production, two sets of freezer stocks with different passages were made from the *Francisella* strains that were obtained from BEI Resources in Table 3.1. Each

individual strain was streaked on BMFC agar and incubated for 24 h at 37°C. A single colony was inoculated into BMFC broth and incubated at 37°C for 225 rpm for 24 h. An aliquot of the overnight culture (750 µl) was mixed with 750 µl of 80% glycerol to make the first passage frozen stock (designated 'A'). The same steps were repeated starting with the 'A' strains to make the second passage frozen stocks (designated 'B'). Plaque assays were performed using BEI 'A' and 'B' passaged stocks to see if the number of passages would affect phage production. The phage lysate and plaque assay were prepared as described previously.

3.2.4. Purification and concentration of phage particles

In order to determine the morphology and nucleic acid type for phage ASC10, large-scale bacteriophage production was carried out as follows. Five ml of *F.t. novicida* NR-575 overnight culture was added to 500 ml of BMFC broth and incubated at 37°C with shaking speed of 150 rpm for 4 h, until the culture reached an OD_{600} of 0.4. Mitomycin C was added to a final concentration of 0.5µg/ml followed by further incubation for 20 h. Two percent of chloroform was added to the bacterial culture and incubated at room temperature for 20 min. Cell debris were collected by centrifugation at 10,000 x g, 10 min, at 4°C. The supernatant fluid was collected and treated with 1µg/ml of DNAse and RNAse for 30 min at room temperature. Sodium chloride was added to the supernatant fluid to give a final concentration of 1 M and kept on ice for one h. The precipitate was collected by centrifugation at 10,000 x g, 15 min, at 4°C. The supernatant was transferred into sterile flasks and 10% PEG8000 (weight/volume) was added and incubated at 4°C for 24 h. The phage particles were collected by centrifugation twice at 10,000 x g, 15 min, at 4°C. The precipitated pellet was suspended in 3 ml of TM buffer (10

mM Tris-HCl, 10 mM MgSO4, pH 7.5). Chloroform (3ml) was added to the phage suspension, mixed and centrifuged at 3000 x g for 15 min, 4°C. The aqueous phase was removed and layered onto a performed cesium chloride step gradient consisting of 4 layers with densities of 1.3, 1.4, 1.5, and 1.7 g/cm^3 in TM buffer, followed by ultracentrifugation at 28,000 rpm for 5 h at 4°C, using the SW-41 Ti Beckman rotor. The bluish phage band was observed at a density close to 1.5 g/cm^3 and it was withdrawn by insertion of a 16-gauge needle through the centrifuge tube wall below the band. The phage band was dialyzed at 4°C for 24 h against 4 L of TM buffer to remove salts and small debris. The purified phage sample was used to determine phage morphology by electron microscopy.

3.2.4.1. Transmission electron microscopy

Dialyzed phage (5 µl) was placed on 200 mesh-formvar/ carbon-coated copper grids for 10 min. The absorbed phages were stained with 2% aqueous uranyl acetate for one min. The grids were then examined using a Hitachi H-7000 transmission electron microscope (TEM) at the University of Wyoming, Laramie, Colorado.

3.2.5. Use of different purified phage preparations in TEM examination

The morphology of the phage still needed to be determined. To avoid exposure of phage to chloroform and the damage caused by it, three methods were used for TEM. First, plaques obtained from previous plaque assays were picked up and suspended in TM buffer. The suspension was passed through a 0.22 µm filter to eliminate bacterial cell debris and stored at

4°C for TEM examination. Second, plaques were picked up and suspended in TM buffer but cell debris was pelleted by centrifugation at 10,000 x g, 15 min, at 4°C. In the third method, 50 µl of TM buffer was placed in the center of plaques obtained from previous overlays. Two hundred mesh formvar carbon-coated grids were placed on top of the buffer drop and left for two minutes to allow absorption of phage particles. Grids were stained with 2% uranyl acetate for one min. Excessive stain was removed with filter paper and grids were allowed to air dry. The grids were directly used for viewing in the TEM. Grids prepared by these three methods had been rendered hydrophilic by glow discharge to enhance absorption. The same staining technique was followed and grids were examined with an FEI Phillips CM100 TEM at the University of Colorado, Boulder, CO

3.2.5 Extraction of phage DNA

In addition to morphology, the nature of the nucleic acid is important to classify a novel phage. In this experiment, 13 strains of *F. t. novicida* (listed in table 3.1) were cultivated overnight in BMFC at 37°C at 225 rpm. An aliquot of 384 µl of each overnight culture was inoculated into 500 ml of BMFC broth and incubated at 37°C, 115 rpm, for 4 h. The bacterial culture reached an OD_{600} of 0.3, and 0.5µg/ml of Mitomycin C (final concentration) was added to the mixed culture and incubation was continued for an additional 20 h. Cell debris was eliminated by centrifugation at 10,000 x g for 15 min at 4°C. Supernatant fluid was treated with DNase and RNase at a final concentration of 1µg/ml and kept at room temperature for 30 min. Phage particles were concentrated by adding 10% polyethylene glycol 8000 (weight/volume) and 0.5 M NaCl and the phage lysate was slowly stirred for 1 h at room temperature. Precipitate was

collected by centrifugation at 10,000 x g for 20 min at 4°C. Supernatant fluid was discarded and the precipitated pellet was suspended in 5 ml 10mM Tris-HCl pH 8. Phage DNA was extracted using a standard phenol-chloroform procedure (Maniatis et al., 1982). The resulting DNA pellet was dissolved in 1ml TE buffer (10mM Tris-HCl, 1mM EDTA pH 8) and stored at -20°C.

Phage DNA was digested with *EcoRI*, *Hind*III, *BamHI* and *ClaI* restriction enzymes for 1h at 37°C. Restriction enzymes were inactivated by incubation for 20 min at 65°C. The uncut DNA and the digested fragments were subjected to electrophoresis on a 0.5% agarose gel in TBE (Tris, borate, EDTA) buffer. DNA standards, fragments of *Hind*III/ bacteriophage λ and a 1kb DNA ladder were used as molecular weight markers. To estimate the genome size of the phage, the gel was stained with ethidium bromide and the DNA bands were viewed with a standard UV transilluminator.

3.2.6 Phage protein analysis

Structural phage proteins were analyzed using sodium dodecyl sultate-polyacrylamide gel electrophoresis (SDS-PAGE). Two phage lysate samples were used in this procedure. The first sample was obtained from the induction of a mixed culture of *F. t. novicida* strains (13 strains) and the other was obtained from induction of *F. t. novicida* NR-575 culture (500 ml). Bacterial strains were grown and induced with mitomycin C as described previously and bacterial cell debris was removed by centrifugation at 10,000 x g for 15 min at 4°C. Each phage lysate was treated with 1μg/ml of DNase and RNase and 0.5 M NaCl and incubated for 1 h at 4°C. The bacterial debris was eliminated by centrifugation at 8000 x g for 10 min at 4°C. PEG6000 was added to the supernatant to final concentration of 10% (weight/volume) and dissolved by slow

stirring at 4°C for 1 h. Phages were precipitated by centrifugation at 10,000 x g for 15 minute at 4°C and the supernatant fluid was discarded. The phage pellet was suspended in 4 ml of phage buffer (20 mM Tris-HCl PH7.4, 0.1 M NaCl, 10m M $MgS0_4$), and kept at 4°C overnight. PEG was extracted by adding an equal volume of chloroform for 1 min, followed by centrifugation at 5000 x g for 15 min. The phage-containing aqueous phase was removed and used for protein analysis. SDS-PAGE of purified phage proteins was performed by using a mini gel system. A gradient mini gel (Novex by Life Technologies, Grand Island, NY) was used to resolve phage proteins within a large range of molecular weights. Samples were prepared and loaded as described in the NuPAGE® Bis-Tris Mini Gels instruction sheet. The Novex® sharp protein standard was used as the molecular weight markers. Samples were subjected to electrophoresis at 150 V for 45 min. The gel was stained with Acqua Stain (Portsmouth, NH) for 15 min with gentle shaking and protein bands were visualized.

3.2.7 Phage stability at different temperatures

A phage ASC10 lysate was prepared as described previously. An aliquot of 3 ml of the phage lysate was added to 7 glass tubes and each tube was incubated for 1 hour at 30, 40, 50, 60, 70, 80, and 90°C. These samples were used in a plaque assay using the *F. t. novicida* NR-584 indicator strain. Overlay plates were incubated at 37°C for 24 h. In addition, phage lysate was stored at 4°C for 12 months and was used in a plaque assay to test phage viability over time.

3.3 Results

3.3.1 Presence of temperate phage in several *F. tularensis* ssp. *novicida* genomes

Prophages were obtained from *F. t. novicida* strains NR-573, NR-574, NR-575, NR-577, NR-580, NR-581, and NR-582 by mitomycin C induction indicating that these are lysogenic bacterial strains. Big turbid plaques were observed on strains listed in table 3.1. (Figure 3.1 A & B). To confirm these results, plaques were stabbed with a sterile toothpick and used to streak lines on soft agar containing the same indicator bacteria and incubated overnight. On the indicator lawn, clear zones surrounding the plaque lines were detected, confirming phage lysis.

Table 3.1: List of *Francisella* lysogen and indicator strains

Lysogen (contain ϕ)	Host (sensitive to the ϕ)
NR-573 *F. novicida*	NR-50 *F. t. tularensis,* NR-582 *F. novicida,* NR-583 *F. novicida*
NR-574 *F. novicida*	NR-13 *F. novidica,* NR-581 *F. novicida,* NR-582 *F. novicida,* NR-583 *F. novicida*
NR-575 *F. novicida*	NR-13 *F. novidica,* NR-574 *F. novicida,* NR-577 *F. novicida,* NR-578 *F. novicida,* NR-580 *F. novicida,* NR-581 *F. novicida,* NR-583 *F. novicida,* NR-584 *F. novicida,* NR-585 *F. t. holarctica*
NR-577 *F. novicida*	NR-13 *F. novidica,* NR-580 *F. novicida,* NR-584 *F. novicida*
NR-580 *F. novicida*	NR-13 *F. novidica,* NR-14 *F. t. holarctica,* NR-579 *F. novicida,*
NR-581 *F. novicida*	R-574 *F. novicida,* NR-580 *F. novicida*
NR-582 *F. novicida*	NR-574 *F. novicida,* NR-578 *F. novicida*

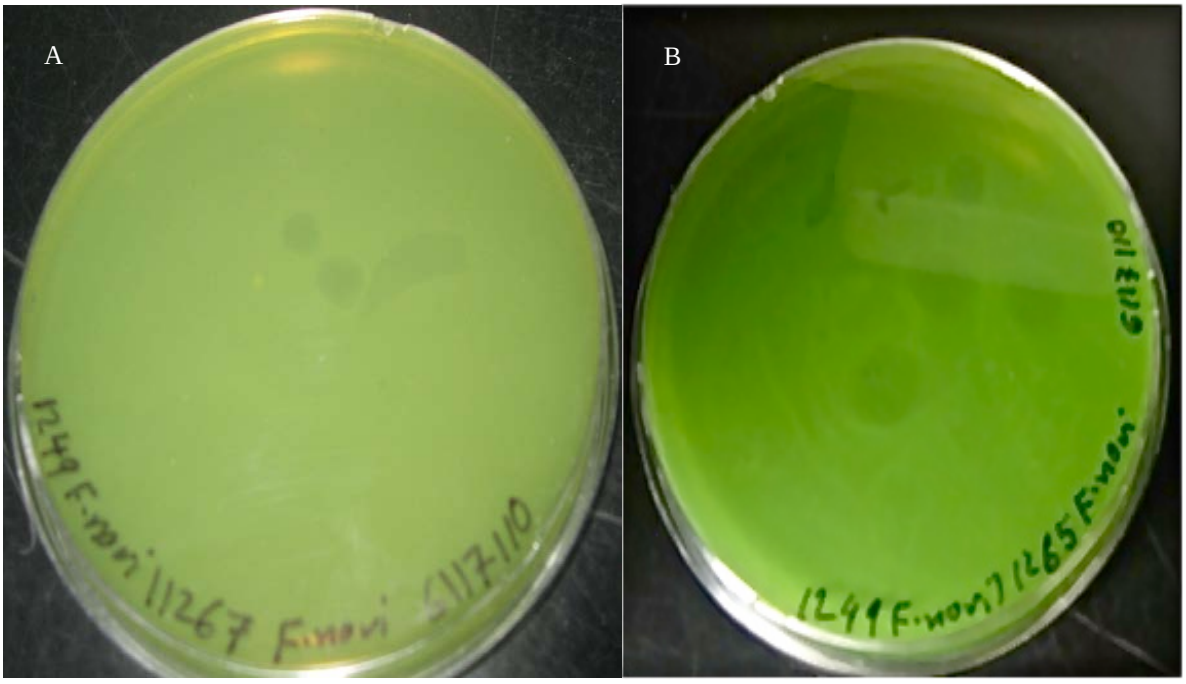

Figure 3.1: Plaques from *F. t. novicida* lysogen strain NR-575 on (A) F. t. novicida NR-584 and (B) on *F. t. novicida* NR-583.

3.3.2 ASC10 phage is specific for *Francisella* strains and active against *F. tularensis* subsp. *tularensis* Schu S4

ASC10 temperate bacteriophage was obtained by mitomycin C induction of *F. t. novicida* strain NR-575. This phage formed plaques on the different *Francisella* strains including *F. tularensis* subsp. *tularensis* Schu S4 (Figure 3.2), *F. tularensis* subsp. *tularensis* strain MA00-2987, and *F. tularensis* subsp. *holarctica*, strain K499-3387. However, plaques were not detected on other gram-negative or positive bacteria (Table 3.2)

Figure 3.2: 2 Phage plaques of ASC10 on *F. t. holarctica, F. t. novicida* and *F. tularensis* subsp. *tularensis Schu S4* indicator strains.

Table 3.2: ASC10 phage host range

***Francisella* strains**	**Plaque formation detected**	**Biosafty level**
F. t. novicida strain NR-13	+	BSL2
F. t. holarctica strain NR-14	_	BSL2
F. t. tularensis strain NR-50	_	BSL2
F. t. novicida strain NR-573	_	BSL2
F. t. novicida strain NR-574	+	BSL2
F. t. novicida strain NR-575	_	BSL2
F. t. novicida strain NR-576	_	BSL2
F. t. novicida strain NR-577	++	BSL2
F. t. novicida strain NR-578	++	BSL2
F. t. novicida strain NR-579	_	BSL2
F. t. novicida strain NR-580	+	BSL2
F. t. novicida strain NR-581	+	BSL2
F. t. novicida strain NR-582	+	BSL2
F. t. novicida strain NR-583	++	BSL2
F. t. novicida strain NR-584	+++	BSL2
F. t. holarctica strain NR-585	++	BSL2
F. t. holarctica strain NR-597	_	BSL2
F. t. holarctica strain NR-646	_	BSL2
F. tularensis subsp. *tularensis* Schu S4	++	BSL3
F. tularensis subsp. *tularensis* strain WY96-3418	_	BSL3
F. tularensis subsp. *holarctica*, strain OR96-0246	_	BSL3
F. tularensis subsp. *tularensis* strain MA00-2987	+	BSL3
F. tularensis subsp. *holarctica*, strain K499-3387	+	BSL3
E.coli LE392	_	BSL2
E. coli HB 101	_	BSL2
Yersinia pestis A1122	_	BSL2
Salmonella enterica 001-01749	_	BSL2
Listeria monocytogenes	_	BSL2
Corynebacterium pseudotuberculosis	_	BSL2
Bordetella bronchispeptica	_	BSL2
Mycobacteria smegmatis	_	BSL2
Pseudomonas aeruginosa	_	BSL2

+, plaque formation detected; _ , no plaque formation detected

3.3.3.1 *F. t. novicida* NR-575 collected at different time points after mitomycin C induction does not produce phage plaques

Mitomycin C is a prophage-inducing chemical, and is used in small concentration in lysogenic bacterial cultures. Lysogenic cells are usually collected a short time after mitomycin C treatment. In initial experiments, *F. t. novicida* NR-575 aliquots collected at different times post induction did not contain phage according to plaque assays. This may have been due to an insufficient concentration of mitomycin C during the induction process or the mitomycin C may have lost its potency over time. Therefore, another experiment was designed to test the stability of mitomycin C when stored at 4°C over time.

3.3.3.2 Mitomycin C suspended in water loses its potency over time when stored at 4°C

Mitomycin C was used in our laboratory to induce bacteriophage ASC10. Varied results were obtained using the same mitomycin C stock. *E.coli* K-12 was used to test four different vials of mitomycin C, each of which was suspended in water and stored at 4°C for over a year. The most recently suspended vial (#4) yielded 10^8 PFU/ml while the other 3 vials gave a reduced number of PFU (Figure 3.3). These results indicate that mitomycin C loses its activity when suspended in water and stored at 4°C. Technicians in the chemical division department of the Fisher company suggested that mitomycin C could be suspended with DMSO. From then on, water was substituted with DMSO and mitomycin C dissolved rapidly with no precipitation.

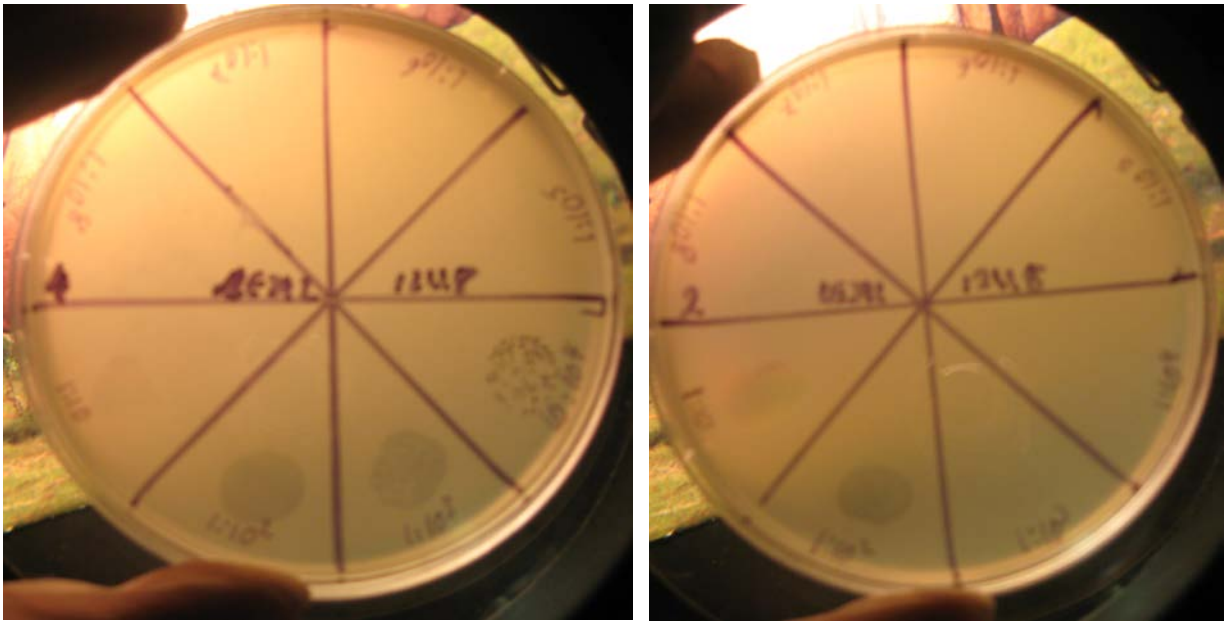

Figure 3.3: Lambda phage lysates were induced by mitomycin C, serially diluted, and plated on *E. coli* LE392. Number 2 (left photo) shows that mitomycin C lost the ability to induce phage (as visualized by plaque production) after 6 months of storage, while Number 4 (right photo) shows that induction of lambda phage immediately after suspension of mitomycin C in water results in more efficient phage induction

3.3.3.3 Phleomycin improves plaque clarity

One of the attempts to improve phage production was to test two antitumor agents, phleomycin and hydrogen peroxide, for phage induction. Phage lysates from phleomycin- and hydrogen peroxide-induced from *F. t. novicida* NR-575 were used in a plaque assay. One plaque was obtained from the phleomycin-treated bacteria. No plaques were observed by induction with hydrogen peroxide. This plaque was more clear and round than the plaques typically observed (Figure 3.4). Therefore, a final concentration of 0.2 μg/ml of phleomycin was added with mitomycin C for the induction procedures that were performed later.

Figure 3.4: Phage plaque resulting from induction with phleomycin. Note clarity of the plaque

3.3.3.3 *F. t. novicida* NR-575 mutation rate affects phage production

To see if the mutation rate of *F. t. novicida* NR-575 and the number of passages affect phage production, three frozen stocks were tested: 1) the original stock from BEI Resources, 2) 'A' strains (passage one), and 3) 'B' strains (passage two). Each stock was used induced and observed for plaques using the plaque assay. The phage lysate that was prepared directly from the BEI stock revealed a total of 20 plaques while the one prepared from the "A" stock and passaged once gave 5 plaques. No plaques were observed from the induced "B" stock that had been passaged twice (Figure 3.5). These results indicate that limiting the passage of the lysogen on growth media enhances plaque production.

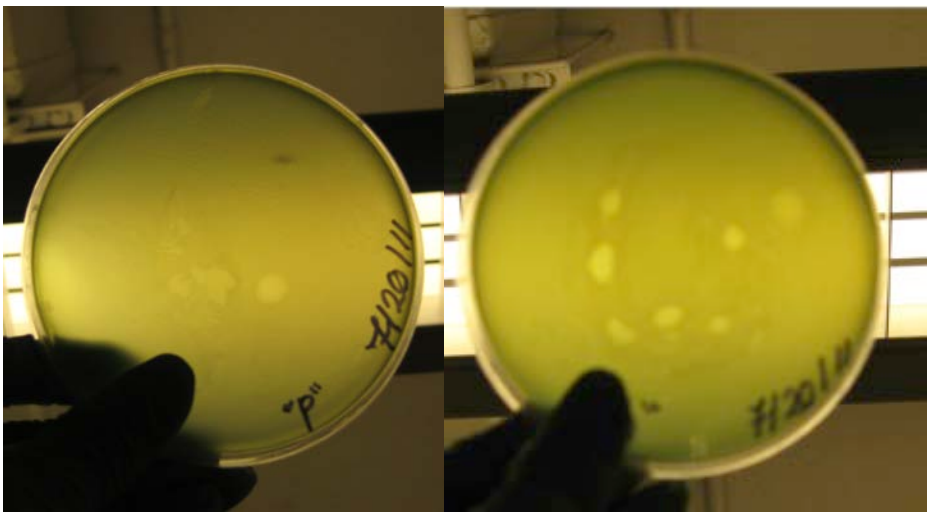

Figure 3.5: Mutation rate effect on phage production; left photo shows plaques from induced *F. t. novicida* NR575 "A" stock culture (one passage), and right photo show plaques from the induced, original BEI strain (no passages).

3.3.4 Different purification methods have a direct effect on ASC10 shape and structure

Since ASCl0 is a prophage and is produced in a low titer, large-scale purification methods were followed to obtain a sufficient amount of phage particles for characterization experiments. Concentration of phage particles from large volume phage lysates required the use of PEG, followed by chloroform extraction. The TEM image of phage particles obtained by this procedure showed that the phage structure was altered due to the use of chloroform (Figure 3.6) indicating that the use of the organic solvents such as chloroform denatured the proteins associated with the phage capsid and tail structure. Filtration of plaque suspensions through 0.22 μm pore-size filters led to phage tail breakage as observed in TEM images (Figure 3.7). On the other hand, centrifugation of plaque suspensions yielded intact phage particles with a lot of cell debris (Figure 3.8). The image of the grids that were placed in buffer in the center of plaques showed bacterial cells only, indicating that this method is not applicable in our case due to plaque turbidity. Based on the dimensions measured by TEM (Figure 3.9), bacteriophage ASC10 possessed an icosahedral head approximately 114 nm in diameter and non-contractile tail of 92 nm in length. These measurements place ASC10 into the family *Siphoviridae* based on the International Committee on Taxonomy of viruses.

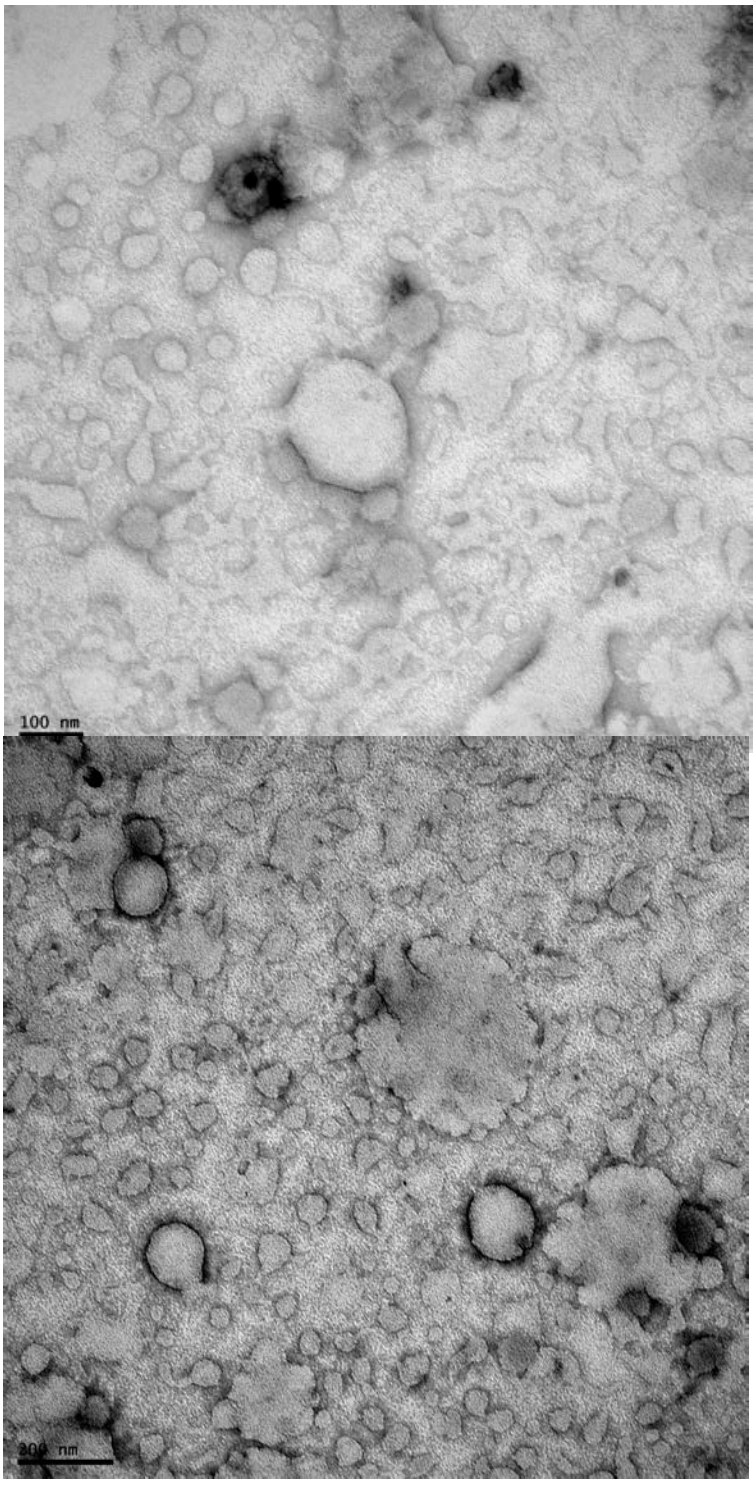

Figure 3.6: Transmission electron micrographs of bacteriophage ASC10 demonstrate the effect of chloroform on the phage structure.

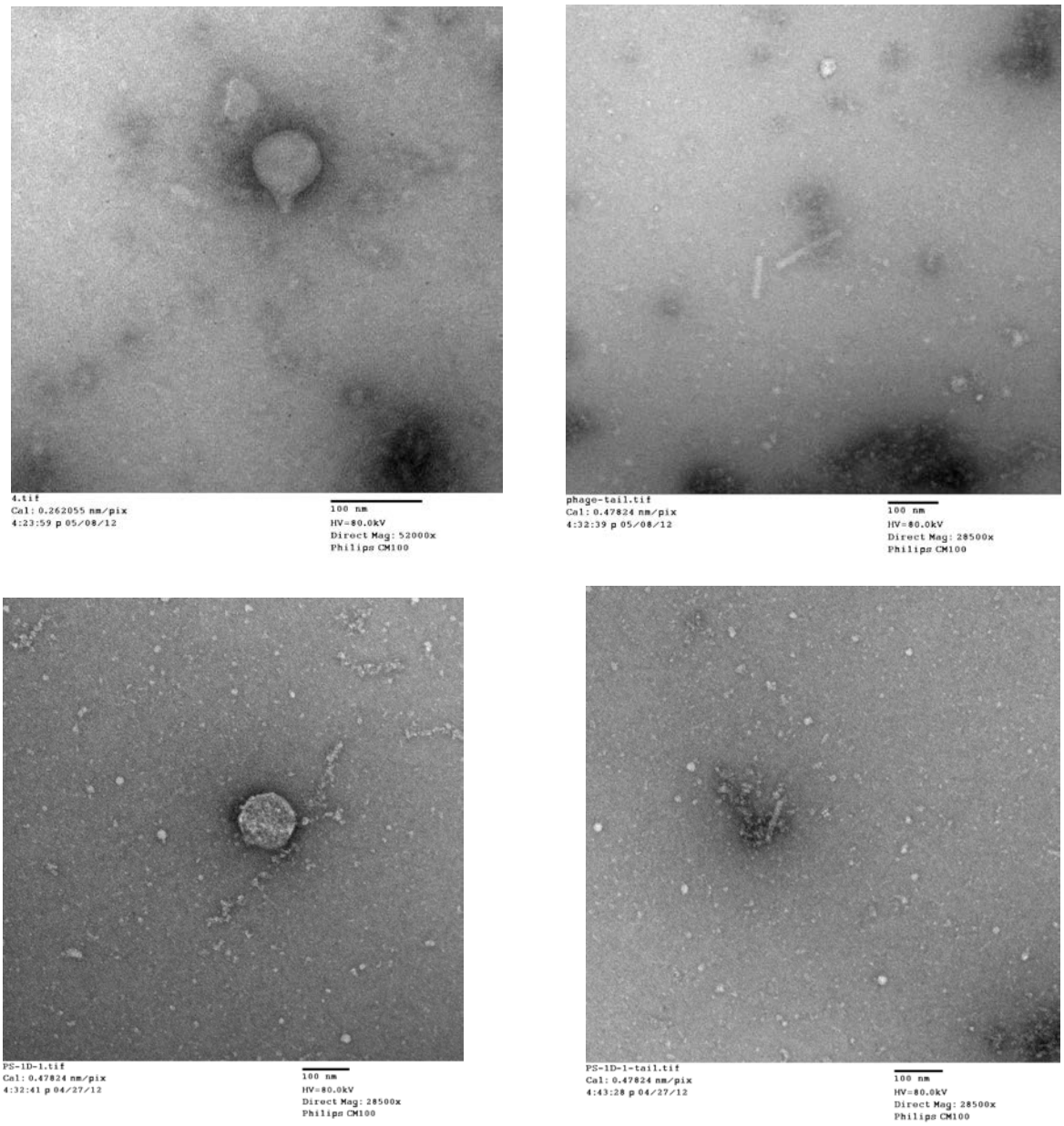

Figure 3.7: Electron microscope image of bacteriophage ASC10 showing tail breakage due to filtration. The bacteriophage was stained with 2% uranyl acetate. Final magnification is 28500X.

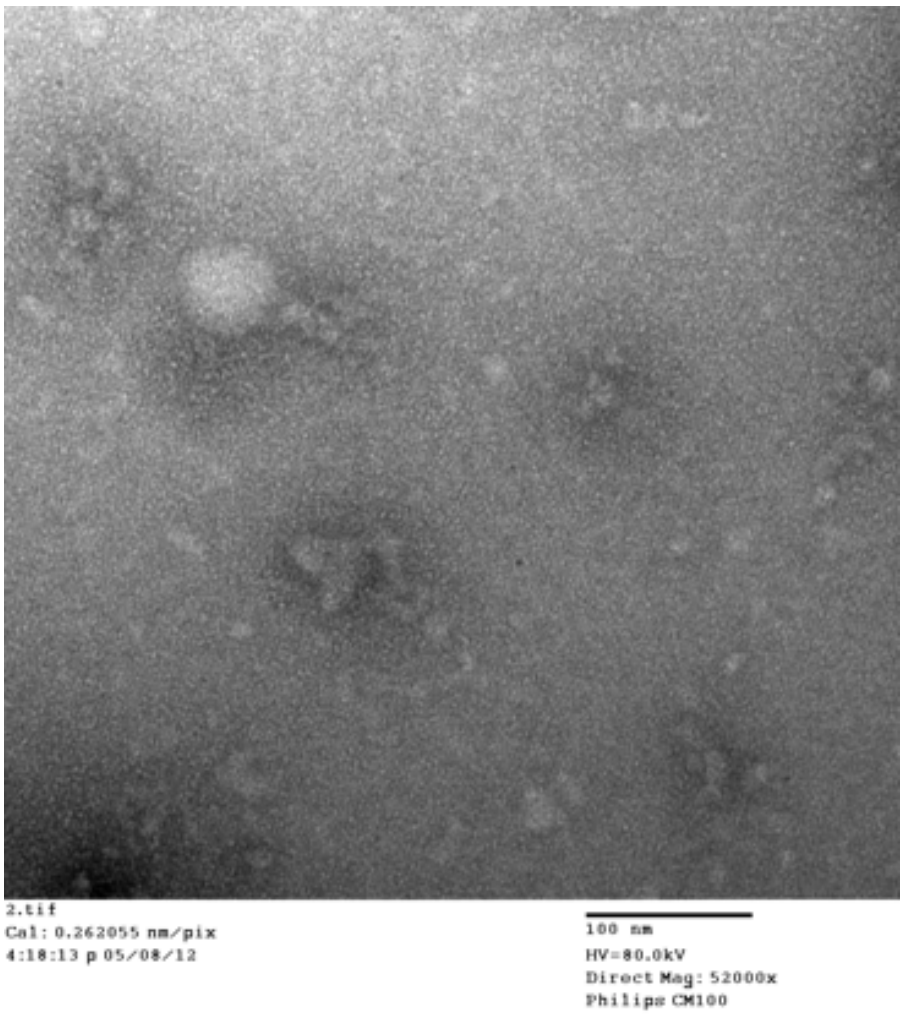

Figure 3.8: Electron microscope images of bacteriophage ASC10 demonstrate that bacteriophage ASC10 and the tail remain attached due to the elimination of filtration

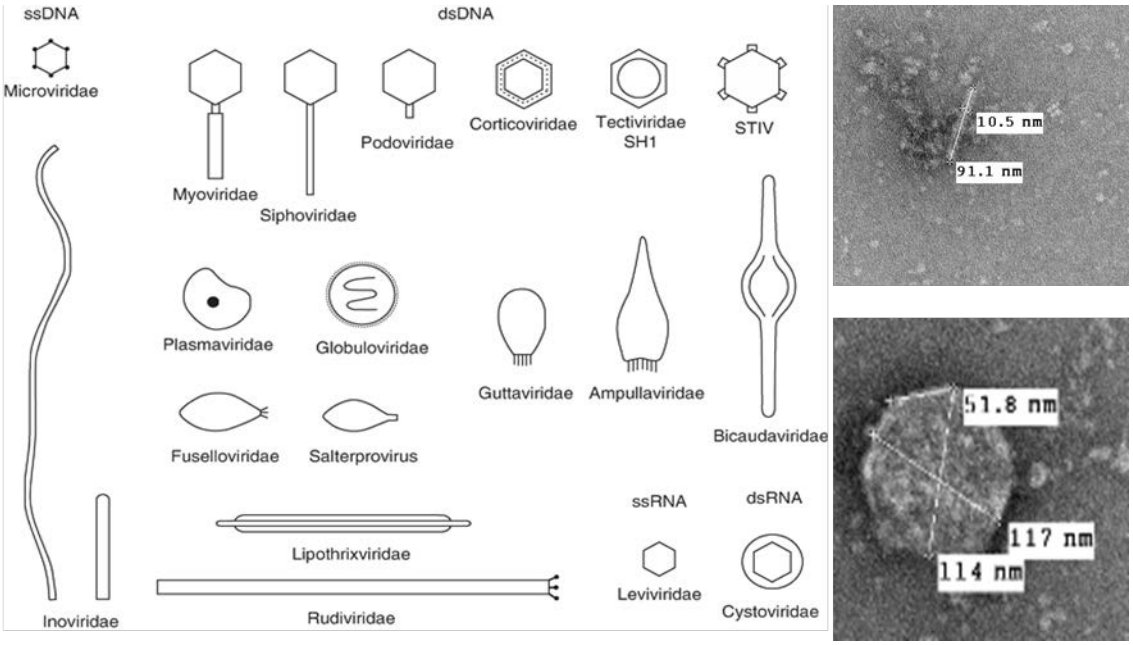

Figure 3.9: Dimensions, measurements and classification of phage ASC10.

3.3.5 ASCl0 DNA Analysis

The size of the restriction fragments of phage ASC10 DNA obtained from *Bam*H digestion, and the uncut DNA were similar in size. Agarose gel electrophoresis of ASCl0 DNA revealed two bands of DNA when digested with *Bam*HI and one band for the uncut DNA, indicating that ASCl0 is a double-stranded DNA phage (Figure 3.10). The genome size was estimated to range between 20 and 25 kb which is the range for the *Siphoviridae* family.

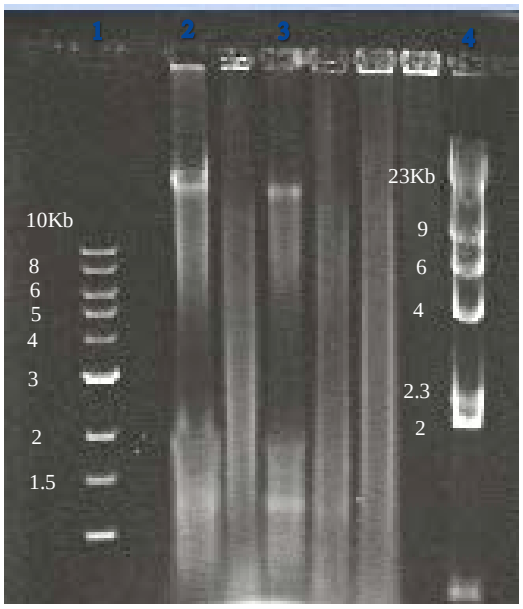

Figure 3.10: Agarose gel of ASC10 DNA digested with restriction enzymes. *Lane* 1 and 4: 1Kb and λ DNA digested with HindIII ladders; *Lane* 2 undigested ASC10 DNA; *Lane* 3 ASC 10 DNA digested with *Bam*HI.

3.3.6 SDS-PAGE protein profiles of ASCl0 phage

Protein preparation of all phage samples, revealed three intensive protein bands corresponding to the phage structural proteins of the capsid and the tail. The first two bands had a size of 30 and 37 kDa, which corresponds to known phage major capsid proteins. The third band had a size of 22 kDa, which corresponds to sizes of phage tail proteins. (Figure3.11). *Siphoviridae* phages with icosahedral heads and non-contractile tails share a range of 30 to 50 KDa for capsid major proteins and a range of 16 to 28 KDa for tail proteins.

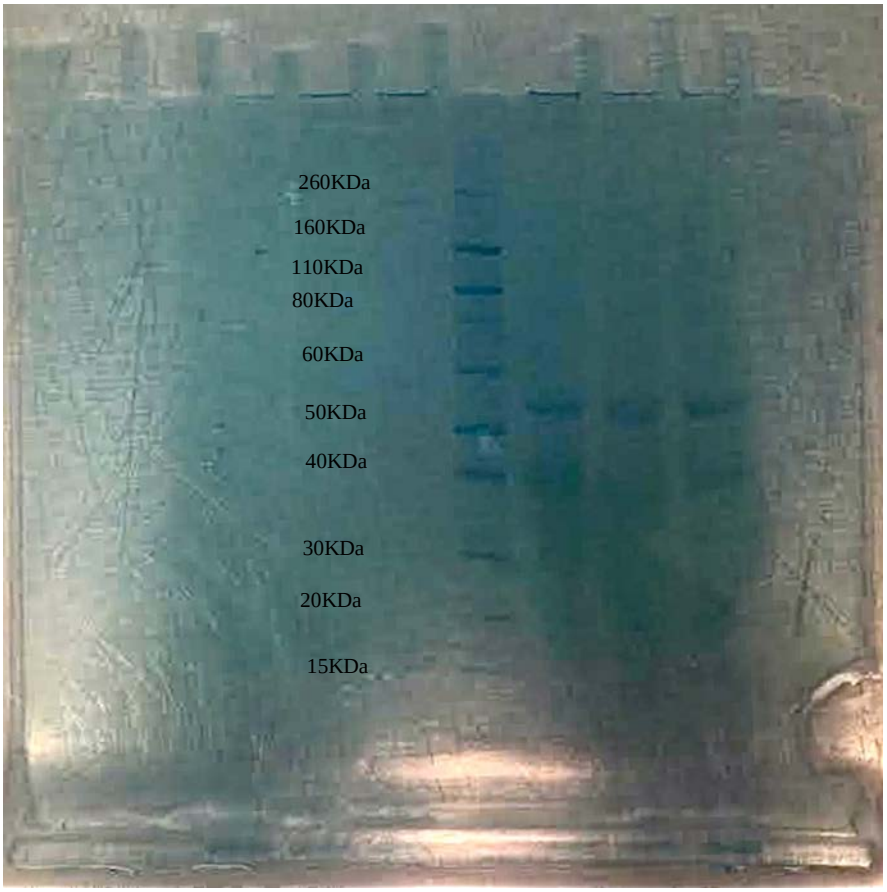

Figure3.11: SDS-PAGE analysis of ASCl0 phage proteins; bands had a size of 22, 30 and 37 kDa.

3.3.7 ASCl0 is stable at low temperature

For long term storage of ASCl0 bacteriophage, purified phage lysate was kept in screw capped tubes at 4°C for 1 year. Plaques were obtained from this phage lysate (Figure 3.12). Almost complete inactivation of the bacteriophage occurred when it was exposed to chloroform indicating that it probably contains lipid. The bacteriophage was stable at temperatures below 45°C but was completely inactivated at temperatures above 50°C.

Figure 3.12, Plaques obtained from phage lysate that was stored at 4°C for one year.

3.4 Discussion

Research investigating the pathogenicity of *Francisella tularensis* has progressed significantly over the past few years. However, our knowledge of *Francisella* phage is narrow and certainly behind that of other bacterial phages. Furthermore, there have not been any *F.tularensis* phages isolated until now. In this chapter, a novel *F. tularensis* phage was isolated from *F.t. novicida* NR-575 and proved to be active against different *Francisella* strains including *F. tularensis* subspecies *holarctica* strain *LVS, novicida* and *philomiragia.* Furthermore, this phage, designated ASC10, has the ability to lyse *F. tularensis* subsp. *tularensis* strain MA00-2987, *F. tularensis* subsp. *holarctica,* strain K499-3387 and *F. tularensis* subsp. *tularensis* Schu S4. Phage ASC10 must represent a new species since no phage have been documented that lyse *Francisella* strains.

In general, bacteriophage share very limited similarities, thus there is no uniform criteria that can be followed exactly to identify a novel phage. In addition to genomic approaches, biological experiments are important to confirm the viability and activity of a new phage. Procedures to isolate, concentrate, and purify bacteriophages cover a range of parameters and each of these parameters has a direct effect on bacterial-phage interactions. Alteration and modification of these parameters is needed to establish the best method for purification and maintenance of each specific phage.

The majority of research articles investigating the efficiency of mitomycin C (MMC), focused on the antitumor activity of this agent. In scientific articles on temperate phages, mitomycin C was used at a final concentration of 0.5 to 1 μg/ml without detailed information about its preparation. Mitomycin C is stable in acid or base solvent at a pH ranging from 6 to 9. However, water suspensions of mitomycin C had a pH of 4. The use of *E.coli* to test mitomycin

C efficiency over time proved that mitomycin C in water loses its potency within 2 to 3 weeks. DMSO was recommended as a solvent instead of water for dissolving mitomycin C.

Mutations occur spontaneously during bacterial cell division representing a form of bacterial adaptation and survival mechanisms. In case of lysogenic cells these mutations could have a direct effect on the integrated prophage genome. Therefore, limiting the mutation rate of the lysogenic cells would prevent alteration in the prophage-encoded genome. Furthermore, limiting the number of passages for *F.novicida* NR-575 improved phage production.

Large-scale purification was undertaken for phage ASC10 electron microscope examination, DNA and protein analysis. This procedure included PEG concentration followed by chloroform extraction. Phage ASC10 showed sensitivity to chloroform and TEM images showed capsid protein denaturation. These images were comparable to TEM images of PAV1 virus-like particles after chloroform exposure. Geslin et al reported a comparison of the morphology of PAV1 virus-like particles after exposure to different chemicals and concluded that the sensitivity of phage to lipid solvents such as chloroform does not prove the presence of lipid. Alternative approaches were carried out to purify phage particles. Filtered and centrifuged plaque suspensions were examined under the electron microscope. Filtration caused tail breakage while centrifugation revealed intact phage particles with interfere cell debris. An evaluation of the use of transmission electron microscopy to analyze the viral community structure, reported that *Siphoviridae* phage were highly susceptible to tail loss (32-76%) during the purification processes (Williamson et al., 2012).

Phage ASC10 possessed an icosahedral head approximately 114 nm in diameter and a non-contractile tail of 92 nm in length. These measurements place ASC10 into the family *Siphoviridae.* Agarose gel electrophoresis of ASC10 DNA revealed two DNA bands when

digested with *Bam*HI, indicating that this phage has double-stranded DNA and the genome size, estimated based on the gel, ranges between 20 and 25 kb. BCJA1 is a temperate bacteriophage that was isolated by Jarrel in 1997 and its genome size was compared to two other phages based on the correlation between the head dimensions and the genome size. For example, TP-15 phage has a head of 52 nm and a genome size of 35 kb, phage γ has a head of 59 nm and a genome size of 40 kb, while BCJA1 has a head of 62 nm and a genome size ranging between 32-35 kb. Since phage ASC10 has a head of 114 nm the genome size should be at least 75 kb. Therefore, pulsed-field gel electrophoresis needs to be done to determine the accurate size of the ASC10 genome.

Analysis of the protein profile of purified phage indicated the presence of three protein bands with an estimated size of 22, 30 and 37 kDa. These proteins are likely to represent major head and tail proteins. The 30 and 27 kDA protein bands correspond to the major capsid protein while the 22 kDa protein corresponds to the tail protein of phage in the *Siphoviridae* family.

Phage ASC10 was stable when stored at 4°C for a period of 1 year and was stable in low temperatures. Plaques were observed when the temperature was as high as 50°C but not above this temperature. In conclusion, this study provides the basic knowledge of a novel *Francisella* bacteriophage and introduces the first phage proven to be active against most of the *Francisella* strains. Perhaps bacteriophage ASC10, shown here to be a temperate phage, can be used as the basis of a transduction system for *Francisella tularensis.*

This phage could possibly be used as a therapeutic agent. In general, a lytic phage that has no lysogenic cycle is more favorable for use as a therapeutic phage than a temperate phage. On the other hand, there have been no reported phages for *Francisella* until this study. Therefore, for phage therapy against *F.tularensis,* temperate phage ASC10 may be considered as a therapeutic candidate.

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Chapter 4

Introduction to the genomic analysis of bacteriophage ASC10

4.1 Introduction

Bacteriophages are widely distributed among bacterial genomes, and they influence the adaptive capabilities and genomic evolution of their hosts. Bacterial genome nucleotide sequences have revealed that prophage genomes constitute as much as 20% of the bacterium's chromosome (Casjens, 2003). In February 2013, more than 6500 bacterial genome sequences were included in the GenBank database (Edward et al., 2013) and many of these sequences contained prophage-encoded genes. Although genome/protein sequences of many phages are present in the NCBI databases, the number of sequences phage genomes is low in comparison to the number of sequenced bacterial and eukaryotic viral genes. The relatively low number of phage genes/proteins available for homology searches and the fact that bacteriophage often consist of mosaics of genes from several different phages, is a challenge to using homology searches to identify phage genomes in bacterial chromosomes. However, these tools can assist in this process.

In our laboratory, ASC10 phage was isolated from *F. t. novicida* NR-575 by induction, and *F. t. novicida* NR-584 was selected as a reliable indicator strain for this phage. Surprisingly, both of these strains are derivatives of *F. t. novicida* wild type strain U112, suggesting that there must be a difference between them that would overcome phage immunity and allow the phage to replicate on the closely related strain NR-584. *F. t. novicida* NR-575 is a spontaneous *mgIA* mutant of the wild type strain U112. *F. t. novicida* NR-584 is a transposon mutant of *F. t.*

novicida U112 and has the *pdpA* region with Tn*MaX2* transposon as described in the BEI Resources database. Chromosomal DNA extraction, sequencing, and genome alignment was performed on both strains to identify the differences between *F. t. novicida* NR-575 and NR-584 that would account for the ability of phage ASC10 to replicate in *F. t. novicida* NR-584.

To identify the location of the ASC10 phage in the *F. t. novicida* U112 genome, the basic BLAST online tool available from NCBI was used to identify proteins homologous to known phage proteins in protein databases. Protein similarity searches revealed clusters of proteins that shared similarities to known phage proteins, and these clusters were analyzed further to determine whether they were indicative of prophage in the genome.

ASC10 is a prophage with its DNA integrated into the bacterial chromosome. There are several genes involved in maintaining the lysogenic state such as prophage repressors *cI* and other transcriptional regulators. Mutations in these regulators have been shown to generate lytic phages from temperate phages such as λ phage. A literature review of temperate phage studies showed that lytic phages could be generated from temperate phage by either spontaneous or genetically engineered mutations in the repressor or other regulatory genes. Since recovery of a lytic phage would make production of phage lysates and characterization of phage ASC10 easier to accomplish, a transposon mutant library available from BEI Resources was screened for the presence of lytic phage. The recovery of lytic phage would suggest that the transposon insertions mutated phage regulators associated with the phage genome.

In addition to the above experiments, the effect of the CRISPR-Cas system *in F. t. novicida* U112 (and derivatives) on phage production was studied. Since phages are the most abundant entities on earth, bacteria have evolved resistance mechanisms against phage infection such as abortive phage infection proteins and the CRISPR-Cas system (**c**lustered **r**egularly

interspaced **s**hort **p**alindromic **r**epeats). To understand whether or not these systems inhibit the production of phage ASC10, the effect of different mutations in the CRISPR-Cas sytem on phage production was studied.

In this chapter a combination of experimental and genomic approaches were used to identify the ASC10 phage genome within the bacterial chromosome and to examine bacterial-phage interactions, focusing on phage resistance mechanisms.

4.2 Materials and Methods

4.2.1 DNA extraction and sequencing of *F. t. novicida* NR-575 and NR-584 genomic DNA

In order to fully understand the lysogenic state of ASC10 phage and the phage-bacteria interaction on the indicator strain, genomic DNA was isolated and sequenced for both the *F. t. novicida* NR-575 lysogen and *F. t. novicida* NR-584 indicator strains. DNA was purified from both of these strains using the Gentra Purgene Yeast/Bact. Kit (Gram-Negative Bacteria). *F. t. novicida* strains NR-575 and NR-584 were inoculated directly from the BEI Resources frozen stocks into BMFC broth and incubated at 37°C with agitation at 225 rpm, for 24 h. Cell counts were performed and the OD_{600} was measured on these cultures. 500 µl from these cultures (containing 2×10^9 cfu/ml) was transferred into a 1.5 ml microfuge tube and stored on ice for 15 min. Bacterial cells were pelleted by centrifugation at 16000 x g for 10 min. Supernatant fluid was discarded and 300µl of cell lysis solution was added, followed by incubation at 80°C for 5 min. RNase was added (1.5µl) and samples were incubated at 37°C for 1h and then transferred to ice and incubated for 1min. Protein precipitate solution (100 µl) was added to the samples,

followed by centrifugation for 3 min at 16000 x g. The supernatant fluid was then transferred into a clean 1.5 microfuge tube and 300 µl of isopropanol was added to the samples followed by centrifugation for 1 min at 16000 x g. The supernatant fluid was discarded and 70% ethanol (300 µl) was added. The samples were centrifuged at 16000 x g for 1 min, the supernatant fluid was discarded, and the pellet was allowed to air dry. DNA hydration solution was added (100 µl) and mixed with the pellet. DNA suspensions were incubated at 65°C for 1 h, followed by incubation at room temperature for 24 h. DNA samples were stored at −20°C and then sent to the CSU Next Generation Sequencing Core, Fort Collins, CO. The genomic DNA was sequenced using an Ion Torrent semiconductor sequencer.

4.2.2 DNA sequencing alignment of *F.t. novicida* NR-575 and NR-584 strains and search for proteins homologous to known phage proteins

The *F. t. novicida* NR-575 and NR-584 genome sequences were aligned and analyzed using NextGen software (NextGen Software, Inc., Aurora, CO) and compared with the parent, wild-type strain U112 genome. The aligned sequences were compared visually for deletions and insertions and a list of point mutations was generated by the software program. Except for point mutations scattered throughout the genome, the *F. t. novicida* NR-575 genome was almost identical to the U112 genome available through GenBank, and based on this information, the *F. t. novicida* U112 genome was used for further genetic analysis.

Genetic analysis of the *F. t. novicida* U112 genome for proteins homologous to known phage proteins was carried out using the NCBI database. The *F. t. novicida* U112 sequenced

genome, available in the NCBI database, was used to perform a search for homology to known phage proteins in the non-redundant protein database, using the BLASTP algorithm with program default values. An e-value of 0.005 was used as the cut-off value for homology, unless phage proteins were not identified that showed an e-value below this number. In this case, the e-value and the corresponding phage protein was reported.

4.2.3 Attempts to identify a lytic ASC10 phage from a transposon mutant library

F. t. novicida U112 transposon mutant library was used in this experiment to screen for mutants that produce lytic phage. This library is composed of sequence-defined transposon insertion mutants of *F. t. novicida* U112. BMFC agar media was prepared and poured into OmniTray polystyrene flat plates obtained from Thermo Fisher Scientific, Inc. (Waltham, MA). Bacterial strains were spotted on duplicate BMFC agar plates using a 48-pin replicator. After incubation at 37°C for 48 h, a plaque assay was performed as described previously with slight modifications. The soft agar (8 ml for each plate) was prepared with a final concentration of 0.8% agar, 300 µl of the indicator strain *F. t. novicida* NR-584 (overnight culture), and 0.5µg/ml (final concentration) of mitomycin C. The mixture was poured over the agar plates containing 42 ml 1% BMFC agar. Bacterial colonies were then spotted onto these plates, incubated at 37°C for 48 h, and observed for clearance around the colonies as an indication of phage production.

4.2.4 Using *F. t. novicida* U112 with mutations in CRISPR loci as an indicator host for ASC10 phage

A range of 2-10 plaque forming units (pfu)/ml were usually obtained when *F. t. novicida* NR-584 strain was infected with phage ASC10 lysates. This low number of plaques could be due to the high homology between the lysogen and the indicator strain resulting in insertion of the temperate phage ASC10 into the genome rather than entering the lytic cycle. Another explanation is that the innate bacterial immunity against phage infection prevents replication and cell lysis. To investigate if either of these explanations was true, our laboratory collaborated with Dr. David S. Weiss's laboratory at Emory University, Atlanta, GA. The Weiss lab constructed five *F. t. novicida* U112 strains with CRISPR-Cas mutations in individual loci. These strains were sent to our laboratory, and experiments were performed on these mutants in conjunction with Hanna Ratner, a graduate student in Dr. Weiss's lab. An ASC10 phage lysate was prepared as described previously. These strains were grown in BMFC broth at 37°C with agitation of 225 rpm for 24 h. Plaque assays were performed as described before using each of the *F. t. novicida* U112 CRISPR-Cas mutants as an indicator host strain, to determine the effect of each mutation on plaque production.

4.3 Results

4.3.1 The indicator strain for ASC10 phage (*F. t. novicida* NR-584) has a deletion in its genome area that encodes for the, phage infection system

F. t. novicida NR-575 and NR-584 genomic DNAs were sequenced and aligned to *F. t. novicida* U112, the reference genome. *F. t. novicida* NR-575, the lysogenic strain, has point mutations scattered throughout the genome. Major deletions or insertions were not recorded in this strain. On the other hand, a large deletion area (from 749,273-760,569 nucleotides) was detected in the *F. t. novicida* NR-584 genome (the indicator strain), (Figure 4.1). A list of the genes included in the deleted region is presented in table 4.1. This region includes two bacterial defense mechanisms against phage infection, the abortive infection bacteriophage resistance protein and a restriction- modification system. These results support our selection of this strain as an appropriate phage indicator.

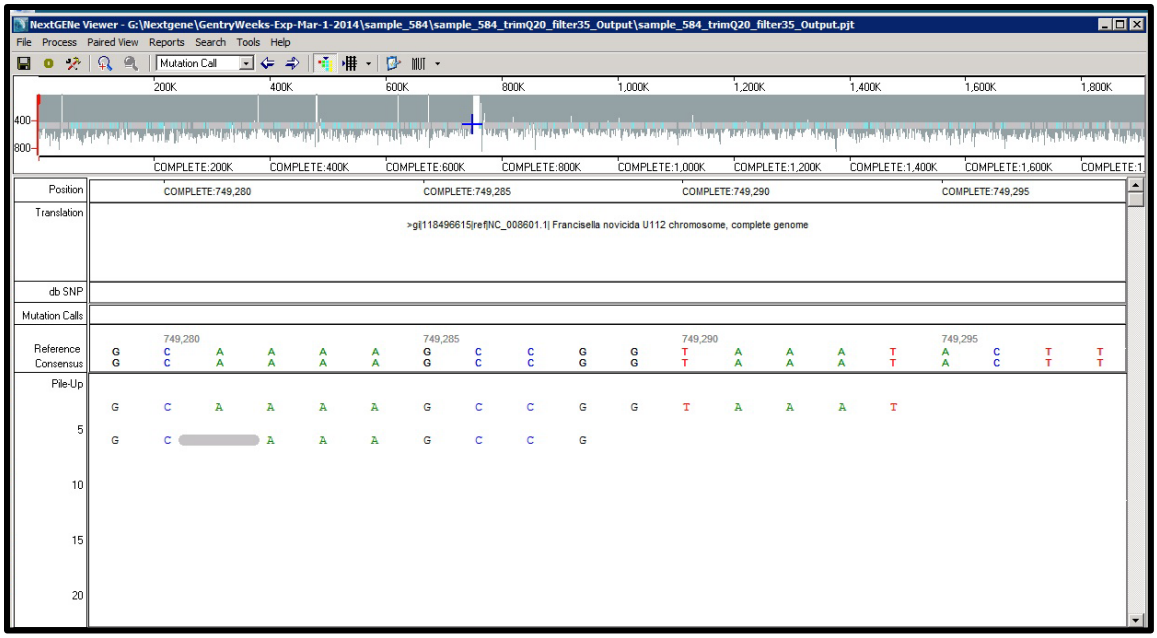

Figure 4.1: *F. t. novicida* NR-584 deletion in the genome area that encodes for the abortive phage infection system

Table 4.1: Genes included in the 1 *F. t. novicida* NR-584 deletion area in the genome

Locus Tag	Description
FTN_0705	Abortive infection phage resistance protein
FTN_0706	Hypothetical protein
FTN_0707	Type I restriction-modification system subunit S
FTN_0708	Hypothetical protein
FTN_0709	Hypothetical protein
FTN_0710	Type I restriction-modification system subunit R
FTN_0711	Metal dependent hydrolase
FTN_0712	Heat shock protein
FTN_0713	OstA organic solvent tolerance protein
FTN_0714	Hypothetical protein

4.3.2 Prediction of prophage sequence area within the bacterial genome

There was no significant difference between the NCBI-published *F. t. novicida* U112 genome sequence and our lysogenic strain NR-575 (containing phage ASC10) DNA sequences. Therefore, we decided to use the published DNA sequence of *F. t. novicida* U112 to perform a BLASTP search to identify clusters of encoded proteins that share similarities with proteins of known phages. Based on proteins revealed by homology searches with protein databases, one predicted prophage genomic area was predicted (Figure 4. 2). This region contains essential phage genes such as integrase (see table 4.2).

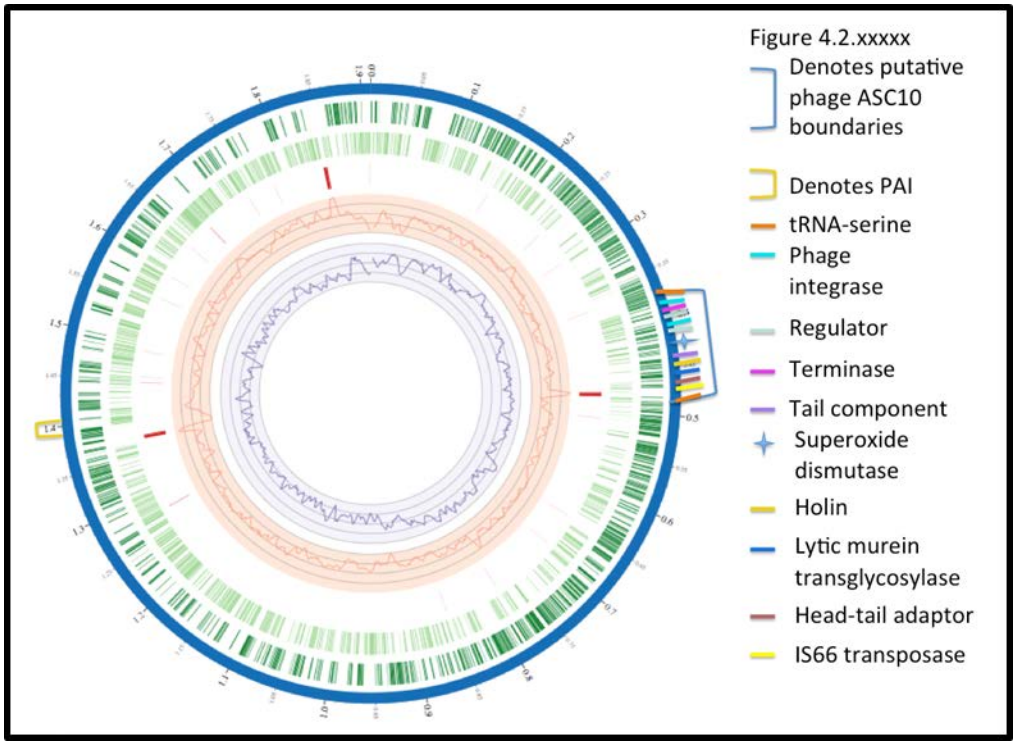

Figure 4.2: Predicted prophage area in the *F. t. novicida* U112 genome is delineated by a blue bracket and interrupts a tRNA-serine gene

Table 4.2: Genes that encode proteins homologous to those of known phages

Locus	Similar phage protein in database	*e* value	% identity
FTN_0367	*E. coli* phage integrase family	6e-77	23
FTN_0368	Phage DNA packaging protein Nu1 (terminase)		
FTN_0369	Putative repressor *Leuconostoc* phage phiLN25	0.065*	22
FTN_0370	Bacterial addiction module (toxin-antitoxin)	3e-27	33
FTN_0371	Phage-associated homing endonuclease (*Vibriophage* phi-pp2)	4.8*	30
FTN_0372	AlpA transcriptional regulator *Enterobacteria* phage P4	8e-20	40
FTN_0373	*E. coli* phage integrase family	1e-75	24
FTN_0374	*Burkholderia* phage BcepIL02	5e-18	34
FTN_0375	NSM		
FTN_0377	Pseudogene – IS insertion		
FTN_0378	*E. coli* CP4-57 prophage uncharacterized protein	4e-116	17
FTN_0379	*Photobacterium phosphoreum* ANT220 conserved hypothetical phage protein	0.63*	30
FTN_0380	*Lactobacillus gasseri* XRE family transcriptional regulator (phage repressor)	2e-18	26
FTN_0381	NSM		
FTN_0382	*Leptospira weilii* tail tape measure protein, TIGR phage 176 phage family	1e-4	12
FTN_0383	NSM		
FTN_0384	*Lysinibacillus sphaerus* phage tail length tape measure protein	3e-4	13
FTN_0385	*Lactobacillus equi* terminase	0.099*	21
FTN_0386	NSM		
FTN_0387			
FTN_0388	*Vibrio* phage nt-1 hypothetical protein VPFG	4e-6	40
FTN_0389	*Salmonella* phage SPN3U5 putative virion structural protein 1	0.006*	19
FTN_0392	*Burkholderia* phage phi 1026b gp58 LysR transcriptional regulator	5e-55	18
FTN_0393	*Colletotrichum gloeosporoides* Cg-14 HK97 family prohead protease	5e-14	15
FTN_0394	*Clostridiales* putative phage tail component domain protein	4e-140	28
FTN_0395	*Lactobacillus animalis* phage shock operon rhodanese PspE and *Natrinema altunense* phage Phi H1 repressor protein	2e-6	13
FTN_0396	*Synechococcus* phage S-CBP2 hyothetical protein SXHG 00051	1e-11	21
FTN_0397	*Methanobrevibacter smithii* putative phage tail component domain protein	3e-13	19

FTN_0398	*Dorea longicatena* putative phage holin protein, LL-H family	0.006	
FTN_0399	*Mycobacterium* phage Kostya gp118	4e-25	17
FTN_401	NSM		
FTN_403	*Staphylococcus* phage SA11 hypothetical protein	0.005	19
FTN_405	*Salmonella enterica* phage superoxide dismutase	3e-46	57
FTN_406	*Vibrio parahaemolytica* hypothetical protein VP1492	3e-40	48
FTN_407	*E. coli* phage S-methylmethionine transporter	5e-101	32
FTN_408	*Bacillus* phage Fah mannose 6-phosphate isomerase	6e-16	13
FTN_409	*Synechococcus* phage S-SM2 zinc containing alcohol dehydrogenase superfamily protein	4e-75	26
FTN_0410	*Petnophilus lacrimalis* AAT-like putative phage tail component domain	2e-146	47
FTN_0411	*Aneurinibacillus aneurinilyticus* phage holin LL-H family	6e-56	17
FTN_0412	*Enterobacteria* phage CP-1639 RecN protein	2e-100	34
FTN_0413	*Deftia* phage phi W-14 hypothetical protein	1e-10	48
FTN_0414	*Deftia* phage phi W-14 hypothetical protein	2e-7	47
FTN_0415	*Campylobacter* phage CPt10 hypothetical phage protein		
FTN_0416	NSM		
FTN_0418	*Bacillus* phage vB BanS-Tsama hypothetical protein	5e-10	23
FTN_0419	*Prochlorococcus* phage P-SSM2 phosphoribosylaminoimidazole synthetase	6e-121	36
FTN_0420	*Prochlorococcus* phage Syn 1 phosphoribosylaminoimidazaole-succinocarboxamide synthase	1e-66	24
FTN_0421	*Bacillus thuringiensis* phage protein	2e-80	32
FTN_0422	*Mitsuokella sp.* oral taxon 131 putative phage head-tail adaptor	0.003	19
FTN_0423	*Synechococcus* phage S-DBM2 30S ribosomal protein S6 modification protein	8e-14	14
FTN_0424	*Synechococcus* phage hypothetical protein P60	3e-6	29
FTN_0425	*Capnocytophaga sputigena* phage minor structural protein, N-terminal protein domain	?	26
FTN_0426	NSM		
FTN_0427	*Pseudomonas* sp. GM67 tail protein	0.009*	21
FTN_0428	*Streptococcus parauberis* phage tail length tape-measure protein	4e-4	13
FTN_0429	*Clostridium* sp. ASF502 phage tail tape measure protein TP901 family, core region	6e-6	6
FTN_0430	NSM		
FTN_0431	*Staphylococcus* phage phiR57	2e-7	15
FTN_0432	*Anabaena* phage A-4L	4e-5	15
FTN_0433	*Halomonoas* phiHAP-1 phage partitioning protein	2e-38	25
FTN_0434	*Catenibacterium mitsuokai* putative phage tail component	1e-51	24

	tail domain protein		
FTN_0435	*Klebsiella pneumoniae subsp. pneumoniae* ST258-K28BO phage endopeptidase	2e-48	19
FTN_0436	*Staphylococcus* phage minor structural protein	2e-7	12
FTN_0437	*Vibrio* phage VP2 hydrolase	2e-8	23
FTN_0438	*Clostridiales* bacterium oral taxon 876 putative phage head-tail adaptor protein	4e-8	17
FTN_0439	*Synechococcus* phage S-ShM2 hypothetical protein	3.8*	
FTN_0440	*Streptococcus sanguinis* phage infection protein	0.016	
FTN_0441	*Staphylococcus* phage Twort ORF001	0.015	17
FTN_0442	*Klebsiella* phage KP15 hypothetical protein KP-KP15 gp123	1e-6	15
FTN_0443	*Escherichia phage* bV EcoS AKFV33 thymidylate synthase	0.03	19
FTN_0444	*Synechococcus* phage S-CMB2 structural protein	0.026	
FTN_0445	*Vibrio parahaemolytica* murein bound lytic murein transglycosylase C	1e-18	17
FTN_0446	*Chlamydia pneumoniae* putative phage virion morphogenesis protein/GTP binding protein	5e-31	20
FTN_0447	Phage baseplate assembly protein	0.17*	20
FTN_0448	NSM		
FTN_0449	*Streptococcus pyogenes* type I site-specific DNAse	6e-6	10
FTN_0450	*Bacillus* phage vB BceM-Bc431v3 putative methyltransferase	3e-7	21
FTN-0451	*Eubacterium yurii* putative phage head-tail adaptor protein	2e-145	29
FTN_0452	*Leptospira* phage LnoZ CZZ14 IS66 family element transposase	7e-4	26
FTN_0453	*Acinetobacter* CPS-53 prophage bactoprenol glucosyl transferase	5e-22	12
FTN_0454	*Leptospira* phage LbrZ 5399 HlyD family secretion protein	2e-19	14
FTN_0455	*Capnocytophaga gingivalis* putative phage head-tail adaptor	2e-13	8
FTN_0456	*Eubacterium yurii* putative phage head-tail adaptor	5e-148	25
FTN_0457	*Thermus* phage phi OH2 predicted amidophosphoribosyltransferase	2e-11	20
FTN_0458	*Streptomyces* phage phiSASD1 gp4	1e-20	25
FTN_0459	*Ruminococcus callidus* phage tail component protein	9e-6	17
FTN_0460	*Bifidobacterium adolescentis* site-specific recombinase phage integrase family	0.37*	16
FTN_0461	*Leuconostoc* phage LLC-1 methyltransferase	4e-16	23
FTN_0462	*Parascardovia denticolus* putative phage prohead protease HK97 family	6e-19	20
FTN_0463	NSM		
FTN_0464	NSM		

FTN_0465	*Synechococcus* phage S-SSMS carbamoyltransferase	0.15*	21
FTN_ 0466	NSM		
FTN_0467	*Pasteurella multocida* phage protein	1e-21	19
FTN_0468	*Ruminococcus torques* phage portal protein	0.19*	17

4.3.3 Lytic phage was not recovered from the *F. t. novicida* U112 transposon mutant library

A 16,508-member transposon mutant library of *F. t. novicida* U112 was screened for lytic phage since mutagenesis of phage repressors and regulators encoded by temperate phages results in the generation of a lytic phage. Clear zones surrounded the strains listed in table 4.3, indicating that specific mutants have the enhanced ability to produce phage. Two mutants (P6-E08 and P18-D6) showed distinct zones of clearing and are mutated in genes encoding cell wall/LPS/capsule production and signal transduction and regulation, respectively. Clear zones were also observed around P17-B7 and P18-C7 mutants that are mutated in carbohydrate and nucleoside transport, respectively (Figure 4.3). In addition, mutant P32- F8, which lacks a restriction-modification enzyme system, exhibited enhanced clearing around the colony. These preliminary results were not confirmed when each mutant was grown individually in broth and induced with mitomycin C. Phage lysates from all mutants listed in table 4.3 were tested individually in a plaque assay using *F. t. novicida* NR-584 as the indicator strain and no plaques were detected.

Figure 4.3: Clear zones around mitomycin C induced mutated strains

Table 4.3, Transposon Type and mutated gene in the strains with surrounded clear zones

Plate#	Well	Transposon Type	Gene	Description	Function
6	E08	T20	wbtF	NAD dependent epimerase	Cell wall/LPS/capsule
17	B07	T20	−	Sugar transporter	Transport carbohydrate
17	B05	T20	−	Major facilitator transport protein	Transport
18	C06	T20	−	Dienelactone hydrlase	Putative enzyme
18	D6	T20	−	Trp repressor binding protein	Signal transduction and regulation
18	C07	T20	−	Nucleoside permease	Transport
18	D07	T20	−	Hypothetical membrane protein	Hypothetical-novel
32	F08	T18	recJ	Single-stranded-DNA	Restriction/modification

4.3.4 CRISPR-Cas mutations of *F. t. novicida* U112 is an improved indicator for phage ASC10.

Bacterial cells evolved the CRISPR system to defend themselves against phage infection. This system targets foreign genome (phage/plasmid) and degrades it. The presence of the CRISPR system in the indicator cells, *F. t. novicida* NR-584, could help explain the low phage titers in phage lysates and plaque turbidity. In collaboration with Dr. David S. Weiss's laboratory in Emory University, Atlanta, GA, a plaque assay was carried out by adding phage ASC10 lysate to each of five CRISPR-mutated, *F. t. novicida* U112 strains. Forty-three plaques from 10 plates were detected on two of these strains (Figure 4.4), indicating that these strains are relatively more sensitive to ASC10 infection because of a mutation in their CRISPR-Cas function.

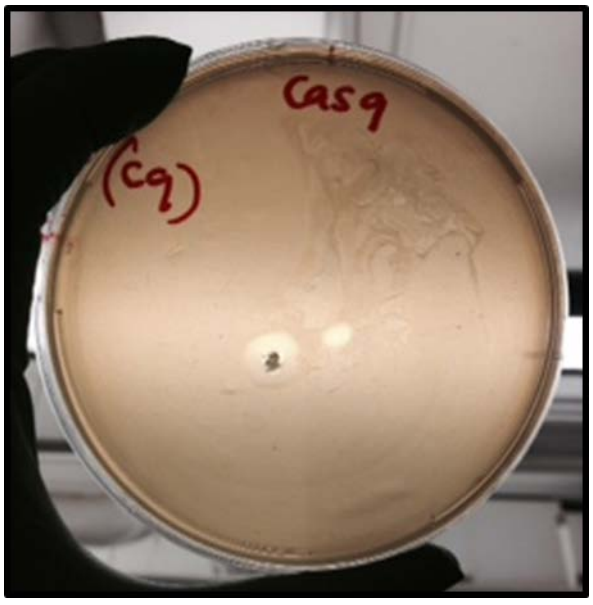

Figure 4.4: Phage ASC10 plaques on a CRISPR-Cas mutant of *F. t. novicida* U112 strain

4.4 Discussion

Phage ASC10 was isolated successfully and characterized based on its morphology when visualized by transmission electron microscopy. In this chapter, basic genomic analyses were carried out using several genomic approaches and experimental studies. Comparison of the *F. t. novicida* NR-575 lysogen genome to the *F. t. novicida* NR-584 indicator genome revealed that the indicator host contained a deletion in several genes for proteins involved in inhibition of phage replication (abortive phage infection protein and a restriction-modification system). This information suggests that although *F. t. novicida* NR-584 and NR-575 are both derivatives of *F. t. novicida* U112, the loss of these genes/proteins from *F. t. novicida* NR-584 allows replication of phage induced from *F. t. novicida* NR-575. Although the current recovery of phage ASC10 is very low, additional mutations in the *F. t. novicida* U112 genome may lead to an increase in the phage titer.

A BLASTP search for proteins homologous to known phage proteins indicated that *F. t. novicida* U112 contains three integrase genes in its genome. This finding is important since phage integrase genes are more conserved as compared to other phage proteins and they can be used to predict (in conjunction with other methods) the presence of a phage genome. Phages genomes often consist of genes that are mosaics from different phages, making identification by screening for homologous phage proteins challenging. So far, more than 500 phage genome sequences have been deposited in GenBank (Canchaya et al., 2003). By comparing each protein encoded on the *F. t. novicida* U112 genome to known phage proteins, a region of 105,707 nucleotides (spanning from nucleotide 369,143 to nucleotide 779,775 on the *F.t. novicida* U112 genome) was found to contain a cluster of genes that were significantly homologous to known

phage proteins. This region was flanked by tRNA-serine genes, one of the most common areas for phage integration in a bacterial genome, and contained two integrase genes in close proximity. Prophages normally integrate into the bacterial genome by site-specific recombination catalyzed by integrases, and therefore integrases are considered a marker for prophage.

Critical phage genes that maintain the lysogenic state in other well-known phages include the *cI* prophage repressor and *ArsR* transcriptional regulators. Mutations in these three genes may generate lytic phage. A commercially available transposon mutant library of *F. t. novicida* U112 was used to screen for lytic phage. Clear zones were detected around eight mitomycin C-induced colonies of the mutant library. These bacteria varied in their mutated genes and further experiments are needed to determine if mutations in specific putative phage repressor genes result in identification of a lytic variant of phage ASC10.

Bacterial defense mechanisms against phage infection are provided by the CRISPR-Cas system. In collaboration with Dr. David Weiss's laboratory at Emory University, Atlanta, GA, CRISPR mutants were tested their ability to support growth of phage ASC10. These strains showed relatively high sensitivity to phage ASC10 and approximately 5-fold more plaques were obtained when using these mutants as indicator hosts.

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