Defining the gene expression of five predicted DNA binding proteins in *Ehrlichia chaffeensis* cultured in macrophages and tick cells

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Cheyenne Autumn Knox

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Approved by:

Major Professor
Roman R. Ganta

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Abstract

Tick-borne diseases (TBDs) have been emerging as a major concern for the health of people and various vertebrate hosts. Pathogens such as viruses, bacteria, and parasites are transmitted from infected ticks during their bloodmeal acquisition from hosts and cause TBDs. One of the important TBDs impacting people and dogs in the USA is caused by the bacterial pathogen *Ehrlichia chaffeensis*. This pathogen is an obligate intracellular organism which is well adapted to tick and vertebrate hosts. Understanding how this pathogen can survive in dual hosts is important in devising methods of controlling the disease caused by it. We have been investigating how this pathogen is able to overcome the clearance by both tick and vertebrate hosts. Specifically, we use laboratory-based research involving molecular biology experiments to determine how the bacterium survives in a host. We isolated the bacterial genetic material (DNA) with proteins bound to it by performing an experiment called chromatin immunoprecipitation (CHiP). This method allows for identification of what parts of the DNA play a critical role in the bacterial survival in dual hosts. This research involved growing the bacteria using cell culture systems and by performing several molecular manipulation experiments to support our understanding of bacterial gene regulation. We focused on investigating the DNA binding proteins: MerR and EcxR, for their role in aiding the bacterial survival. We anticipate that this research will facilitate advancing the research for defining better methods of disease control and prevention.

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Dedication

This paper is dedicated to my loving mother who is the backbone of who I am today.

Chapter 1 - Review of Literature

Infectious Diseases

Infectious Diseases are responsible for about one-third of deaths worldwide. These diseases remain a constant threat to the human population and to various animal species (Holmes et al., 2017). Bacteria, fungi, and viruses can enter the body of animals or humans to replicate and cause infections leading to illness (https://www.cdc.gov/ncezid/who-we-are/index.html). The disease-causing agents can be spread from air or food, or from biological vectors, such as mosquitos, ticks and various other arthropods, and flukes. An important historical example of an infectious diseases is smallpox caused by a pox virus. This disease is described in the 4th century caused about 30% mortality with a death toll of ~500 million worldwide (https://www.cdc.gov/smallpox/history/history.html). Although smallpox was eradicated by the 1970s, infectious diseases caused by several viral pathogens continue to emerge. For example, SARS-CoV-2 viral infections rapidly emerged as a major pandemic in a very short time span (reported for the first time in 2019). This new virus has caused several million deaths worldwide and is responsible for the recent and active pandemic (https://covid19.who.int). New infectious diseases have been discovered frequently during the past two decades and finding solutions to contain them remains a high priority (Fonkwo, 2008). Influenza virus is a good example of an infectious virus as it is a highly contagious respiratory virus responsible for causing significant morbidities and mortalities. Due to rapid changes in its genome resulting in the generation of new variants, annual vaccine improvements and vaccinations are needed to contain the disease (https://www.cdc.gov/flu/about/viruses/change.htm) (Fonkwo, 2008).

Vector-Borne Diseases (VBDs)

Pathogens transmitted from vectors account for about 17% of all known human infectious diseases in the world. Vector-borne diseases are also a major concern for food animal health and are responsible for major economic losses. Similarly, vector-borne diseases impact companion animal health. A biological vector is defined as an organism responsible for acquiring, harboring, and transmitting an infectious agent from one vertebrate host to another. The most common vectors are arthropods which depend on hosts for a bloodmeal. During their blood feeding. Ectoparasitic arthropods can acquire pathogens from infected hosts. Such pathogens are then maintained in the arthropod hosts, thus serving as vectors and the pathogens are subsequently transmitted during the subsequent blood feeding times of infected arthropods to a naïve host. Mosquitos are widely known arthropod vectors, while ticks, flies, body lice, sand flies, and kissing bugs also contribute to vector-borne diseases (OMS, 2014). One of the first recognized vector transmitted diseases (filariasis) was discovered in 1877 by Sir Patrick Manson. It is caused by the mosquito-borne nematode parasite *Wuchereria bancrofti* (Chernin, 1983). Since then, many more vector-borne diseases have been discovered, including important mosquito-borne illnesses such as malaria, Yellow fever, Dengue, Chikungunya, Zika, and West Nile viral disease (https://www.cdc.gov/niosh/topics/outdoor/mosquito-borne/default.html). Similarly, tick-borne diseases have emerged as the second major concern for human health as well as for food and companion animals. The major tick-borne diseases include the Lyme diseases and various rickettsial diseases caused by the pathogens of the genera Anaplasma, *Ehrlichia*, and *Rickettsia*.

Tick-Borne Diseases (TBDs)

Ticks transmit several types of pathogens globally (Durden, 2006; Pfäffle et al., 2013). Ticks are a part of a diverse group of species with at least 898 recognized species representing from three families: Ixodidae, Argasidae, and Nuttalliellidae. Ticks parasitize a large range of vertebrates including humans. Lyme disease is the most prevalent tick-borne disease in the USA followed by various rickettsial diseases; ehrlichiosis, anaplasmosis, and rickettsiosis. Hard ticks have different stages of life cycle; egg, larvae, nymph, and adults. The later three stages require complete bloodmeals from a host to progress from one stage of life to another (Cotte et. al., 2008)(X. Y. Liu & Bonnet, 2014). Pathogens can be acquired during bloodmeals at any one of the three stages. While most pathogens progress from one developmental stage to another (transstadial), few ticks are passed on from adult female to eggs and then to larvae (transovarial). In transstadial infections, non-infected larvae and nymphs can obtain infection through a bloodmeal from an infected host which then be passed on to the next developmental stage. Infected nymphs and adults can then serve as the transmitting vectors (Walker et al., 2004). Some infected hosts can serve as the reservoir hosts supporting the continuous maintenance of a pathogen in nature, while incidental hosts may develop a disease, they may or may not facilitate the spread of the pathogen (Paddock & Childs, 2003).

Rocky Mountain Spotted Fever

Rocky Mountain spotted fever (RMSF) is a life-threatening tick-borne disease caused by *Rickettsia rickettsii* in people and dogs. This Gram-negative intracellular bacterium is prevalent in most of North, South and Central Americas. In the USA, most cases of the RMSF are documented from North Carolina, Arkansas, Oklahoma, Tennessee, Missouri, and Arizona (Thorner et al., 1998) (https://www.cdc.gov/ticks/tickbornediseases/rmsf.html). The disease

diagnosis can be a challenge as its symptoms resemble many viral diseases, such as fever, body pains, and headache (Alvarez-Hernandez et al., 2017) (https://www.cdc.gov/rmsf/symptoms/index.html). In severe cases, a patient may develop rashes, myalgia, edema, and gastrointestinal problems. Late stages of this disease can be severe where the skin turns dark and necrotic. Based on this symptom, the disease used to be called as "black measles" (https://www.niaid.nih.gov/diseases-conditions/rocky-mountain-spotted-fever) (Thorner et al., 1998). Mortality rate for RMSF varies greatly depending on the geographic locations; it can vary from 30-80% in untreated patients, while it can be lower to ~5% in patients offered treatments early. Antibiotic treatment may include the use of doxycycline or chloramphenicol (Wisseman and Ordonez 1986)(Breitschwerdt et al., 1991)(Thorner et al., 1998).

The history for RMSF began in 1896 when the first description of the disease is reported by Edward E. Maxey in Idaho (Dantas-Torres, 2007). The patient may develop high fever and eruptions on the skin which spread rapidly to various parts of the body. A 1902 study described more than 100 reports of RMSF with 69% case fatalities (Thorner et al., 1998). The disease is spread by *Dermacentor* species ticks (Dantas-Torres, 2007). *Dermacentor variabilis*, the American dog tick, is the most commonly identified vector in the eastern parts of the United States and some from the Western coastal areas (Dantas-Torres et al., 2012; Thorner et al., 1998). *Dermacentor andersoni*, the Rocky Mountain wood tick, is the primary vector in Canada and in the Rocky Mountain region. *Rhipicephalus sanguineo*us, the brown dog tick, is also presumed the major vector in Mexico and more recently, it has been shown to transmit the pathogen in Arizona (Bustamante and Varela 1947). *Amblyomma cajennense*, the Cayenne tick, is likely the major vector in Central and South America (Chapman et al., 2006) (Dantas-Torres,

2007). *Amblyomma aureolatuum*, the yellow dog tick, has recently been found as another vector in Brazil (Pinter & Labruna, 2006)(Dantas-Torres, 2007). A*mblyomma Americanum*, the lone star tick, is also implicated as the vector for RMSF (Levin et al., 2017)

Ticks acquire *R. rickettsii* infection during a bloodmeal from infected animals which then is maintained during their molting to the next stage of life cycle. Infected ticks serve as vectors for transmitting the pathogen to naïve hosts, such as dogs and people. RMSF pathogen infects vascular endothelium where it replicates and leading to the vascular damage leading to rashes and cause damage to other organs of the body and ultimately progress to the life-threatening outcome. The cell injury of the endothelium increases vascular permeability that causes edema, hypotension, hypovolemia, and hypoalbuminemia (Thorner et al., 1998) (https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5504a1.htm#top). The disease may progress to severe outcome within two weeks following tick transmission of the pathogen. Diagnosis is mainly based on the history and physical examinations of the patient. It is important to be thorough when identifying previous exposure to potential tick bites, treat with chemicals to kill ticks in areas where ticks are commonly found like wooded or high grass areas where the disease is endemic. RMSF can be difficult to diagnose during early stages of the illness because of non-specific signs (https://www.cdc.gov/rmsf/healthcare-providers/ClinLab-Diagnosis.html). Direct immunofluorescence assay targeting to the bacterial antigen detection or staining of skin biopsies with immunoperoxidase have been used for detecting *R. rickettsii.* The later method is limited to only patients experiencing rashes. Serological studies have been used for clinical diagnosis as well but are not always useful during the early stages of the disease (https://www.cdc.gov/rmsf/healthcare-providers/ClinLab-Diagnosis.html) (Thorner et al., 1998)

There are no current vaccines available to prevent this pathogen in people or dogs. It is complicated to develop vaccines because there are not many studies on understanding what *R. rickettsii* antigens are involved in protective immunity and how the protective host response functions. More recently, our research team has investigated the efficacy of two experimental vaccines, a subunit vaccine containing two outer membrane proteins as antigens (RCA) and a whole cell inactivated antigen (WCA), in providing protection against *R. rickettsii* infection challenge in a canine model for RMSF. The study demonstrated that the WCA vaccine confers complete protection from intravenous *R. rickettsii* infection (Alhassan et al., 2019).

Anaplasma phagocytophilum

Anaplasma phagocytophilum is an emerging obligate intracellular tick-borne pathogen impacting animals and humans worldwide (Jin et al., 2012). This organism is initially identified as the causative agent of tick-borne fever (TBF) in sheep in Scotland (Gordon et al. 1940) (Woldehiwet, 2010). Since then, it has also been described in cattle, goats, and sheep in 1940 (Carrade et al., 2009; Woldehiwet, 2010) (Foggie, 1951). *A. phagocytophilum* causes disease in cattle and sheep in parts of the UK, Europe, Scandinavia, and Ireland. Infections of this organism in dogs are reported in 1982 in California, which is now known as canine granulocytic anaplasmosis (CGA) and the disease in dogs is documented from various parts of the United States, Europe, and the United Kingdom. (Carrade et al., 2009)(Matei et al., 2019). The first report of fatal human granulocytic anaplasmosis (HGA) is reported in 1990 due to a severe febrile illness after being bitten by a tick two weeks prior (Dawson et al., 1991) (Dumler et al., 2005).

Anaplasma phagocytophilum is a small, Gram-negative obligate intracellular pathogen that infects cytoplasmic membrane-bound vacuoles of neutrophils (Jin et al., 2012; Rikihisa, 2011; Woldehiwet, 2010). This bacterium is transmitted by *Ixodes* species ticks which can lead to inflammatory lesions (Jin et al., 2012). Reservoir hosts for *A. phagocytophilum* vary depending on their location geographically. A variety of small wild mammals, deer, and birds can act as these hosts (Woldehiwet, 2010). For the eastern and midwestern United States, chipmunks and white-footed mice are identified as reservoirs, while in western states, gray squirrels and dusky-footed woodrats are also regarded as reservoirs of infection, while people and dogs are accidental hosts (Carrade et al., 2009).

Ixodes scapularis, the black-legged tick, is the vector of *A. phagocytophilum* distributed among south, mid, and northeastern United States. Primary hosts of immature *I. scapularis* ticks are reptiles, birds, and small mammals, whereas adults feed on large animals like deer and dog. This species of ticks also feed well on humans (Parola et al., 2004). *Ixodes pacificus*, the western black-legged tick, is the main vector for the Pacific coastal regions spanning from Canada to California. The primary hosts are the same as *I. scapularis* except that nymphs and adults can feed on humans (Parola et al., 2004). The main vector of *A. phagocytophilum* in Europe is *I. ricinus*, although the transmission to mammals is not clear (Jin et al., 2012; Thomas et al., 2009). *I. persulcatus* is suggested as the primary vector in Asia, while *I. ovatus* ticks are also regarded as acquiring *A. phagocytophilum* infections naturally (Jin et al., 2012).

Human granulocytic anaplasmosis is now the second major tick-borne disease in the USA (Dumler et al., 2005). The first case of HGA in Europe is identified in 1997 and since then it is most commonly identified in causing seasonal febrile illness during spring and summer months (Parola et al., 2004)(Olano & Walker, 2002). Anaplasmosis cases have increased since its earlier

description from 348 cases in year 2000 to 5,655 cases in 2019 (https://www.cdc.gov/anaplasmosis/stats/index.html). HGA symptoms include chills, fever, headache, hematological abnormalities, increased serum aminotransferase liver activity and myalgia (Rikihisa, 2011). It is important to diagnose HGA early and start antibiotic therapy quickly to reduce the risk of fatal outcome. Doxycycline is the treatment of choice (Jin et al., 2012). Vaccines are yet to be developed to control A. *phagocytophilum* infections (Stuen et al., 2013).

Anaplasma marginale

Bovine anaplasmosis (formally known as gall sickness) is caused by the bacterium *Anaplasma marginale* and is a hemolytic disease of cattle that can cause weight loss, adult mortality, abortion, and reduction in performance (Aubry & Geale, 2011; Khumalo et al., 2016; Spare et al., 2020). *A. marginale* is present globally in tropical and subtropical regions, including South and Central America, nearly all parts of the United States, southern Europe, Africa, Asia, and Australia (https://www.merckvetmanual.com/circulatory-system/blood-parasites/anaplasmosis?query=bovine%20anaplasmosis) (Aubry & Geale, 2011). This disease is widespread in cattle, but the pathogen can also infect other ruminants including water buffalos, bison, wildebeest, African antelopes, white-tailed deer, black-tailed deer, Rocky Mountain elk, and mule deer (Aubry & Geale, 2011; Khumalo et al., 2016; Kocan et al., 2004; Kuttler, 1984).

There are several modes of transmission for *A. marginale* including transmission by ticks, mechanical transmission from biting flies and by blood-contaminated fomites such as infected needles or tattooing/surgical instruments. More than 20 different species of hard ticks are identified as the vectors for this pathogen (Hosseini-Vasoukolaei et al., 2014). Tick vectors that transmit *A. marginale* include some *Dermacentor* spp., *Boophilus* spp., *Ixodes Ricinus*, and

Rhipicephalus spp. (Kocan et al., 2003). Trans placental infection transmission from an infected cow to calf during gestation is also documented (Kocan et al., 2003) (Ewing, 1981) (Norton et al. 1983).

Anaplasma marginale replicates within the erythrocytes which leads to the development of anemia resulting from the loss of red blood cells (Kocan et al., 2003). The severity of the disease and clinical symptoms associated with it vary depending on the age of the cattle. Calves under 6 months of age are less susceptible to illness. Animals of the ages 6 months to one year develop a mild disease (Aubry & Geale, 2011; Kocan et al., 2003) (Richey, 1991). For adult cattle over one year, the disease is often fatal. Clinical symptoms may include fever, weight loss, abortion, lethargy, and icterus. Additionally, independent of the age of an animal, *A. marginale* infection persists indefinitely in all infected animals (Kocan et al., 2003) (Ristic, 1997).

Bovine anaplasmosis can be diagnosed in Giemsa- or polychromatic-stained blood smears to find *A. marginale* inclusions from clinically ill infected animals. This method is not reliable for the detection of carrier animals or before symptoms appear. For these type of cases, diagnosis usually uses additional detection methods like complement fixation test, indirect fluorescent antibody test, various enzyme linked immunosorbent assays, such as competitive ELISA, and by molecular methods, such as PCR and real-time quantitative PCR (Aubry & Geale, 2011). Oxytetracycline is approved for parenteral use for no more than four days total in beef and dairy cattle. Chlortetracycline is approved for continuous use in feed (Reinbold et al., 2010).

Ehrlichia canis

Ehrlichia canis is a tick-borne rickettsial pathogen responsible for a potentially fatal disease in dogs; canine monocytic ehrlichiosis (CME). It has also been reported to cause infections in people (Alhassan et al., 2021; Cohn, 2003; Harrus et al., 1999). This pathogen was the first reported in 1935 in Algeria (Donatien and Lestoquard 1935) and subsequently reported worldwide. It is endemic in every continent except from Australia (Harrus et al., 1999; Mylonakis et al., 2019)

The principal vector of *E. canis* is *Rhipicephalus sanguineous* commonly known as the brown dog tick (Harrus et al., 1999; Harrus & Waner, 2011; Mylonakis et al., 2019). Infections with this bacterium are more prevalent in warmer climates when the ticks are active (Cohn, 2003; Harrus & Waner, 2011; Wen et al., 1997). Infections with *E. canis* are described clinically in three stages: acute, subclinical, and chronic infection (Cohn, 2003; Mylonakis et al., 2019). The acute stage of infection typically occurs from 1 to 3 weeks post an infected tick bite and is usually a mild illness (Harrus et al., 1999). The bacterial organisms will invade and replicate in the mononuclear cells. Hematological changes usually occur during the acute stage like thrombocytopenia resulting from vascular endothelial inflammation. Although clinical signs vary, illness may include lethargy, anorexia, weight loss, fever, and splenomegaly. Some dogs may recover from the clinical disease, but the majority progress in developing the subclinical disease (Neer, 1998) (Cohn, 2003; Harrus et al., 1999). The subclinical disease ranges from several weeks to years and the factors that influence the subclinical stage to enter the chronic stage is not readily known. Hematologic changes can be detected during this stage of infection but no clinical signs may be evident (Codner et al. 1986) (Cohn, 2003; Harrus & Waner, 2011). During the chronic stage of infection, canine ehrlichiosis resulting from *E. canis* can be difficult

to diagnose by molecular methods, while serological methods aid in the detection of circulating bacterial antibodies (Cohn, 2003; Harrus & Waner, 2011).

Dogs are treated with tetracyclines (Mylonakis et al., 2019), although the pathogen may not be cleared completely (Wen et al., 1997) (Harrus et al. 2012). Molecular detection methods are used to detect the infections, such as a PCR assay targeting 16S rRNA gene (Sirigireddy and Ganta 2005) (Alhassan et al., 2021) and the immunofluorescent-antibody (IFA) test, which is the most widely used test for diagnosis (Wen et al., 1997).

Ehrlichia ewingii

The first report of granulocytic ehrlichiosis in dogs is documented in 1971 where it is presumed to be an infection caused by *E. canis* (Goodman et al., 2003). *Ehrlichia ewingii* as the agent of canine granulocytic ehrlichiosis is established in 1992 (Anderson et al., 1992). *E. ewingii* and *A. phaogctophilum* are two known ehrlichial agents known to cause granulocytic infections (Cohn, 2003). It is difficult to differentiate granulocytic morulae between *A. phagocytophilum* and *E. ewingii* infections due to morphological similarities of morulae and having cell tropism to neutrophils. *E. ewingii* is predominantly prevalent in the southeastern and central United States where its tick vector; *A. americanum* is more prevalent. The most common clinical symptoms of dogs with *E. ewingii* infections are lameness and fever associated with joint swelling. Other symptoms include lethargy, vomiting, anorexia, weight loss, diarrhea, and head tilt (Goodman et al., 2003).

E. ewingii infections in humans are also recognized for the first time in 1999 (Buller et al., 1999; Parola et al., 2004). Clinical symptoms observed in people include fever, headache, and thrombocytopenia (Buller et al., 1999). Several documented cases of *E. ewingii* infections

represent HIV infected patients exhibiting fever, headache, nausea, and vomiting (Parola et al., 2004; Regan et al., 2013). Fatal infections are rare and clinical signs subside rapidly with appropriate therapies, such as treating with doxycycline (Cohn, 2003). *E. ewingii* infections transmitted through transfusion of platelets is also reported (Mcquiston et al., 2000; Regan et al., 2013). PCR and serological testing can be used for diagnosis of *E. ewingii* infections and patients can us doxycycline for treatment (Buller et al., 1999). Despite the public health importance, research is limited on human ewingii ehrlichiosis caused by *E. ewingii.* This is primarily because of the lack of an established cell culture system to grow the organism *in vitro.* Few studies documented the infection assessment of the pathogen using blood stabilate infections (De la Fuente et al., 2002; Nair et al., 2021).

Ehrlichia chaffeensis

In 1987, infection with an *Ehrlichia* organism in monocytes and having high homology to the canine ehrlichiosis agent, *E. canis,* is reported in a human patient with a history of tick bites (Maeda et al., 1987) (Dawson et al., 1991) (Anderson et al., 1991). Subsequently, detailed molecular characterization defined the human infection is caused by a new species, *Ehrlichia chaffeensis* (Anderson et al., 1991). Subsequently, the transmitting tick vector, *A. americanum*, and the reservoir host of the pathogen, white-tailed deer, are identified (Anderson et al., 1993) (Dawson et al., 1994) (Lockhart et al., 1997). Human monocytic ehrlichiosis caused by *E. chaffeensis* is now a well-recognized emerging disease in several parts of the US, particularly in southeastern states where the *A. americanum* tick is the most abundant species (Dumler, 1998) (Whitlock et al., 2000). The HME is also documented from all parts of the USA and from many countries around the world (Demma et al., 2005) (Paddock & Childs, 2003). *E. chaffeensis*

infection is also reported from several other vertebrate hosts including dogs, coyotes, and goats (Kocan et al., 2000) (Dawson, 1996; H. Liu & Ganta, 2019; Lockhart et al., 1997; Paddock et al., 2001; Wei et al., 2021). People infected with this pathogen may experience acute flu-like illness with symptoms like fever, headache, myalgia, chills, and anorexia (Walker et al., 2008) (H. Liu & Ganta, 2019; Paddock & Childs, 2003). There are several methods used to diagnose HME including serological testing, such as immunofluorescence assay (IFA), Western blot, enzyme linked-immunosorbent assay (ELISA), and PCR (Sirigireddy and Ganta, 2005) (Rikihisa, 2010) (Paddock & Childs, 2003). *E. chaffeensis* is treated with doxycycline (Everett et al. 1994)(Fishbein et al. 1994), while it is resistant to several antibiotics; ciprofloxacin, penicillin, erythromycin and telithromycin (Bakken and Dumler 1999) (Rolain et al., 2000) (Brouqui & Raoult, 1992)(Paddock & Childs, 2003). Little is known as what contributes to the resistance of *E. chaffeensis* to these antimicrobials. Doxycycline is the treatment of choice for people experiencing HME. The number of HME cases have been steadily increasing over the years. In the year 2000, about 200 cases of HME were reported and this number increased in 2019 to over 2,000 cases. Additionally, fatality due to HME has also increased averaging about 1% of cases (https://www.cdc.gov/ehrlichiosis/stats/index.html).

The life cycle of *E. chaffeensis* begins with the feeding of the lone star tick vector, *A. americanum*. This tick larvae acquires infections from an infected white-tailed deer, which is its major reservoir (Walker et al., 2004). The fed larvae molt into infected nymphs. The infected nymphs transmit *E. chaffeensis* to susceptible hosts during bloodmeal acquisition. Nymphal ticks can also acquire infection from an infected host and the infection is progressed to adult stage ticks serving also as the vectors for the pathogen transmission (Paddock & Childs, 2003). There are two forms of *Ehrlichia* that include the dense-cored cells (DC) and the reticulate cells

(RC) which live inside the vacuoles of mammalian cells (Zhang et al., 2007). DCs are the infectious form typically found near host cells or inside the early phagosomal vacuoles. DCs transform to RCs which then replicate within the phagosomal vacuoles (Popov et al., 1995; Zhang et al., 2007). The lifecycle of *E. chaffeensis* in macrophages and tick cells is very similar in having RC and DC forms (Dedonder et al. 2012). The pathogen progression stages include the attachment of the organism to the host cell membrane, its engulfment, replication within a morula by binary fission, and release of the organisms from infected host cells by complete host cell lysis or by exocytosis (Dedonder et al. 2012).

Bacterial Gene Regulation

Gene expression in both Gram-negative and Gram-positive bacteria is primarily controlled at the transcription level of a gene. An RNA polymerase (RNAP) core enzyme with a sigma (σ) factor provides a means for bacteria to rapidly accommodate diverse environmental changes suited for modifying the transcriptional profiles (Gruber & Gross, 2003) (Browning & Busby, 2016; Gunesekere et al., 2006). An RNAP holoenzyme is a multi-subunit complex having the core enzyme containing several subunits; two alpha (α), a beta (β), a beta′ (β′) and an omega (ω) and inclusion of a sigma (σ) factor (Chamberlin et al., 1983). A σ factor permits a core enzyme to specifically bind to a gene promoter region for initiating transcription to generate messenger RNA. The mRNA is then translated to produce the gene product--a protein. The number of σ factors differ depending on the environmental diversification of a bacterium (Kill et al., 2005). For example, *E. coli* contains 7 σ factors, while 109 σ factors are present in *Sorangium cellulosum* (Tripathi et al., 2014). Obligate intracellular bacteria having reduced genomes contain fewer σ factors (Darby et al., 2007). For example, *E. chaffeensis* has only two

σ factor genes; the primary housekeeping σ^{70} encoded by *rpoD* gene (ECH_0760) and the alternate σ^{32} gene made from *rpoH* gene (ECH_0655)(Dunning Hotopp et al., 2006) (GenBank # NC_007799.1).

To study gene regulation of σ^{70} in *E. chaffeensis*, our research team described an *in vitro* transcription system and an *E. coli* surrogate system (Faburay et al., 2011; H. Liu et al., 2013, 2016). The prior research revealed that the RNAP binding motifs (-10 and -35 regions) of *E. chaffeensis* genes share extensive homology and that they are recognized by RNAP with either one of its only two sigma factors; σ^{32} or σ^{70}, while affinities vary for the different gene promoters (H. Liu et al., 2013). Our research group reported that the *E. chaffeensis* chaperonin gene (Ech_0471) encoding for DnaK protein is transcribed primarily by σ^{32} (H. Liu et al., 2013). Genes regulated by σ^{32} respond to cellular responses under various stress conditions during the bacterial growth and are likely critical for *E. chaffeensis* survival in its hostile host environments similar to other Gram-negative bacteria (Chakrabarti et al., 1999; Delory et al., 2006; Du et al., 2005; Matsui et al., 2008; Sahu et al., 1994; Slamti et al., 2007) (Spector & Kenyon, 2012).

Much remains to be defined regarding the gene regulation of intracellular pathogens, such as *E. chaffeensis*. Specifically, regulation of gene expression to overcome host stress and adaptation to host environmental changes within its arthropod (tick) and vertebrate hosts for *E. chaffeensis* remain largely unknown. To extend our knowledge on how *E. chaffeensis* regulates its gene expression, we continued investigations in defining the functions of its RNAP holoenzyme comprising σ^{32} or σ^{70}. Transcription derived by an RNAP is typically implicated in recognizing and binding to DNA sequence motifs of a promoter; -10 and -35 regions, and the spacer sequences located between the two motifs of a gene promoter (Gross et al., 1998) (Paget & Helmann, 2003). Recently, our group described the mapping of *dnaK* gene promoter

recognized primarily by the *E. chaffeensis* RNAP containing σ^{32} using the previously developed *E. coli* surrogate system in the strain, CAG57101 (Koo et al., 2009a) (H. Liu et al., 2013). In *E. coli* CAG57101, its native *rpoH* gene (encoding for σ^{32}) is inactivated (Koo et al., 2009a) and in its place, *E. chaffeensis* σ^{32} from a plasmid is expressed which aided in mapping the bacterial *dnaK* promoter. Functional domains of *E. chaffeensis* σ^{32} are likely important for the RNAP function and its interactions with the -10 motif and the spacer sequence of *dnaK* have been reported (H. Liu & Ganta, 2019).

Chapter 2 - Scope of Thesis

Scope of the research

Despite the substantial progress made by our research team, the role of transcriptional regulators for RNAP function remains to be defined for *E. chaffeensis.* Transcriptional regulation in bacteria is accomplished by the interaction between the binding of *trans* factors to *cis*-regulatory elements present in a gene. In bacterial chromosomes, a transcriptional unit includes a regulatory region, the 5' untranslated region, a protein coding region and the downstream transcriptional termination segment (Balleza et al., 2009). A gene promoter segment contains *cis*-regulatory elements where transcriptional regulators typically bind to regulate gene expression (Browning & Busby, 2004). Transcriptional regulators facilitate altering gene expression in bacteria, including sensing host environment to support pathogenic bacterial differential gene expression (Browning & Busby, 2016). Understanding how transcriptional regulators influence gene expression in bacterial organisms, including obligate pathogenic bacteria such as *E. chaffeensis*, is critical in developing methods of control.

E. chaffeensis exists in two distinct forms: a smaller dense core cell (DC) and a larger reticulate cell (RC) (Rikihisa, 2006; Zhang et al., 2007). The infectious DC enters a host cell through phagocytosis, which then transforms into RC form. RCs replicate within a phagosome and then mature to DCs prior to the release and progressing to the new infectious cycle (Dedonder et al., 2012) (Zhang et al., 2007). Little is known about how this bacterium's phenotypic transformations occur. Bacterial gene expression is primarily regulated through controlling the transcription profiles. The transcriptional regulators contribute to differential gene expression by transforming bacteria from DC to RC and RC to DC forms, as well as in sensing tick and macrophage cell environments. *E. chaffeensis* genome has a limited number of

predicted transcription regulators; EcxR, CtrA, HU, MerR and Tr1 (Dunning Hotopp et al., 2006). Recent studies also suggest that EcxR, CtrA and Tr1 are active transcriptional regulators in *E. chaffeensis* (Z. Cheng et al., 2008, 2011; Duan et al., 2021). The homologs of EcxR; ApxR in *Anaplasma phagocytophilum* and ErxR in *Ehrlichia ruminantium* are identified as active in contributing to transcriptional regulation (Moumène et al., 2018; X. Wang, Cheng, et al., 2007; X. Wang, Kikuchi, et al., 2007). EcxR is shown to regulate type IV secretion apparatus genes during its intracellular development and auto regulates its gene expression (Z. Cheng et al., 2008). ApxR induces the putative transcription factor, Tr1, and a membrane protein gene encoding for the immunodominant pleomorphic 44 kDa protein, in addition to auto regulating its expression (X. Wang, Cheng, et al., 2007; X. Wang, Kikuchi, et al., 2007). ErxR in *E. ruminantium* is also found to regulate type IV secretion system protein gene (*virB*), a major antigenic protein gene and tr1 gene (Moumène et al., 2018). The EcxR homolog is also present in *Wolbachia* species and binds to the type IV secretion system gene promoters (Li & Carlow, 2012).

CtrA is known to control many cell-cycle events such as the initiation of DNA replication, methylation, flagellar biogenesis, and cell division (Domian et al., 1999). It belongs to the two component signal transduction systems response regulator family (Domian et al., 1999). CtrA *Caulobacter crescentus* is identified as serving the molecular switch controlling the coordination of the cell cycle and morphogenesis (Hallez et al., 2004). CtrA is identified as a response regulator engaged in facilitating rapid environmental changes within a bacterial cell (Wuichet et al., 2010). The first evidence of CtrA as a response regulator in *E. chaffeensis* was reported during its development to DC form (Z. Cheng et al., 2011). *E. chaffeensis* CtrA binds to

the promoter regions of several genes, however, there is still much to be discovered on how *E. chaffeensis* regulates its gene expression in a host to support its growth.

MerR is the regulator in Gram-negative bacterial mercury resistance (*mer*) operons under the MerR family of transcriptional activators (Brown et al., 1984;1986) (Barrineau et al., 1984;1985) (Brown et al., 2003). It activates or represses *mer* genes by binding within the spacer region located between the -35 and -10 promoter elements (RNAP binding motifs). Activation of transcription by MerR involves its bending at an unusually long spacer region in order to realign the two promoter elements (Heltzel et al., 1987; Lund & Brown, 1989) (O'Halloran & Walsh, 1986). MerR family regulators are also known to respond to metal (Brocklehurst et al., 1999; Outten et al., 2000; Rutherford et al., 1999). The role of MerR for *E. chaffeensi*s gene regulation is yet to be defined.

Tr1 is a transcriptional regulator that is predicted to regulate the expression of 28 kDa outer membrane proteins in *E. chaffeensis* (Duan et al., 2021). This regulatory ortholog is conserved in both *Ehrlichia* and *Anaplasma* species pathogens. It contains the DNA-binding motifs of the helix-turn-helix and is highly conserved. Understanding the role of Tr1 in gene expression and host adaptation will aid in developing novel therapeutic targets to prevent infections caused by these intracellular bacteria (Duan et al., 2021).

HU belongs to histone binding protein (HBP) family. Histones are small DNA binding proteins that are highly conserved in prokaryotes and facilitate DNA packaging as nucleosomes (McMacken et al. 1987). HU is a small, abundant DNA-binding protein that can wrap DNA. It is highly conserved in many bacteria (Drlica & Rouviere-Yaniv, 1987). HU binds to double and single strand DNAs as well as to RNA (Ghosh, 2004; Rouviere Yaniv & Gros, 1975).

Despite recent limited studies in defining *E. chaffeensis* transcriptional regulation, particularly, the CtrA and EcxR, much remains to be defined regarding how and when the five regulatory proteins are expressed and how they contribute to bacterial gene expression. My current research as partial fulfillment of the masters degree seeks to address this gap of knowledge.

Chapter 3 - Materials and Methods

Cultivation of *E. chaffeensis*

E. chaffeensis Arkansas isolate (ATCC # CRL-10389) was cultivated in either DH82 macrophage cells or ISE6 embryonic tick cells. These cell types were grown and sustained as described previously (C. Cheng & Ganta, 2008). The host cells were infected with either mutant (MerR-HA, EcxR-HA, or HU-HA) or wild-type *E. chaffeensis* and grown to 80-90% infectivity and used for subsequent experiments. Infected cultures were monitored using Cytopro® 7620 Cytocentrifuge Rotor and stained using Hema 3 solutions (C. Cheng & Ganta, 2008).

DNA isolation

DNA was isolated from host bacteria using DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) from a single T25 DH82 or ISE6 cell culture flask (Invitrogen Life Technologies, Carlsbad, CA).

PCR for validation of tagged mutagenesis experiments

Purified DNA samples from the cultures of MerR-HA, EcxR-HA, and HU-HA were assessed by polymerase chain reaction to validate the tagged mutagenesis experiments. Primers used for these three PCR experiments are listed in Table 1. The PCR analysis was performed in a 20 µl reaction mixture using Q5® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) shown in Table 2. The reaction was carried out under these conditions: initial denaturation at 98°C for 30 seconds, 98°C for 10 seconds, followed by 35 amplification cycles of 57-60°C for 30 seconds, 72°C for 2 minutes and a final extension at 72°C for 2 minutes and hold at 12°C until needed.

Table 1. Primers used in PCR to detect gene targets from tagged mutagenesis experiments

Primer sequences

Primers	Sequence (5' to 3')	Orientation	Target Gene
RG919	TTATACAACTGCTACACCAAGA	Forward	*merR*
RG94	AAGCAAATGCTTTAGGTGCAT	Forward	*merR/ecxR*
RG925	CACTAGCTGCAGGAGCTTCT	Reverse	*merR*
RG97	TCCGCAGGATGTTTCACATA	Reverse	*merR/ecxR*
RG1472	CCTGGATGTTGAACAATATGTAC	Forward	*merR*
RG1470	CCCTTAAGCTTAACATATCATCTAG	Reverse	*merR*
RG2153	TTATGCATAATCAGGAACATCATAAG	Reverse	*merR-HA*
RG907	GCAAGCACAGTTTGATCC	Forward	*ecxR*
RG912	GTCTATTTTCATATTGACCAGAAGA	Reverse	*ecxR*
RG913	CTTTCTCTATTTAAGTACAGCA	Forward	*hup*
RG918	AAGGTTAGGTATAGATAGAGTAT	Reverse	*hup*

Table 2. PCR Reaction Mixture

Reagents	Volume (µL)
5X Q5 Reaction Buffer	5
10 mM dNTPs	0.5
10 µM Forward Primer	1.25
10 µM Reverse Primer	1.25
Template DNA	2
Q5 High-Fidelity DNA Polymerase	0.25

Nuclease-Free Water	14.75

RNA isolation

RNA was isolated from host cell-free bacteria (described in later section) using Invitrogen TRIzol™ Reagent from a single T25 DH82 or ISE6 cell culture flask (Invitrogen Life Technologies, Carlsbad, CA). One milliliter of cell suspension from either DH82 or ISE6 tick cell culture was centrifuged at 18,000 x g for 10 minutes at 4°C. The pelleted samples were resuspended in one milliliter of Trizol reagent and stored at -80°C until needed. For RNA isolation, frozen samples were thawed and 200 µl of chloroform was added then agitated for 15 seconds and left to stand for 15 minutes at room temperature. Samples were centrifuged at 12,000 x g for 15 minutes using the Avanti J-26 XPI high speed centrifuge (Beckman Coulter Life Sciences, Indianapolis, IN) at 4°C to allow for phase separation. The colorless aqueous phase was transferred to a new tube and 500 µl of isopropanol was added then allowed to stand for 10 minutes at room temperature. The collected aqueous phase was then centrifuged at 12,000 x g for 10 minutes at 4°C. After centrifugation, the supernatant was discarded, and the RNA pellet was mixed with 1 milliliter of 75% ethanol then centrifuged at 7,500 x g for 5 minutes at 4°C. The supernatant was discarded, and the RNA pellet was air-dried for 5-10 minutes, resuspended in 50 µl of nuclease-free water, and stored at -80°C until needed. Isolated RNA was further purified using the Monarch® RNA Cleanup Kit (New England Biolabs, Ipswich, MA) as per manufacturer specifications. Frozen RNA samples were thawed and 100 µl of RNA Cleanup Binding Buffer was added. The mixture was then resuspended with 150 µl of 100% ethanol and loaded onto a column containing a collection tube. The RNA sample was then centrifuged at 16,000 x g for 1 minute at room temperature. The flow-through was discarded and 500 µl of

RNA Cleanup Wash Buffer was added to wash the sample then centrifuged at 16,000 x g for 1 minute at room temperature. The sample was washed one additional time and centrifuged again to remove excess traces of ethanol and salt. The column was then transferred to a fresh tube and the sample was eluted using 20 µl of nuclease-free water and stored at -80°C until needed.

Table 3. Primers used in qRT-PCR for detecting the expression of the five gene regulators in *E. chaffeensis*

Primer sequences

Primers	Sequence (5' to 3')	Orientation	Target Gene
RG2162	GCA CTA GAA ACT TCT CAT CTA A	Forward	*hup*
RG2163	GAG CTA GTG TTT CTA ACT TCC	Reverse	*hup*
RG2165	GTG ATG ATA GAG GAG GAT ATA GA	Forward	*ctrA*
RG2166	TG CTT CCT CAA CAT ACT TT	Reverse	*ctrA*
RG2168	GGA ATC ACT TTC CAA CAA GTA	Forward	*tr1*
RG2169	AAC GTT AAG TAC GCT TGC	Reverse	*tr1*
RG2171	AAT GAT TAC GGC ACT AAG TAT AA	Forward	*ecxR*
RG2172	GGT CTA CGC CCA GTA TC	Reverse	*ecxR*
RG2174	TTT CCT CAG GTT AAT CCA AT	Forward	*merR*
RG2175	TGC TGC ACA CCT TTA ATC	Reverse	*merR*
RG2180	CAA GTC GAA CGG ACA AT	Forward	*16s*
RG2182	T TCT AAT GGC TAT TCC ATA CTA C	Reverse	*16s*

Table 4. Probes used in qRT-PCR for detecting the expression of the five gene regulators in *E. chaffeensis*

Probe sequences

Probes	Sequence (5'- 3')	Target Gene
RG2164	/56-FAM/TG GAA CAC T/ZEN/A CAT GTT ATC CAA TGT GCA GA/3IABkFQ/	*hup*
RG2167	/56-FAM/CA ACA TTG G/ZEN/C ACC ACC ATG ATC CC/3IABkFQ/	*ctrA*
RG2170	/56-FAM/AG CCT GCT A/ZEN/A TCA CTA TAC GGT TTG TTC C/3IABkFQ/	*tr1*
RG2173	/56-FAM/TC ACT GGA A/ZEN/C CAA GTA ACC ACA GCA /3IABkFQ/	*ecxR*
RG2176	/56-FAM/AC GTC GTG G/ZEN/T AGA AGA TTG TAT TCA CAA GT/3IABkFQ/	*merR*
RG2182	/56-FAM/CC CGT CTG C/ZEN/C ACT AAC AAT TAT TTA TAA CC/3IABkFQ/	*16s*

Table 5. One-Step qRT-PCR Reaction Mixture

Reagents	Volume (µL)
SuperScript™ III RT/Platinum™ *Taq* Mix	0.4
2X Reaction Mix	10
Forward Primer, 10 µM	0.4
Reverse Primer, 10 µM	0.4
Fluorogenic Probe, 10 µM	.2
Template	4
Nuclease-free water	4.6

DNase treatment of RNA

RNA concentrations were determined using a Nanodrop 8000 (NanoDrop Technologies, Inc., Wilmington, DE). RNA samples were treated using the TURBO DNA-*free*™ Kit (Invitrogen Life Technologies, Carlsbad, CA) to remove residual DNA. DNase treatment of the RNA samples was carried out using a 50 µl reaction mixture including 1µl of TURBO DNase™ enzyme, 10X TURBO DNase™ buffer, and nuclease-free water. The mixture was incubated at 37°C for one hour and following incubation, the sample was resuspended using 5 µl of DNase inactivation reagent followed by incubation at room temperature for 5 minutes with gentle mixing 2-3 times during incubation. Samples were then centrifuged at 10,000 x g for 2 minutes at 4°C. The supernatant was collected in a fresh tube and stored at -80°C until further use.

qRT-PCR for the determination of relative gene expression

Purified RNA samples from time course experiments were assessed by one-step quantitative reverse transcriptase PCR (qRT-PCR) to detect the relative gene expression for five genes, including *ecxR* (ECH_0795), *tr1* (ECH_1118), *ctrA* (ECH_1012), *merR* (ECH_0163) and *hup* (ECH_0804) by using the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA) similar to the method described previously (Sirigireddy & Ganta, 2005). The primer and probe sequences used in these experiments are listed in Table 1 and Table 2. The qRT-PCR analysis was performed in a 20 µl reaction mixture using the SuperScript™ III RT/Platinum™ *Taq* Mix (Invitrogen Life Technologies, Carlsbad, CA) and carried out at these temperature cycles: reverse transcription at 50°C for 30 minutes, 95°C for 3 minutes, followed by 40 amplification cycles of 95°C for 15 seconds, 50°C for 30 seconds and 60°C for 1 minute. The reaction mixture is detailed in Table 3.

Synchronous culture and renografin density gradient purification of cell-free *E. chaffeensis*

E. chaffeensis Arkansas isolate (ATCC # CRL-10389) was cultivated in DH82 cells. The whole cell count was determined using Countless™ II Automated Cell Counter (Thermo Fisher Scientific, Rockford, IL) before harvesting the infected flasks. Ten µl of cell suspension was removed from each flask and added to a microcentrifuge tube and 10 µl of 0.4% trypan blue stain was resuspended with the cell suspension. The stained cell suspension was loaded into a chamber of the sample slide and settled for 30 seconds before viewing. When cells reached greater than 90% of infectivity (10 x 10^6 cells per ml), cells from culture flasks were recovered and centrifuged at 12,000 x g for 5 minutes at 4°C. The supernatant was then discarded, and the cell pellet was resuspended in 5 ml of media. The cell suspension was homogenized using a 27g bent needle (15-20 pushes) to release cell-free bacteria. Cell debris and remaining unbroken cells were removed by centrifugation at 4,000 x g for 5 minutes at 4°C. The supernatant was recovered and filtered using a syringe and a 1.6 µm filter for synchronous culture and 2.0 µm filter for density gradient purification. The filtrate containing the cell-free bacteria was centrifuged at 18,000 x g for 10 minutes at 4°C. The pellet was used for either synchronous culture or density gradient purification.

For synchronous culture, the pellet was resuspended in 10 ml of fresh media and incubated with 5 x 10^6 uninfected DH82 cells at 37°C for 2 hours to obtain synchronous infection. The bacterial-host cell mixture was then washed with cold 2× phosphate-buffered saline (274 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4, 4 mM KH_2PO_4; pH 7.4) three times (2000 g, 5 min) to remove the unbound bacteria. The final pelleted cells were resuspended in 25 ml fresh media, evenly separated into 5 T25 flasks, and incubated at 37°C. Samples were

collected at this time point (designated 0 h post infection [p.i.]) and at 24, 48, 72 and 96 h p.i. by centrifugation at 2,000 × *g* for 5 min. The pellets from 1 ml culture (4 repeats) and 2 ml culture (5 ml culture total) were resuspended in 1 ml Invitrogen TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA) to be stored at -80°C for RNA isolation.

For renografin density gradient method, prepared renografin layers in different concentrations (25%, 35%, and 45%) in sterile ultra-centrifuge tubes. Resuspend the pellet in sterile 1X DPBS (Dulbecco's phosphate buffered saline) and carefully add the cell free suspension on top of the 25% renografin layer. Placed the tube with the layers and cell suspension in the ultracentrifuge buckets (S50-ST swinging bucket rotor) and centrifuged at 100,000 x g for 1 hour at 4°C. Carefully removed the tubes after centrifugation and looked for the turbid layers and collected each layer in 15 ml falcon centrifuge tubes. First removed the top, clear layer, then the second layer (junk), third layer (reticulate cells RC), and fourth layer (dense core cells DC). Washed the layers two times with 5 ml 1XDPBS each and transferred the layers into clean ultra-centrifuge tubes and spun them at 100,000 x g for 15 minutes at 4°C. The supernatant was removed, resuspended the final RC and DC pellets in 1ml 1XDPBS and transferred them to fresh 1.7 ml microcentrifuge tubes. Spun the collected suspension at 18,000 x g for 15 minutes at 4°C. The final pelleted cells were stored at -80°C for further use in Western blot experiments.

SDS-PAGE and Western blot

Purified *E. chaffeensis mutant* (MerR-HA, EcxR-HA, or HU-HA) recovered from DH82 cells were recovered by centrifugation at 18,000 x g for 15 minutes at 4°C. The pellet was resuspended with NP-40 buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40) containing protease inhibitor (Thermo Fisher Scientific, Waltham, MA) and 1 mM pefabloc SC

(MilliporeSigma, Burlington, MS) and homogenized using Fisher Scientific 60 Sonic Cell Dismembrator (Fisher Scientific, Hampton, NH). Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). In brief, a standard curve was prepared using BSA stock solution diluted in 1x PBS with varying protein concentrations. The protein samples were added to a 96-well plate and similarly serially diluted BSA was transferred to the plate. The samples were incubated at 37°C for 30 minutes and then the OD was read using the Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT) at 562 nm wavelength. The protein concentration of unknown samples was determined by comparing with the known BSA standards plotted using MS Excel graph.

The protein samples were resuspended with an appropriate amount of 5X SDS loading buffer and boiled for 5 minutes. The samples were loaded in pre-made Novex™ 10-20% tricine gels (Invitrogen Life Technologies, Carlsbad, CA) and then separated by SDS-PAGE at 125 V for 45 minutes to 1 hour. After electrophoresis, the resolved protein gels were either used for colloidal blue staining or for Western blotting analysis. Gels were stained using a Colloidal Blue Staining Kit (Invitrogen Life Technologies, Carlsbad, CA) as per the manufacturer's protocol. For Western blot analysis, resolved proteins from the gels were transferred to a PVDF 0.2 µm membrane (Thermo Fisher Scientific, Rockford, IL) by subjecting to the voltage; 90 V for 1 hour. The membranes were then placed in 5% blocking buffer (1XTBST with nonfat dry milk) at room temperature for an hour. The primary antibody: Anti-HA (Human influenza hemagglutinin) tag rabbit polyclonal antibody (Abcam, Waltham, MA) was diluted 1:4000 in 5% blocking buffer and added to the membrane and incubated overnight at 4°C. The membrane was then washed five times (5-minutes each time) with 1X TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween® 20 detergent). Post washing, the membrane was incubated with the secondary

antibody: goat anti-rabbit lgG conjugated to horseradish peroxidase (HRP) (Invitrogen Life Technologies, Carlsbad, CA) diluted 1:5000 in 5% blocking buffer for 1 hour at room temperature and followed by washing as described above. The HRP on immunoblots was detected using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL) as per the manufacturer's protocol and visualized using iBright™ CL1500 Imaging System (Thermo Fisher Scientific, Rockford, IL).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation, sequencing, crosslinking and chromatin sonication of cultured cells

The cultivation of *E. chaffeensis* described as above. The host cells (DH82) were infected with wild-type *E. chaffeensis* and grown to 80-90% infectivity for use in subsequent experiments. After the culture media was removed, 30 ml Crosslinking Solution (1% formaldehyde in MEM media lacking FBS) was added directly to the infected flask for crosslinking of proteins with DNA (Mukhopadhyay et al., 2008). The flask was incubated with gentle rocking at room temperature for 30 minutes then 3.3 ml of quenching solution (2.5 M glycine pH 7.5) was added to the crosslinked cells and incubated with rocking for an additional 15 minutes at room temperature. Crosslinked cells were then centrifuged at 3,500 x g for 10 minutes at 4°C. Cells were washed with 10 ml ice-cold 1X PBS buffer twice and centrifuged after each wash as stated previously. After washing, the pellet was resuspended with an appropriate volume of 1x PBS to allow for 10 x 10^6 cells/ml in 1.7 ml microcentrifuge tubes. The resuspended cells were centrifuged at 16,000 x g for 10 minutes at 4°C. The supernatant was removed, and the pellet was stored at -80°C until further use. The crosslinked cells were then resuspended in 500 µl of lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% sodium

deoxycholate, 1% NP-40, 0.1% SDS) using 1.7 ml microcentrifuge tubes and incubated on ice for 10 minutes. The cells were then lysed by sonication using Fisher Scientific 60 Sonic Cell Dismembrator (Fisher Scientific, Hampton, NH) with 15 pulses for 20 seconds each burst at a power setting 4 on ice. Post sonication, the samples were centrifuged at 20,000 x g for 30 minutes at 4°C to clarify lysate. The lysate supernatant was carefully transferred to a fresh 1.7 ml microcentrifuge tube without dislodging and kept on ice.

Validation of chromatin shearing efficiency

Ten µl aliquots of lysate supernatant for each sample were separated into fresh PCR microcentrifuge tubes for validating the efficiency of chromatin sonication and the remaining lysate supernatants were frozen for future experiments. Ninety µl of TE buffer and 1 µl of RNase A were added to 10 ul aliquots of lysate supernatant and vortexed to mix. The samples were then incubated at 37° C for 30 minutes in the T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). After 30 minutes, 1 µl of proteinase K was added to each tube and mixed. The samples were incubated in the thermal cycler at 55° C for 30 minutes and then the temperature was increased to 80° C for 2 hours. The samples were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The concentration of the sample DNA was determined using the Nanodrop 8000 (NanoDrop Technologies, Inc., Wilmington, DE). 500 ng of input DNA was transferred to a PCR microcentrifuge tube and 1 µl of 500 mM NaCl was added and the final volume was adjusted to 10 µl using nuclease-free water. The samples were heated in the Thermal Cycler at 100° C for 20 minutes followed by another incubation at 50° C for 1 minute. Next, the samples were incubated at room temperature for 5 minutes. Purple loading dye was added to each sample and gel electrophoresis was performed on a 1.5% agarose gel. The DNA appeared as a smear between 200 to 1200 bp.

Chromatin Immunoprecipitation

Sonicated chromatin lysates were thawed on ice and samples were centrifuged at 20,000 x g for 2 minutes at 4°C. Protein G sepharose beads (Abcam, Waltham, MA) were prepared by aliquoting 30 µl of beads to a 1.5 ml microcentrifuge tube. 30 µl of TE buffer pH 8.0 was added, mixed well and then centrifuged at 1,250 x g for 1 minute. The supernatant was removed and this step was repeated two more times. The sonicated chromatin lysate was added to the prepared protein G sepharose beads and incubated for 2 hours at 4°C on a rotating incubator for pre-clearing crosslinked lysate. The protein G sepharose beads were pelleted by centrifugation at 1,300 x g for 3 minutes at 4°C. The lysate was carefully removed without disturbing the protein G sepharose bead pellet and transferred to a new tube. The lysate volume was measured and lysis buffer was added to raise the volume to 630 µl. Thirty µl of thawed chromatin lysate was transferred as input DNA into a 1.7ml microcentrifuge tube and store at -20°C. The remaining chromatin lysate was divided into two microcentrifuge tubes: one tube was used as the negative control for ChIP by Rabbit IgG (Abcam, Waltham, MA) and the other tube was used for ChIP by the Anti-HA (Human influenza hemagglutinin) tag antibody (Abcam, Waltham, MA). Each tube contained 300 µl of chromatin lysate. Lysis buffer was added to each 300 µl sample to increase the volume to 600 µl. The samples were incubated overnight at 4°C with rotation.

Protein A sepharose beads were aliquoted (60 µl each) in separate tubes. To which, 180 µl of chilled lysis buffer was added and mixed. The beads were then centrifuged at 1,000 x g for 1 minute at room temperature. Carefully, supernatants were removed without disturbing the beads and this step was repeated two more times. The reactions were incubated overnight 4° C overnight and then centrifuged at 1,250 x g for 1 minute, and the ChIP reaction samples were added to the washed Protein A Sepharose beads. The samples were incubated on an end-to-end

rotator for 3 hours at 4°C to allow for the binding of antibody:protein complexes to beads. Reaction products were then centrifuged at 1,300 x g for 1 minute at 4° C to pellet beads and the supernatant was carefully removed. Protein A Sepharose/antibody:protein complexes were washed several times with the following washes: Two washes were performed using a low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 7.5), then two more washes using a high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris HCl pH 7.5), followed by one wash using LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0), and finally three washes were performed using 1 x TE wash buffer. Protease inhibitor was added to each wash solution (Thermo Fisher Scientific, Waltham, MA) and 1 mM pefabloc SC (MilliporeSigma, Burlington, MA). The washes were performed 5 minutes each time by gently rotating at 4°C. Between each wash, the complexes were centrifuged at 1,000 x g for 1 minute at 4° C to pellet beads and supernatants were discarded.

Reversal of cross-links and DNA purification

Immunoprecipitation (IP) elution buffer (1% SDS, 100 mM $NaHCO_3$,) was prepared and pre-heated at 65° C. 75 µl of IP elution buffer was added to the beads for each sample. Then, the samples were vortexed at low speed for 15 minutes at room temperature by taping the sample tubes to the vortex mixer. The samples were centrifuged at 1,000 x g for 2 minutes to pellet beads and then the supernatant was transferred to a new tube before repeating the elution procedure. This step was repeated one more time. About 150 µl of sample was recovered after the two elutions. 30 µl of input DNA sample was thawed and the volume was increased to 150 µl using IP elution buffer. To reverse-crosslink the samples, 6 µl of 5 M NaCl was added and incubated at 65° C in a dry bath overnight. One µl of RNase A was added to each sample

(including the input DNA) and incubated at 37°C for 30 minutes, the mix was incubated for additional 2 hours at 45° C by adding 3 µl of 0.5 M EDTA, 6 µl of 1 M Tris-HCl (pH6.5), and 2 µl of proteinase K (10 mg/ml). The DNA was purified by using the QIAQuick PCR Purification Kit as per manufacturer specifications. After purification, the DNA was eluted using 50 µl of pre-heated EB buffer, centrifuging at 17,900 x g for 1 minute to collect the DNA, and stored at -80° C for use in downstream applications.

Whole genome amplification for ChIP sequencing

For the ChIP samples, the concentration of the eluted DNA is usually too low to get an accurate quantitation. The entire 50 µl of eluted DNA was lyophilized and resuspended in 10 µl of nuclease-free water. The resuspended DNA was processed by using the Sigma GenomPlex WGA kit (MilliporeSigma, Burlington, MA) for whole genome amplification as per previous study (Henriette O'Geen, etc Bioteches, 2006 November; 41(5): 577-580). After the ChIP DNA and input DNA were quantified using Qubit 2 fluorometer, samples were sent for ChIP-seq at the Genomic High-Throughput Facility, University of California, Irvine.

Statistical Analysis

Statistical analyses were performed using ANOVA and Tukey Test and a P-value of <0.01 and <0.05 was considered significant.

Chapter 4 - Results

Confirming the presence of *E. chaffeensis* tagged mutations with a HA tag at 3' end of MerR, EcxR and HU by PCR cultivated in DH82 cells

The previously described targeted mutagenesis method used by our research team (Y. Wang et al., 2020) was employed to generate tagged mutations to generate C-terminal end insertion of hemagglutinin (HA) sequence as part of the MerR, EcxR, and HU proteins from the respective genes. This work was performed by Dr. Huitao Liu et al. (unpublished data) as depicted in Figures 4.1, 4.2, and 4.3. *E. chaffeensis.* Genomic DNA recovered from the MerR-HA tagged mutant construct cultured in DH82 cells was used to perform PCR analysis to confirm that the correct mutants were used in the study. First, a PCR assay was performed using primers targeting the genomic region upstream to the insertion site and the inserted segment which yielded the predicted amplicon for the mutant-derived genomic DNA (Figure 4.4, lane 1), but not for wild-type *E. chaffeensis* genomic DNA (Figure 4.4, lane 4) (predicted outcome). Similarly, a second PCR assay was performed using primers targeting the downstream region to the insertion site and the inserted segment. The predicted amplicon was found in the mutant, but not in wild-type genomic DNA (predicted outcome, Figure 4.4, lanes 2 and 5, respectively). The 3rd PCR assay performed targeting upstream and downstream of the inserted fragment where a larger fragment is anticipated for the mutant, but not for the wild-type *E. chaffeensis* (Figure 4.4, lanes 3 and 6, respectively). The data confirm that the targeted mutant used in the study is correct for MerR tagged mutant. Similarly, PCR experiments were performed to confirm the EcxR-HA and HU-HA tagged mutant strains, as detailed in Figure 4.5 and 4.6, respectively.

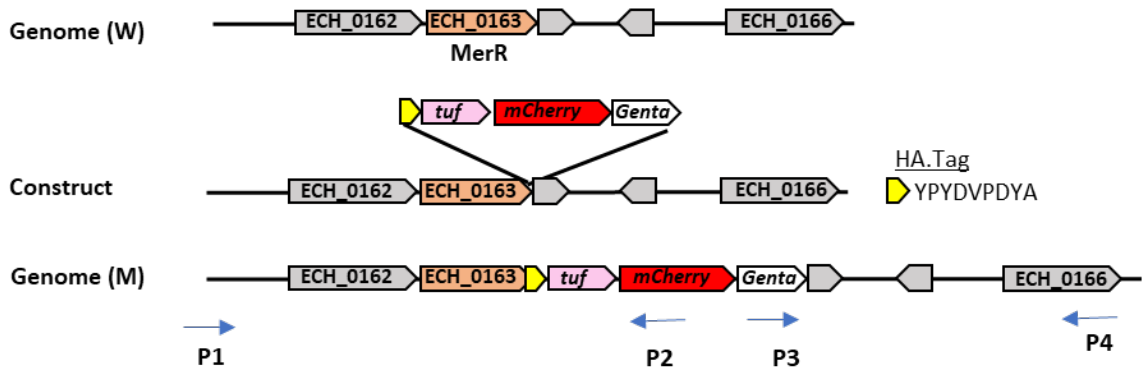

Figure 4.1. Genomic segment illustration of MerR-HA

An illustration depicting the genomic segment spanning the region selected for preparing the allelic exchange construct for MerR-HA. P1, P2, P3, and P4 are the indicated primers; RG919, RG97, RG94, and RG925, respectively, targeting the genomic regions upstream and downstream to the allelic insertion (primers; P1 and P4) and the inserted DNA (primers; P2 and P3)

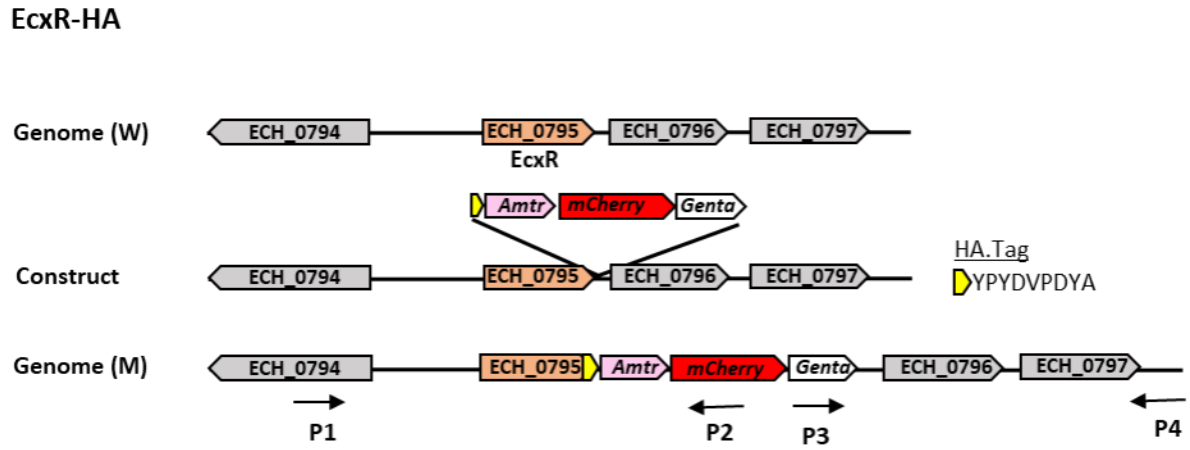

Figure 4.2. Genomic segment illustration of EcxR-HA

An illustration depicting the genomic segment spanning the region selected for preparing the allelic exchange construct for EcxR-HA. P1, P2, P3, and P4 indicate the primers; RG907, RG97, RG94, and RG912, respectively, targeting the genomic regions upstream and downstream to the allelic insertion (primers; P1 and P4) and the inserted DNA ((primers; P2 and P3).

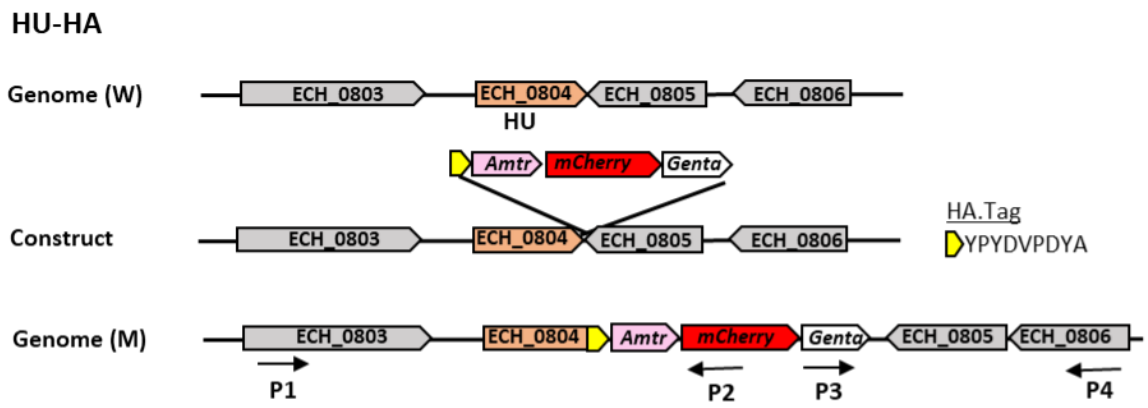

Figure 4.3. Genomic segment illustration of HU-HA

An illustration depicting the genomic segment spanning the region selected for preparing the allelic exchange construct for HU-HA. P1, P2, P3, and P4 are the indicated primers; RG919, RG97, RG94, and RG925, respectively, targeting the genomic regions upstream and downstream to the allelic insertion (primers; P1 and P4) and the inserted DNA (primers; P2 and P3).

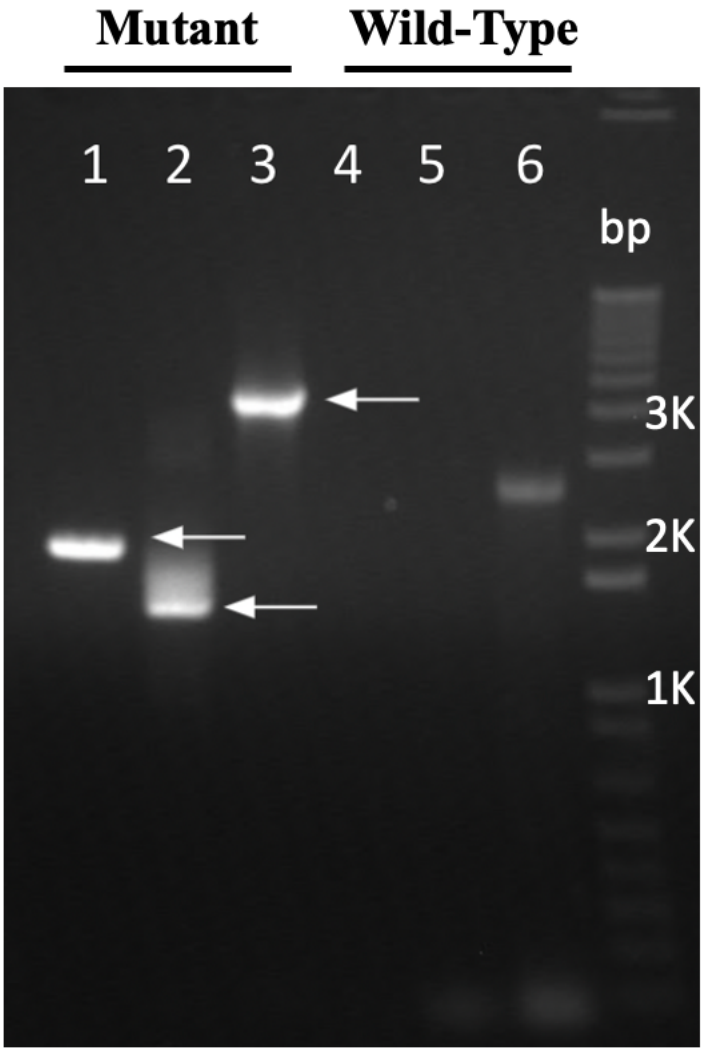

Figure 4.4. Validation of targeted mutagenesis experiments of MerR-HA

Validation of the targeted mutagenesis experiments at the DNA level from DH82 cell line for MerR-HA mutant construct. Lane 1 represents the gene segment upstream from the insertion site using primers P1 and P2 as indicated in Figure 4.1. Lane 2 represents the gene segment downstream from the insertion site using primers P3 and P4 as indicated in Figure 4.1. Lane 3 represents the gene segments upstream and downstream to the insertion site using primers P1 and P4. Lanes 4 through 5 represent the same gene segment targets as stated previously using wild-type *E. chaffeensis* as the template with the same respective primers.

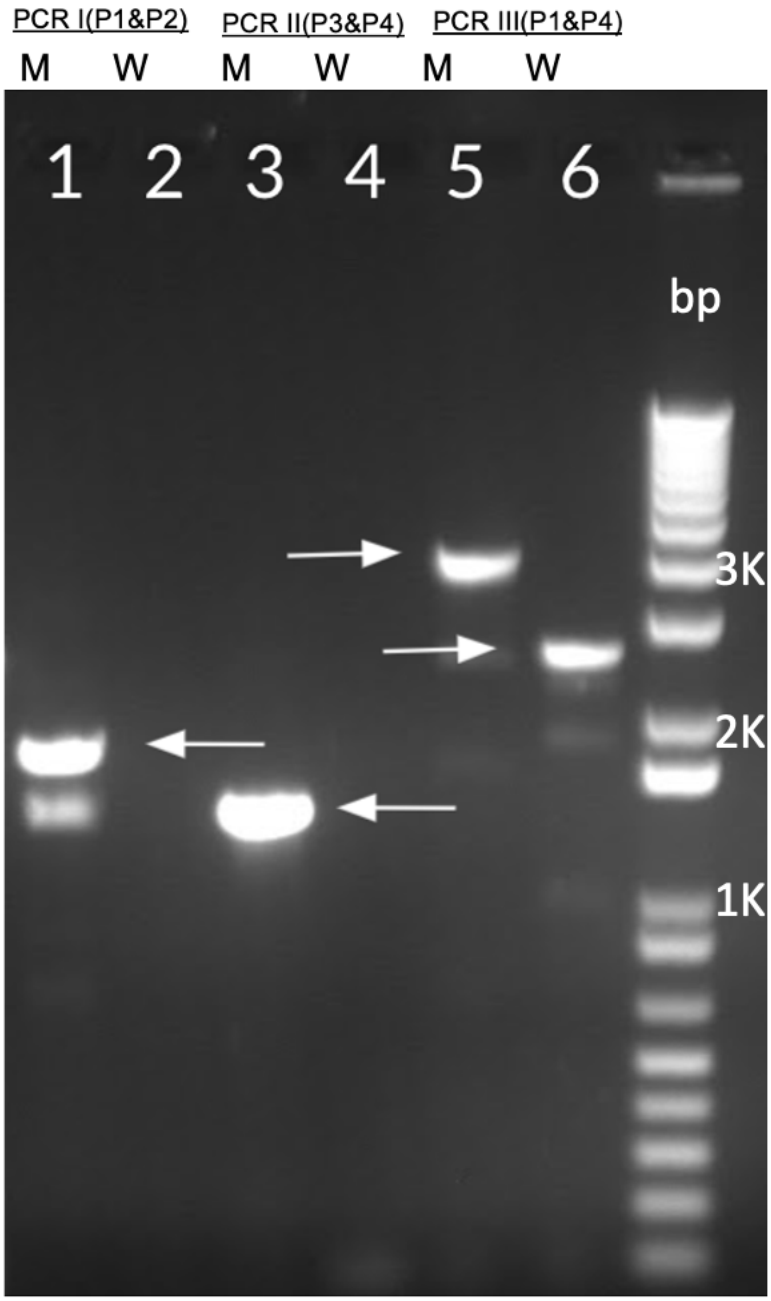

Figure 4.5. Validation of targeted mutagenesis experiments of EcxR-HA

Validation of the targeted mutagenesis experiments at the DNA level from DH82 cell line for EcxR-HA mutant construct. Lane 1 represents the gene segment upstream from the insertion site using primers P1 and P2 as indicated in Figure 4.3. Lane 3 represents the gene segment downstream from the insertion site using primers P3 and P4 as indicated in Figure 4.3. Lane 5 represents the gene segments upstream and downstream to the insertion using primers P1 and P4. Lanes 2, 4, and 6 represent the same gene segment targets respectively as stated previously using wild-type *E. chaffeensis* as the template and the same primers as stated in lane order.

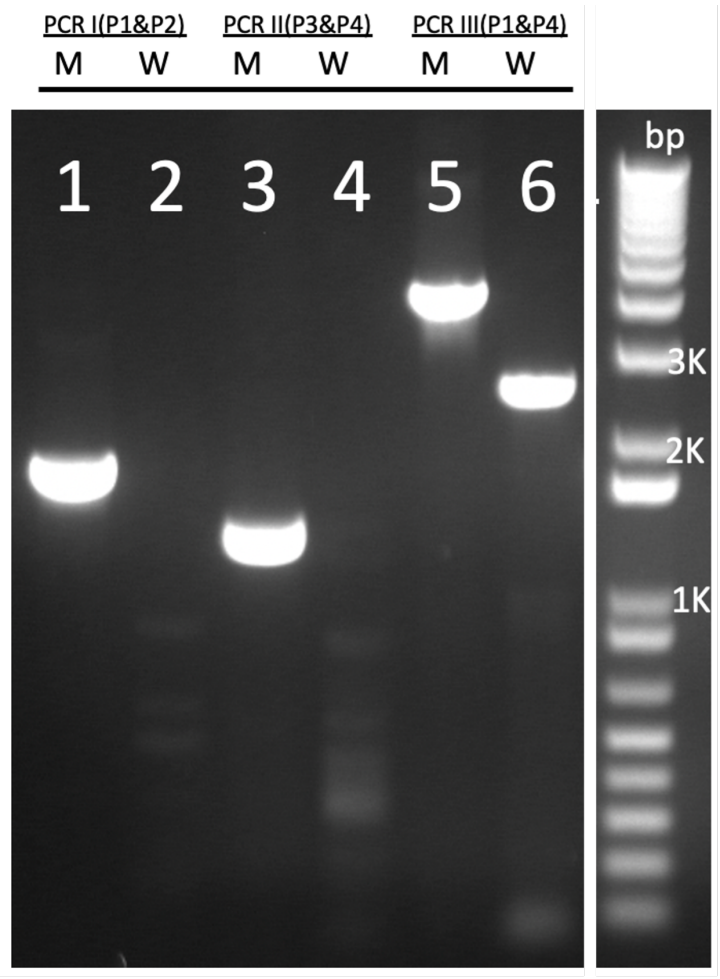

Figure 4.6. Validation of targeted mutagenesis experiments of HU-HA

Validation of the targeted mutagenesis experiments at the DNA level from DH82 cell line for HU-HA mutant construct. Lane 1 represents the gene segment upstream from the insertion site using primers P1 and P2 as indicated in Figure 4.5. Lane 3 represents the gene segment downstream from the insertion site using primers P3 and P4 as indicated in Figure 4.5. Lane 5 Lane 3 represents the gene segments upstream and downstream to the insertion site using primers P1 and P4. Lanes 2, 4, and 6 represent the same gene segment targets respectively as stated previously using wild-type *E. chaffeensis* as the template and the same primers as stated in lane order.

Assessing MerR transcription in *E. chaffeensis* cultured in canine macrophage (DH82) and ISE6 tick cell cultures

MerR-HA tagged *E. chaffeensis* synchronized cultures were grown at different time points and RNA was isolated for use in assessing MerR expression DH82 cultures. Similarly, the bacterial RNA was recovered from ISE6 tick cells. The following time points were selected for the RNA analysis: 0 hpi (hours post infection), 24 hpi, 48 hpi, and 72 hpi. RT-PCR analysis was performed using gene-specific primers (RG1472 and RG2153) and used the following temperature cycles: reverse transcription at 52°C for 30 minutes, 94°C for 2 minutes, followed by 40 amplification cycles of 94°C for 15 seconds, 50°C for 30 seconds, 68°C for 1 minute, a final extension at 68°C for 5 minutes and a 12°C hold at until needed. The reaction mixture is detailed in Table 6.

Table 6. RT-PCR Reaction Mixture

Reagents	**Volume (µL)**
SuperScript™ III RT/Platinum™ *Taq* Mix	0.5
2X Reaction Mix	12.5
Forward Primer, 10 µM	0.5
Reverse Primer, 10 µM	0.5
Template	2
Nuclease-free water	9

This analysis aided in defining the RNA expression. MerR RNA PCR positives were observed only in macrophage cultures (Figure 4.7), but ISE6 tick cell culture-derived RNA was negative for the transcripts for all time points tested (not shown). Quality of tick cell culture-

derived *E. chaffeensis* RNA was confirmed by assessing the pathogen-specific 16S rRNA by RT-PCR (not shown). It is likely that MerR expression is lower or completely absent for the organism cultured in tick cell cultures.

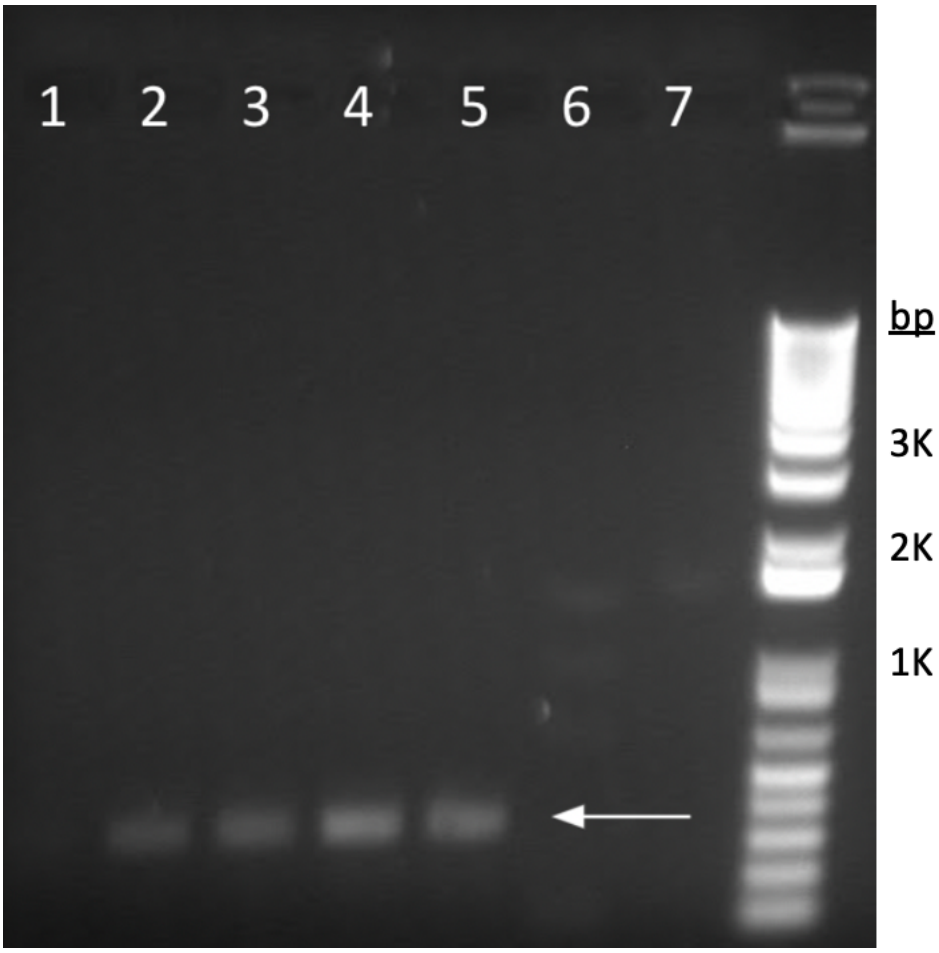

Figure 4.7. Validation of transcription of isolated MerR-HA

Checking for the presence of transcripts for isolated MerR-HA from DH82 cells. Primers RG1470 (P1) and RG2153 (P3) were used to target the *merR*-HA gene. Lanes 1 through 4 indicate MerR-HA RNA recovered from the time points 0 hpi, 24 hpi, 48 hpi, and 72 hpi, respectively. Lane 5 is the positive control for MerR-HA DNA. Lanes 6 and 7 are negative controls indicating WT *E. chaffeensis* and nuclease-free water, respectively.

RNA expression variation for the five predicted transcription regulators (CtrA, EcxR, MerR, Hup, and Tr1) in *E. chaffeensis* during the bacterial *in vitro* replication over time assessed by qRT-PCR

We then assessed the RNA expression of five predicted transcription regulators of *E. chaffeensis* by quantitative reverse transcription-PCR (qRT-PCR). The experiment was performed using *E. chaffeensis* RNA recovered from DH82 and ISE6 cell cultures following the synchronized bacterial growth over different hours post infection (hpi) initiation; 0, 24, 48, 72, and 96). The CtrA expression was observed to have an increased trend at 24 h and 72 h post infection initiation in DH82 cell cultures, while 0, 24 and 96 hpi had similar RNA expression (Figure 4.8). Due to high variation among the three replicates, the data were not significantly different. The CtrA expression in tick cell cultures had a similar higher expression at 24 hpi with a steady decline there after (Figure 4.8). The replicating form, RC, of *E. chaffeensis* is regarded to be higher between 24 to 72 hpi, thus it is likely that the CtrA is expressed more during active bacterial replication. The EcxR expression was the lowest for the 24 hpi and from then on, its expression increased steadily for 48-96 hpi, with the highest expression noted at 72 hpi (Figure 4.9). Similar expression trends were observed in ISE6 cell cultures for EcxR, although the data had high variation among the replicates (Figure 4.9). Expression levels for the Hup remained constant throughout the assessment times for the macrophage cultures, while some variations were noted in tick cell cultures (Figure 4.10). The MerR expression was very similar to CtrA from macrophage cells with higher expression noted at 24 and 72 hpi and lower expressions at 0, 48 and 96 hpi. Similarly, MerR expression in tick cell cultures correlated well with CtrA RNA expression with a peak expression noted at 24 h, while lower expression was noted at all other times (Figure 4.11). The Tr1 RNA expression was the highest at 48 hpi by about 3-fold in

comparison to 24 and 72 hpi and 2-fold in comparison to 96 hpi for the *E. chaffeensis* growth in DH82 cultures (Figure 4.12). Expression levels in the tick cell cultures, however, was the highest at 24 h, although it varied among the replicates due to no patterns between the two culture methods for each mutant constructs, thus making it statistically not significant. While much remains to be defined about how differential RNA expression for the five predicted transcription regulators contributes to the *E. chaffeensis* replication, the current data demonstrate that there are unique gene expression patterns and they are likely be important for bacterial growth at various time points.

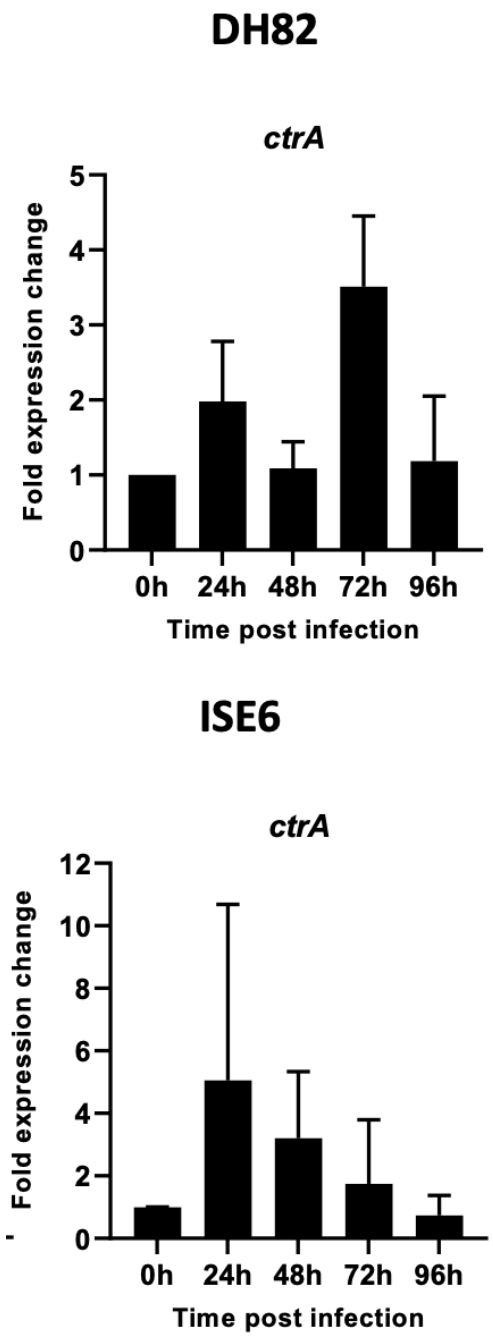

Figure 4.8. qRT-PCR expression levels of gene regulator *ctrA*

Expression levels at various time points for the gene regulator *ctrA* from two host cells. Significant changes in transcript expression were identified with a single asterisk where the P-values were <0.05 and a double asterisk where the P-values were <0.01. RNA samples prepared from synchronously cultured *E. chaffeensis* in DH82 cells or ISE6 cells at different time points and were used to perform quantitative RT-PCR analysis for five regulator genes and normalized

against bacterial 16S rRNA, respectively. Relative values to the amount at 0 h p.i. are shown. Data indicate mean values ± standard deviations from three or two biological replicas with three technical replicates for each biological replicate. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test (*, $P<0.05$; **, $P<0.01$).

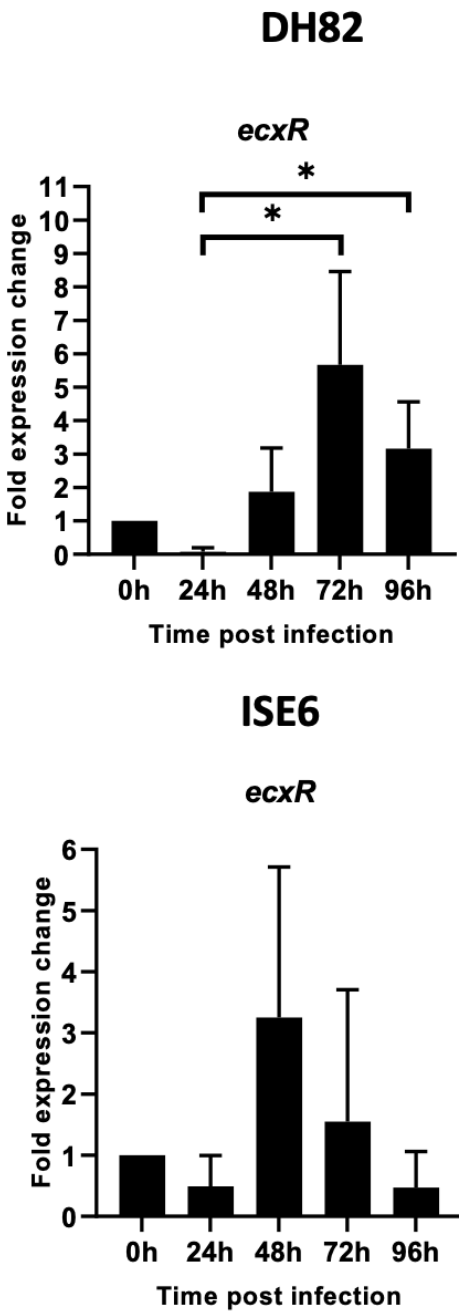

Figure 4.9. qRT-PCR expression levels of gene regulator *ecxR*

Expression levels at various time points for the gene regulator *ecxR* from DH82 and ISE6 cells. Significant changes in transcript expression were identified with a single asterisk where the P-values were <0.05 and a double asterisk where the P-values were <0.01.

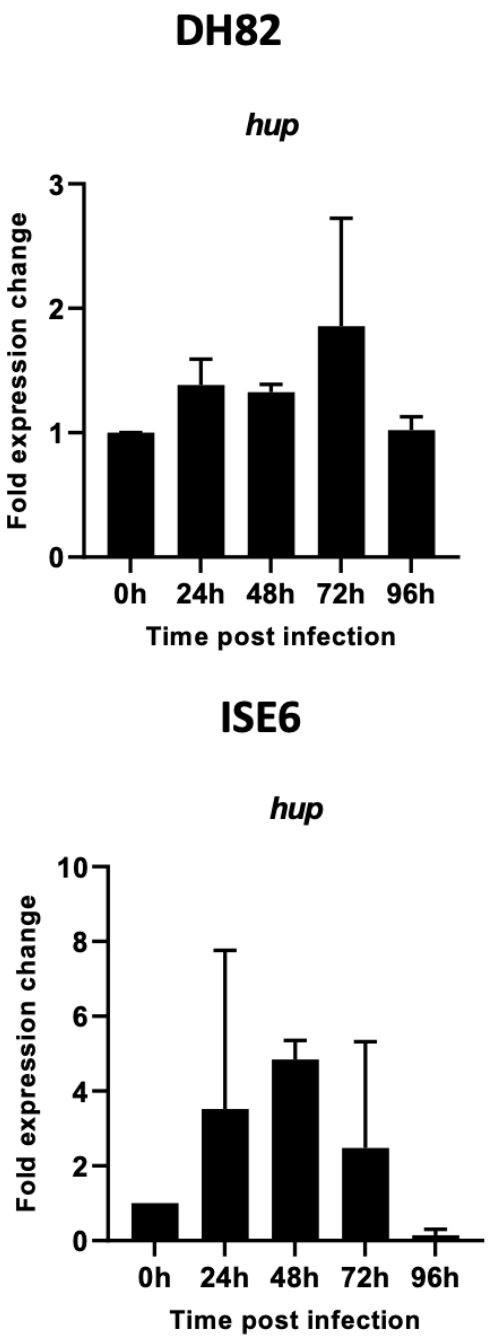

Figure 4.10. qRT-PCR expression levels of gene regulator *hup*

Expression levels at various time points for the gene regulator *hup* from DH82 and ISE6 cells. Significant changes in transcript expression were identified with a single asterisk where the P-values were <0.05 and a double asterisk where the P-values were <0.01.

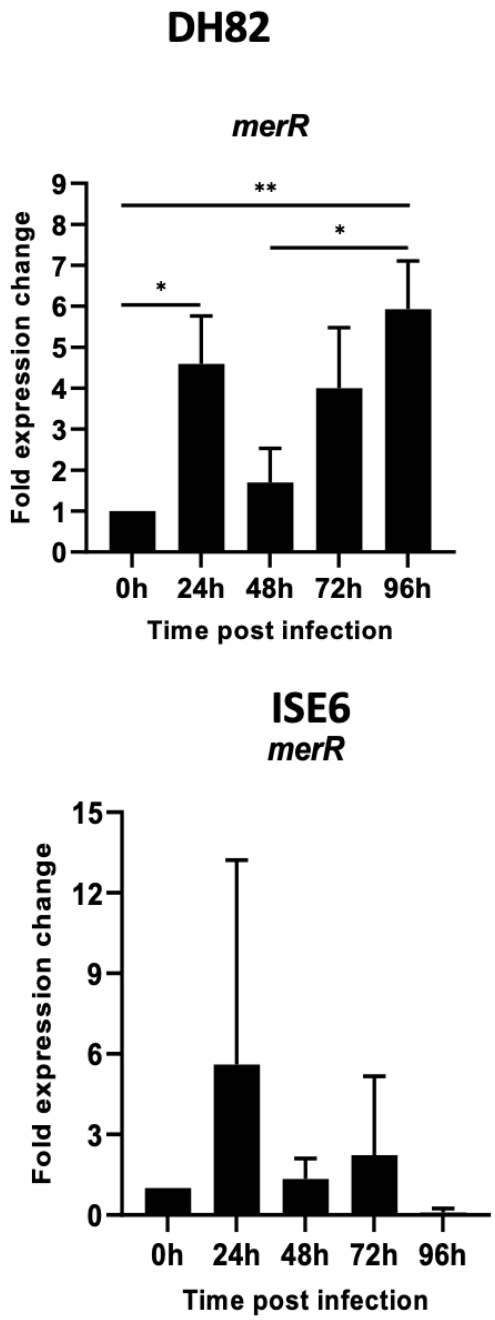

Figure 4.11. qRT-PCR expression levels of gene regulator *merR*

Expression levels at various time points for the gene regulator *merR* from DH82 and ISE6 cells. Significant changes in transcript expression were identified with a single asterisk where the P-values were <0.05 and a double asterisk where the P-values were <0.01.

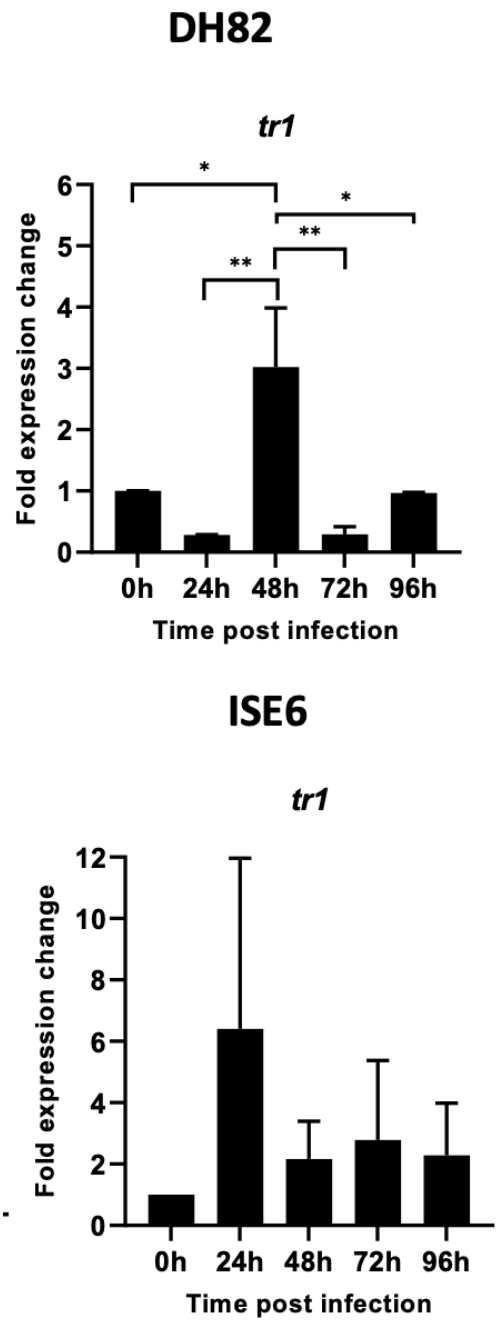

Figure 4.12. qRT-PCR expression levels of gene regulator *tr1*

Expression levels at various time points for the gene regulator *tr1* from DH82 and ISE6 cells. Significant changes in transcript expression were identified with a single asterisk where the P-values were <0.05 and a double asterisk where the P-values were <0.01.

Validating protein expression of *E. chaffeensis* DNA binding proteins

Purified RC and DC proteins of soluble and insoluble fractions of *E. chaffeensis* DNA binding protein MerR having the HA-tag were assessed by SDS-PAGE and Western blot analysis using HA-specific antibody (Figures 4.13 and 4.14). Despite the detection of transcripts in ISE6 cell cultures, MerR protein expression was not evident. A protein band equivalent to MerR was visible in *E. chaffeensis* proteins recovered from macrophage cultures (DH82). Its expression was noted in both RC and DC fractions (Figure 4.14, Lanes 3 and 4). Protein expression for EcxR and HU were also assessed using EcxR-HA or HU-HA tagged mutant culture using HA monoclonal antibody. Western blot analysis of *E. chaffeensis* purified proteins revealed the predicted protein in DH82 cells and ISE6 cells for Hup (Figure 4.15 Lane 2 and Figure 4.16 Lane 3, respectively). These results demonstrate the expression of these DNA binding proteins in *E. chaffeensis.*

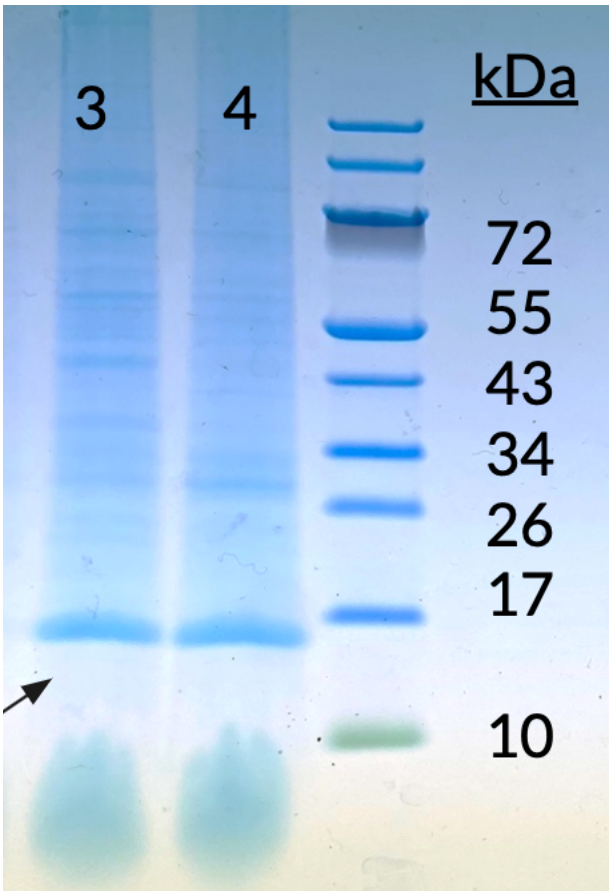

Figure 4.13. SDS-PAGE protein expression of MerR-HA RC/DC

Proteins resolved on an SDS-PAGE tricine gel to observe the expression of MerR-HA RC/DC fractions. Lanes 3 and 4 indicate RC and DC, respectively. Protein bands at 15 kDa are shown (arrow) for MerR-HA for the insoluble protein fractions of both forms.

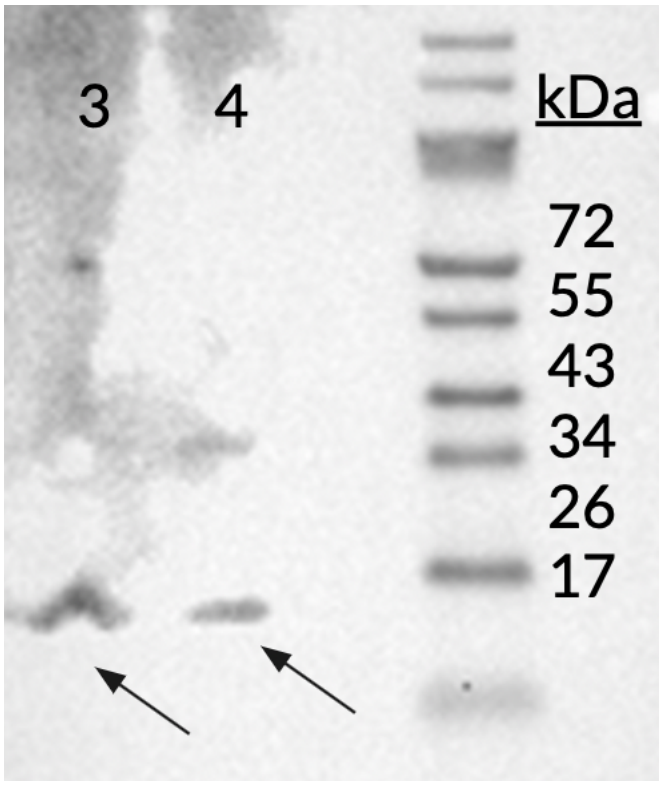

Figure 4.14. Western Blot protein expression of MerR-HA

Validation of the replicate (RC) and infectious (DC) forms of bacteria expressing MerR-HA protein cultivated in dog macrophage cultures. Lanes 3 and 4 indicate the 15 kDa MerR-HA protein is present in both RC and DC forms (arrows) .

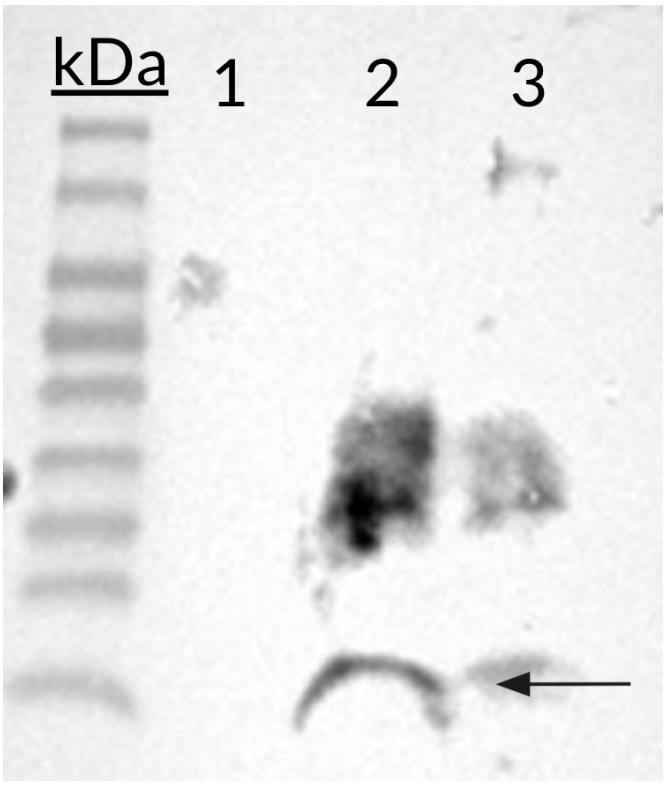

Figure 4.15. Western Blot protein expression of EcxR-HA

Validation of EcxR-HA protein cultivated in dog macrophage cultures. Lane 2 indicates EcxR-HA cell-free protein at its respective size of 13 kDa.

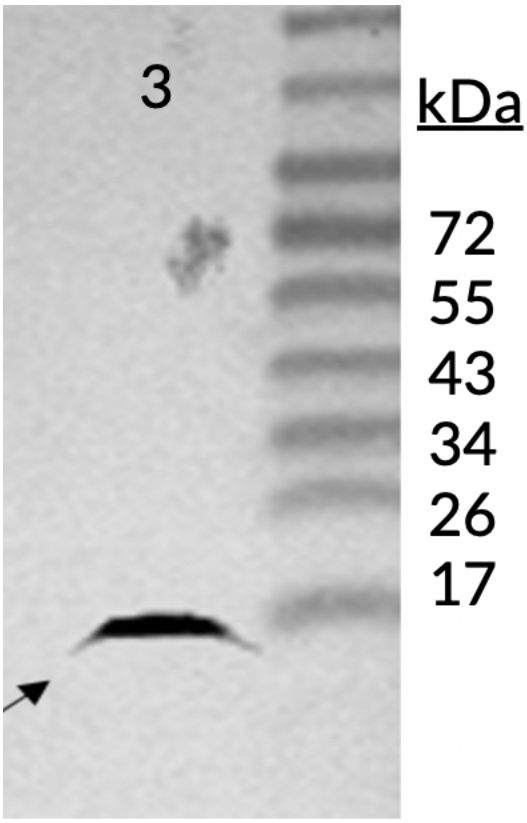

Figure 4.16. Western Blot protein expression of HU-HA

Validation of total and cell-free bacteria expressing HU-HA protein cultivated in dog macrophage cultures. Lane 3 indicates HU-HA cell-free protein expressed at its respective size 12 kDa.

Shearing of crosslinked DNA from Chromatin immunoprecipitation experiments (ChIP)

Mutant cultures of MerR-HA were grown in DH82 and ISE6 cells and DNA was isolated from these cultures to be crosslinked to bound DNA. The crosslinked DNA and protein complexes were sheared by sonication and resolved on an agarose gel to view the DNA smears. The smears are visible around 500 bp which was expected for both MerR-HA in DH82 and ISE6 cells (Figure 4.17 and 4.18, respectively). Similar experiments were performed for EcxR-HA and HU-HA DNA-Protein complexes (Figures 4.19 and 4.20, respectively). Specifically, MerR-

HA (from DH82 and tick cells), HU-HA (from DH82 cells), and EcxR-HA (from DH82 cells) were sent for ChIP-seq analysis to the Genomic High-Throughput Facility, University of California, Irvine.

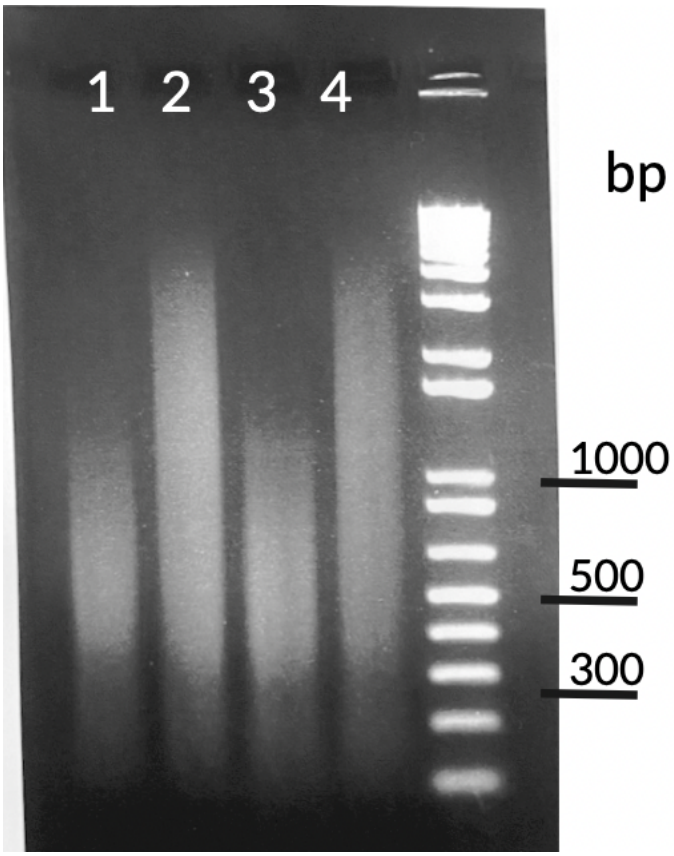

Figure 4.17. Sheared cross-linked MerR-HA DNA from DH82 cells

Sheared cross-linked MerR-HA DNA from DH82 cells resolved on an agarose gel. DNA smears are shown at 500bp. Lanes 1 and 2 indicate DNA-Protein complexes crosslinking times of 20 minutes and resolved with and without the presence of NaCl on a gel, respectively. Lanes 3 and 4 indicate crosslinking times of 30 minutes and sheared DNA was resolved with and without the presence of NaCl, respectively.

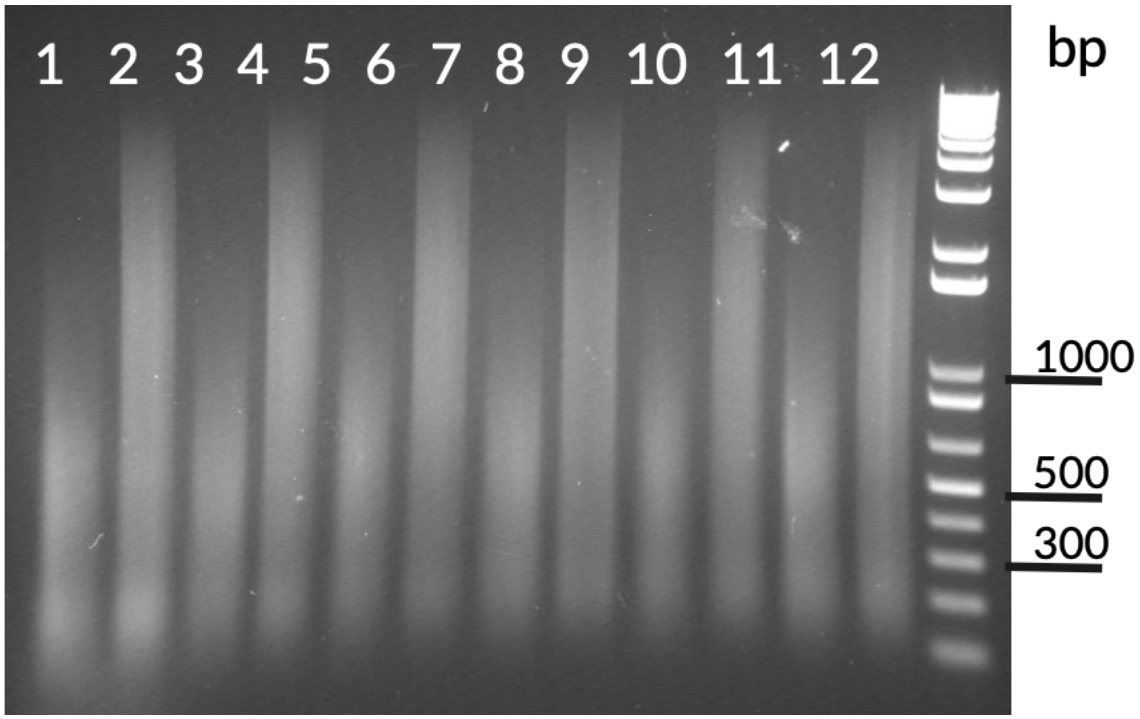

Figure 4.18. Sheared crosslinked MerR-HA DNA from ISE6 cells

Sheared crosslinked MerR-HA DNA from ISE6 cells resolved on an agarose gel. DNA smears are shown at 500bp. Lanes 1, 3, and 5 indicate DNA-Protein complexes crosslinking times of 30 minutes. Lanes 7, 9, and 11 indicate DNA-Protein complexes crosslinking times of 20 minutes and the DNA was resolved on the gel in the presence of NaCl, respectively. Lanes 2, 4, and 6 indicate crosslinking times of 30 minutes and lanes 8, 10, and 12 indicate crosslinking times of 20 minutes and sheared DNA was resolved without the presence of NaCl, respectively.

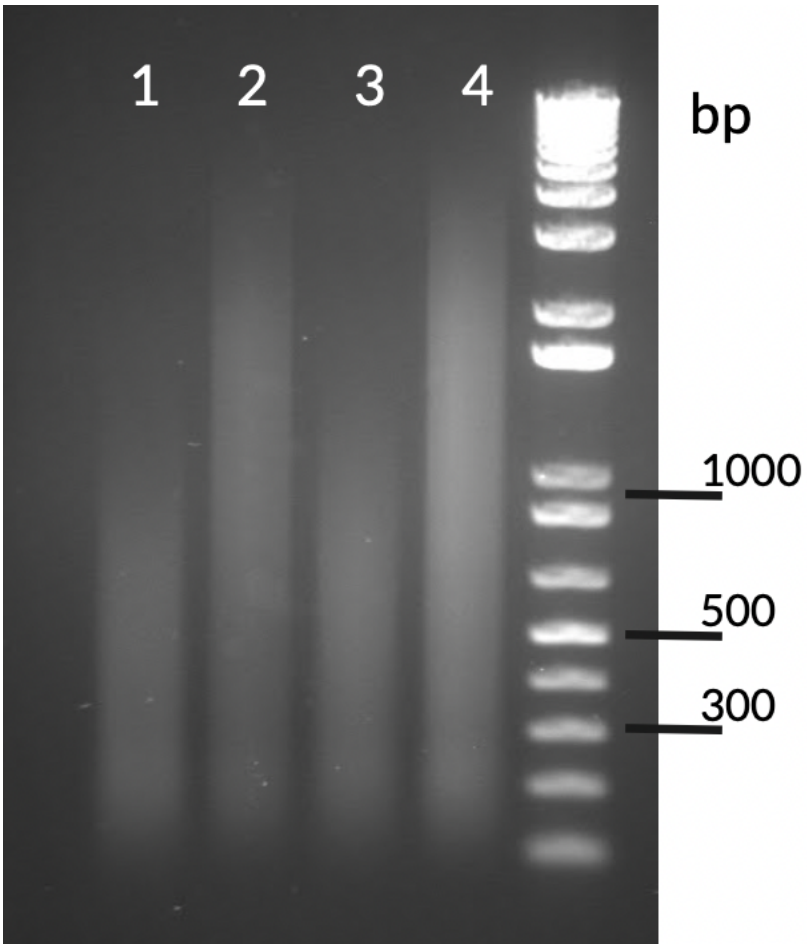

Figure 4.19. Sheared crosslinked EcxR-HA DNA from DH82 cells

Sheared crosslinked EcxR-HA DNA from DH82 cells resolved on an agarose gel. DNA smears are shown at 500bp. Lanes 1 and 2 indicate DNA-Protein complexes crosslinking times of 20 minutes with and without the presence of NaCl, respectively. Lanes 2 and 4 indicate crosslinking times of 30 minutes with and without the presence of NaCl and the DNA was resolved on the gel, respectively.

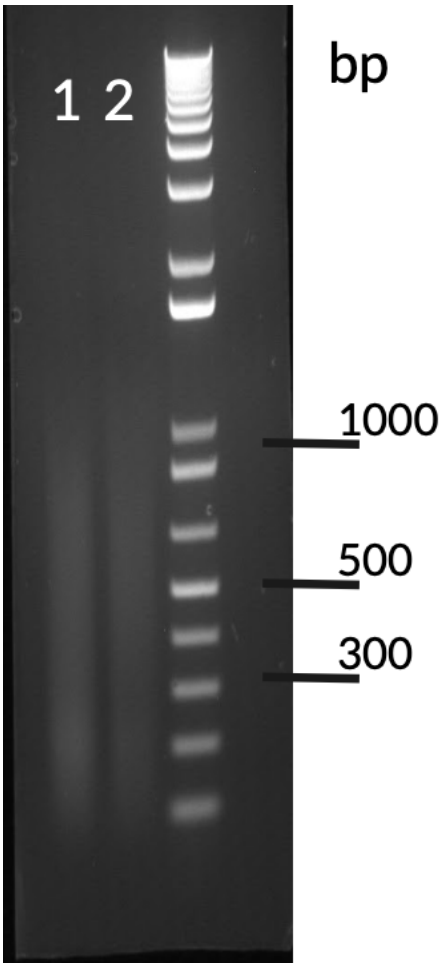

Figure 4.20. Sheared crosslinked HU-HA DNA from DH82 cells

Sheared crosslinked HU-HA DNA from DH82 cells resolved on an agarose gel. DNA smears are shown at 500bp. Lanes 1 and 2 indicate DNA-Protein complexes crosslinking times of 30 minutes and 20 minutes with the presence of NaCl, respectively.

Whole genome amplification for ChIP sequencing of MerR-HA, HU-HA, and EcxR-HA DNA samples

The top 10 genes that were bound to the proteins of interest from our ChIP-seq data set are listed for each of the three regulators MerR, EcxR, and HU in Figures 4.21, 4.22, and 4.23, respectively. The consensus motifs for each regulator MerR, EcxR and HU are listed in figures 4.24, 4.25, and 4.26m, respectively. The consensus motifs represent aligned sequences of the

genes to the *E. chaffeensis* genome that could potentially be conserved. The ChIP sequence data demonstrate that multiple genomic regions are recognized by the three DNA binding proteins. These data are the first in defining the DNA protein interactions of three of the five *E. chaffeensis* predicted DNA binding proteins (DBPs). The importance of the multiple DNA regions where the predicted DBPs were found to bind requires follow up studies that are currently in progress.

Gene	Gene description	Position
ECH_0649	pyridine nucleotide-disulphide oxidoreductase family protein	Overlaps start
ECH_0633	chaperone protein HscA	Overlaps start
ECH_1008	preprotein translocase, YajC subunit	upstream
ECH_0508	PQQ repeat-containing protein	Overlaps start
ECH_0678	hypothetical protein ECH_0678	Overlaps start
ECH_0646	triosephosphate isomerase	upstream
ECH_0584	seryl-tRNA synthetase	Overlaps start
ECH_0480	uroporphyrinogen-III synthase	Overlaps end
ECH_0538	isoleucyl-tRNA synthetase	Inside
ECH_0568	phage minor structural protein	Inside

Figure 4.21. Top 10 genes bound most frequently to MerR-HA

Repeat	Gene	Gene description	Position
R1&R2	ECH_1083	pentapeptide repeat-containing protein	upstream
R1&R2	ECH_0693	GTP-binding protein TypA	upstream
R1&R2	ECH_1068	C-type cytochrome family protein	Overlaps start
R1&R2	ECH_0162	integration host factor, alpha subunit	upstream
R1	ECH_0347	M48 family peptidase	inside
R1	ECH_0933	hypothetical protein ECH_0933	upstream
R1	ECH_0313	ABC transporter, permease/ATP-binding protein	overlaps start
R1	ECH_0326	alpha/beta fold family hydrolase	inside
R1	ECH_0719	hypothetical protein ECH_0719	overlaps end
R2	ECH_1121	major outer membrane protein Omp-1N	overlaps start

Figure 4.22. Top 10 genes that bound most frequently to EcxR-HA

Gene	Gene description	Position
ECH_0159	hypothetical protein ECH_0159	Inside
ECH_0974	trans-2-enoyl-ACP reductase II	Inside
ECH_0221	putative hydrolase	upstream
ECH_1121	major outer membrane protein Omp-1N	Overlaps start
ECH_1050	hflK protein	Overlaps start
ECH_0106	hypothetical protein ECH_0106	upstream
ECH_0535	hypothetical protein ECH_0535	Inside
ECH_0695	hypothetical protein ECH_0695	Inside
ECH_0011	glyceraldehyde-3-phosphate dehydrogenase, type I	downstream
ECH_0360	hypothetical protein ECH_0360	upstream

Figure 4.23. Top 10 genes bound most frequently to HU-HA

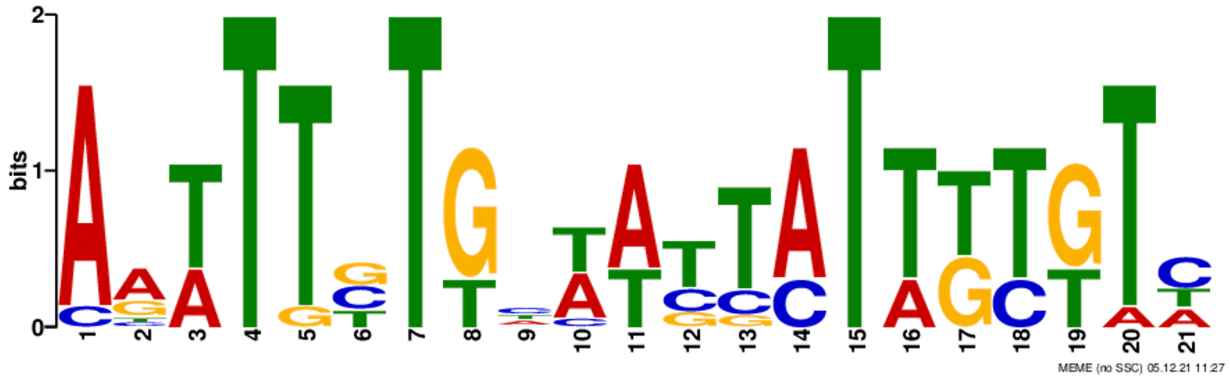

Figure 4.24. Consensus motif for MerR-HA

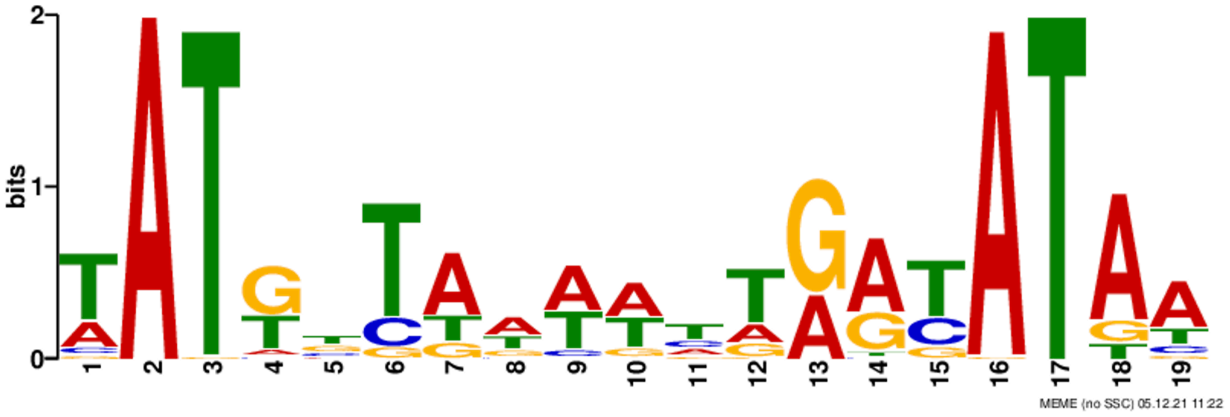

Figure 4.25. Consensus motif for EcxR-HA

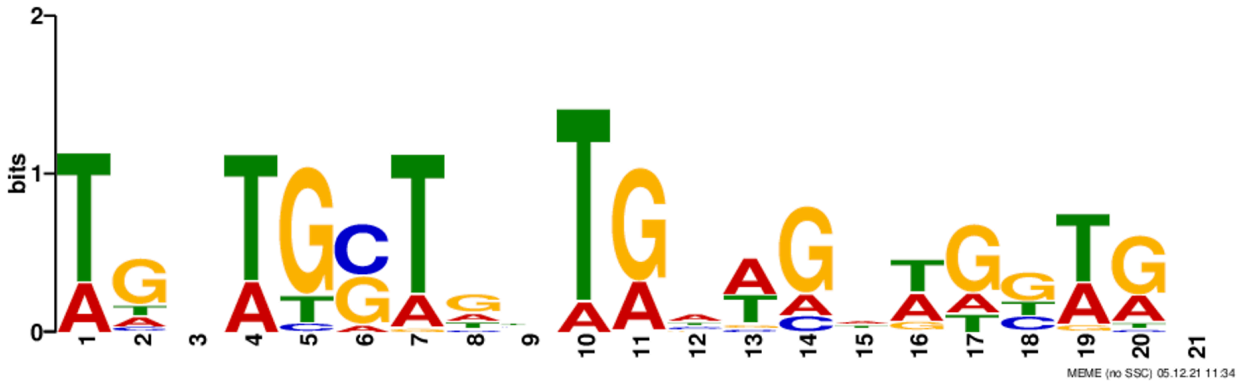

Figure 4.26. Consensus motif for HU-HA

Chapter 5 - Discussion

In the current study, we performed experiments to define the importance of the five predicted DNA binding proteins; HU, CtrA, MerR, EcxR and Tr1, of *E. chaffeensis* for their contributions to the bacterial gene regulation. Specifically, we performed experiments to define the expression of the five DBP genes at RNA levels and the RNA expression changes over time during the bacterial replication. We also performed chromatin immune precipitation experiments to define the interactions of three of the five DBPs; MerR, HU and EcxR. This study identified several interacting segments of *E. chaffeensis* genes. The research results presented in this study are novel and allow new research advances regarding how *E. chaffeensis* and other related tick-borne pathogens regulate gene expression. Considering that minimal knowledge exists regarding the gene expression by these pathogens in vertebrate and tick hosts, it is important to study differential gene regulation by these vector-borne pathogens. The research will be important in identifying unique strategies employed by the tick-borne rickettsial pathogens for their adaptation to tick and vertebrate hosts. Understanding how the bacteria sense the vertebrate and tick hosts in altering gene expression will aid in identifying novel drugs to control the pathogen infections.

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