TRANSCRIPTIONAL ANALYSIS AND PROMOTER CHARACTERIZATION OF TWO DIFFERENTIALLY EXPRESSED OUTER MEMBRANE PROTEIN GENES OF *EHRLICHIA CHAFFEENSIS*

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Ehrlichia chaffeensis is a Gram negative, rickettsial organism responsible for human monocytic ehrlichiosis, an emerging disease in people. *E. chaffeensis* infection to a vertebrate host occurs when the pathogen is inoculated by an infected tick, *Amblyomma americanum.* White-tailed deer is a reservoir host for this pathogen. The strategies employed by *E. chaffeensis* in support of its dual host adaptation and persistence are not clear. One of the possible mechanisms by which the pathogen adapts and persists, is by altering its gene expression in response to its host cell environments. Recently, we reported that *E. chaffeensis* protein expression including from a 28 kDa outer membrane protein multigene locus (p28-Omp), is influenced by macrophage and tick cell environments. *E. chaffeensis* expresses p28-Omp gene 14 product predominantly when it is grown in tick cells and p28-Omp gene 19 protein in macrophages. We hypothesize that *E. chaffeensis* achieves its host-specific gene expression by employing transcriptional regulation by sensing the host cell signals. In support of this hypothesis, transcriptional analysis of genes 14 and 19 was performed utilizing several RNA analysis methods. The results supported our hypothesis that the gene regulation occurs at mRNA level in a host cell-specific manner. This analysis also identified transcription start sites and located putative promoters for these genes. Promoter regions of genes 14 and 19 were mapped to identify gene-specific differences, RNA polymerase binding sequences and the putative regulatory elements that may influence the promoter activities. Electrophoretic mobility shift assays revealed interaction of *E. chaffeensis* proteins with gene 14 and 19 promoters. Several *E. chaffeensis* putative regulatory proteins were expressed as recombinants and their effects on a p28-Omp gene promoter activity were evaluated.

In summary, we demonstrated that the differences in the *E. chaffeensis* p28-Omp genes 14 and 19 are the result of their regulation at transcriptional level in response to the host cell environment. We also identified RNA polymerase binding regions and several DNA sequences that influenced promoter activity. This is the first description of a transcriptional machinery of *E. chaffeensis*. The data from these studies provide important insights about molecular mechanisms of gene regulation in *E. chaffeensis*.

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Dedication

I dedicate this dissertation to all those who have made a positive impact on my life

Chapter 1

Literature Review

Vector-borne diseases

An infectious disease is a deviation from normal functioning caused by a pathogenic organism. The disease causing agents are transmitted to a susceptible host by various means. Some of the common modes of disease transmission include direct physical contact, inhalation (air-borne), ingestion of contaminated food or water (food-borne), or by means of vectors that carry the infectious agents (vector-borne).

Vectors of disease transmission

A vector is a living organism that transmits a disease causing agent from one host to another. The mode of an infectious agent transmission is said to be mechanical if a pathogen is simply physically carried by a vector to its host without the pathogen undergoing any replication in it. An organism that involves in such mechanical transmission of a disease causing agent is called mechanical vector. Sometimes, non-living objects also aid in mechanical transmission of a disease agent and are referred to as fomites. Some of the examples of mechanical vectors include arthropods such as housefly (cholera) (119,188), cockroach (toxoplasmosis) (125), horse fly (anaplasmosis in horses) (222), mosquitoes (fowl pox) (191), and human body louse (relapsing fever) (317). These vectors physically carry the organism in their legs, mouthparts, or through other body parts to another host. They may also contaminate food or feed which then can serve as a source of infection. Vertebrate animals such as migratory birds and dogs can also serve as mechanical vectors in transmitting diseases to a vertebrate host. For example, aspergillosis and toxoplasmosis are transmitted by birds and dogs, respectively (311,410). An organism is considered as a biological vector when it supports the replication of an infectious agent and aids in its transmission to a suitable host. Invertebrate organisms which act as biological vectors include mosquitoes, ticks, fleas, snails, and flukes; vertebrate animals like bat,

skunk, and dogs also serve as biological vectors. A few examples of diseases transmitted by biological vectors listed above include: yellow fever, malaria (mosquitoes) (70,323); anaplasmosis, ehrlichiosis (ticks) (47,289,325). bartonellosis, plague, murine typhus, feline leukemia (fleas) (14,64,144,404); schistosomiasis (snails) (101); neorickettosis (flukes) (127); dogs, bats and skunks transmit rabies (403).

Despite the great advances in human and animal medicine and improved methods of vector-based disease prevention and control strategies, vector-borne diseases are responsible for a major portion of infectious diseases burden in the world in animals and people (142,148,156,257). More than half of total morbidity and mortality reported during the last four centuries are due to vector-borne diseases. They include plague, malaria, yellow fever, dengue, epidemic typhus, trypanosomiasis, filariasis, and leishmaniasis (146). Among the vector-borne infections, the majority of the infectious agents known to date are transmitted by arthropod vectors (140,181). Several important human and animal illnesses transmitted by arthropod vectors are listed in Table 1.1.

The phylum Arthropoda includes the largest number of known species in the animal kingdom. This phylum is characterized by jointed legs, rigid cuticular exoskeleton and open circulatory system (haemocoel). The phylum Arthropoda consists of several subphyla; Trilobita, Crustacea, Uniramia and Chelicerata. The subphylum Trilobita includes extinct group of arthropods which were characterized by a three lobed head and body. Most of the aquatic arthropods such as shrimps, crabs, and barnacles are included under the sub phylum Crustacea. Although, the majority of the members of subphylum Crustacea are marine in habitat, it also includes several freshwater and terrestrial species. Subphylum Uniramia includes centipedes,

millipedes and insects. The class Insecta of Uniramia comprises themajority of all known arthropod species. The subphylum Chelicerata includes arthropods with no antennae, but contain a pair of chelicerae, a pair of pedipalps, and four pairs of walking legs in adults. A few examples of this subphylum are spiders, scorpions, ticks and mites.

Of all known arthropod vectors, blood sucking organisms such as mosquitoes and ticks are the major contributors of diseases in vertebrate animals (118,340,358). The most important mosquito-borne illnesses include bacterial diseases (tularemia) (315), viral infections (yellow fever, dengue fever, West Nile virus, equine encephalitis), protozoal diseases (malaria) and nematodal diseases (filariasis) (156). Most of the mosquito-borne illnesses are concentrated in the tropical regions of the world spanning South and Central America, Asia, and Africa. Malaria, a mosquito-borne protozoan disease, is responsible for about 500 million cases and one million deaths each year in the African continent alone (355). Chickengunya virus, responsible for severe outbreaks for past half a century in Asia and Africa and recent outbreaks in France and other parts of Europe, is transmitted by *Aedes* species of mosquito (104). In developed countries such as in the United States, improved mosquito eradication strategies resulted in a significant decline in mosquito-borne infections (252).

While mosquitoes remain major vectors for the most devastating diseases in animals and people for the past few decades ticks have emerged as the 2nd major arthropod vectors in spreading infections to animals and people (298,373,405). Tick-borne illnesses are of emerging concern in the United States, Europe and other regions of the world. More detailed discussion about ticks, their role as vectors for several animal and human diseases is presented in following sections.

Biology of ticks

Ticks are obligate, blood sucking arthropods that parasitize all classes of terrestrial vertebrates (358). Nearly 850 tick species have been identified to date (276,358). They belong to the subphylum, Chelicerata; class, Arachnida; order, Acari; and grouped into three families: Ixodidae, Argasidae and Nuttalliellidae. The members of the family Ixodidae are known as 'hard ticks'. The name 'hard ticks' is given due to their characteristic hard sclerotized dorsal plate. The family Argasidae, also known as 'soft ticks', have a flexible, leathery cuticular exoskeleton (358). The family Nuttalliellidae is confined to southern parts of Africa and includes only one species, namely, *Nuttalliella namaqua* (185). A detailed diagrammatic representation for classification of ticks is presented in Figure 1.1.

Members of Ixodidae and Argasidae differ in their feeding behavior and life cycle. The 'hard ticks' are slow feeders and stay on a host for a very long period of time, usually for several days, and take blood meals 2-3 times their body weight. In contrast, 'soft ticks' are rapid feeders and consume blood meals 5-10 times their body weight in a few minutes to few hours (358). The bite of Ixodid ticks is relatively painless compared to Argasid ticks. The developmental cycle of a tick typically includes 4 stages namely, egg, larva, nymph and adult. Ixodid ticks contain only one nymphal stage which develops into an adult. The life cycle of Argasid ticks includes several immature nymphal stages and requires multiple hosts for their final maturation into adult ticks. Soft ticks tend to live longer (occasionally up to 20 years) due to their infrequent blood meals, and several nymphal stages in their life cycle. Lifespan of hard ticks is relatively shorter than soft ticks, and they typically live for about 2 to 3 years. Ixodid ticks are most prolific egg layers compared to Argasid ticks (358).

The life cycle of a typical Ixodid tick, from the time of hatching of the larvae to an adult tick, requires three hosts (three-host life cycle). The female tick, upon mating with the male tick and after intake of a blood meal, lays thousands of eggs in a few days. The eggs hatch into larvae under favorable conditions, which then molt into a nymph after obtaining a blood meal from a vertebrate host. The unfed nymphal ticks seek a suitable host to obtain a blood meal. Typically, larvae and nymph feed on small mammals. The adult ticks emerge upon nymphal molt, which then parasitize a vertebrate host to feed, mate, and continue the life cycle. Some exceptions of three-host life cycle exist among members of Ixodidae (358). For example, *Hyalomma* species (*H. anatolicum excavatum*) life cycle requires only two hosts; the larva upon feeding, remain on the host and molt on it to become a nymph. The nymphal tick, after intake of a blood meal from the same host falls onto the ground where it molts to become an adult (two-host life cycle) (358). For some Ixodid ticks, such as *Dermacentor albipictus*, *Boophilus* species, development of a larva to an adult occurs only on one host (one-host life cycle). Argasid ticks, because of the presence of multiple nymphal stages in their developmental cycle, require multiple hosts in order for the completion of their life cycle (358). Figure 1.2 shows a diagrammatic illustration of the life cycle of a three-host ixodid tick species.

Ticks, like other members of Arachnida, are characterized by 6 pairs of appendages, which include a pair is chelicerae that are used for cutting and tearing the host skin, a pair of palps which help to attach to the host skin while feeding, and four pairs of walking legs. Ticks possess some unusual features, which contribute to their vector competency and ability to transmit diseases to a wide range of hosts. These features include their remarkably longer life-span which can range from few to several years, capacity to withstand prolonged periods of starvation, ability to absorb moisture directly from the atmosphere, ability to produce a large

number of eggs (2000 to 25,000), ability to take a blood meal much larger than its body size, intracellular digestion etc. (358). Unlike any arthropod vector, ticks are known to transmit a diverse group of infectious agents such as bacteria, virus, protozoa, Rickettsiae, fungi, and nematodes (160,298,331,357,358). Moreover, ticks are known to harbor and transmit multiple pathogens simultaneously.

Tick-borne infections

Ticks are the vectors to several known arthropod-borne illnesses of animals and humans. They are the second major arthropod vectors after mosquitoes in spreading infectious agents (298,373,405). Some of the important tick-borne animal and human infections to date include Lyme borreliosis, tick-borne encephalitis, Rocky Mountain spotted fever, Colorado tick fever, heartwater fever, east coast fever, and tularemia (358,359). A list of several tick-borne human and animal infections and corresponding causative agents is presented in the Table 1.2. Ticks are also known as the vectors for several recently discovered emerging infectious diseases (358,359,378,405). The number of newly reported tick-borne infections is also on the rise. Some of the emerging tick-borne infections are human babesiosis, Master's disease caused by a spirochete with disease manifestations similar to Lyme disease, Alkhurma viral hemorrhagic fever, and bartonellosis (58,227,236,337). The list of emerging tick-borne human infections also includes several Rickettsial diseases including *Rickettsia parkeri* infection, human granulocytic anaplasmosis, *Ehrlichia ruminantium*-associated human infections, human ewingii ehrlichiosis, and human monocytic ehrlichiosis (3,223,280,358).

Tick-borne rickettsial infections

As described above, ticks are vectors for many pathogens that cause diseases in vertebrate animals including people. In recent years, tick-borne rickettsial infections are of a major health concern to animals and people. Rickettsial organisms are a group of obligate intracellular bacteria that belong to the order Rickettsiales. Besides tick-vectored pathogens, the order Rickettsiales also includes pathogens that are harbored by several other invertebrate vectors, which include lice, mites, fleas, and flukes (97,120). In the following paragraphs, more description about the order Rickettsiales and various diseases of human importance caused by the pathogens that belong to this order are provided.

General introduction of the order Rickettsiales:

The order Rickettsiales includes a group of intracellular organisms requiring eukaryotic cells for their growth and replication (97). Most recent classification of the order Rickettsiales includes two families, *Rickettsiaceae* and *Anaplasmataceae* (97,120). The family *Rickettsiaceae* has two genera *Rickettsia* and *Orientia*. The genus *Rickettsia* includes twenty five species, whereas the genus *Orientia* has only one species, *O. tsutsugamushi* (97). The genus *Rickettsia* is further subdivided into two groups based on the genotypic and phenotypic similarities of the organisms, the spotted fever group rickettsiae (SFGR) and typhus group rickettsiae (TGR) (97,318). Rickettsial organism belong to the spotted fever group can polymerize actin filaments in the host cell cytoplasm. The polymerized actin filaments are then utilized by these organisms to move into the host nucleus or swim across the cytoplasm to spread to neighboring cells. Typhus group of rickettsia, excluding *R. typhi*, lack the ability to polymerize the host actin filaments (153,154).

Organisms that are classified under SFGR are vectored by ticks, whereas lice and fleas serve as the vectors for TGR (298,318,390,406). The only member of the genus *Orientia*, *O. tsutsugamushi* is transmitted to a vertebrate host through the bite of infected mites (332,411). The family *Anaplasmataceae* contains four genera *Wolbachia*, *Neorickettsia*, *Anaplasma* and *Ehrlichia*. Members of the genera *Wolbachia* are endosymbionts in arthropods and nematodes (21,97,176,413). Flukes are the primary vectors for the genus *Neorickettsia*. *Anaplasma* and *Ehrlichia* species are primarily transmitted by Ixodid ticks to wide range of vertebrate hosts (97,152,318,327,411). Many organisms that belong to the order Rickettsiales are responsible for several important diseases of animals and people. Some of the well known and recently discovered emerging diseases of people caused by the members of *Rickettsiaceae* and *Anaplasmataceae* are discussed below.

Diseases caused by the organisms of the family Rickettsiaceae:

Epidemic typhus:

Epidemic typhus, a louse-borne rickettsial infection caused by *Rickettsia prowazekii*, was a serious disease of humans in the 20th century. The flying squirrel serves as the reservoir host for *R. prowazekii* (38). A higher incidence of epidemic typhus is associated with wars, particularly where a large number of people are placed in concentration camps, refuge camps, prisons, etc. (122). Unhygienic conditions in such crowded places lead to easy dissemination human body louse carrying the pathogen, *R. prowazekii*, thereby spreading of the disease very rapidly. In 20th century, the number of deaths resulted due to epidemic typhus are far more than the fatalities resulted from war related injuries (12). After gaining entry into a vertebrate host, *R. prowazekii* infects vascular endothelial cells and macrophages where it multiplies by binary fission (415). The symptoms of epidemic typhus are usually non-specific and may vary with the

immune status of the infected person. The most commonly reported symptoms are high chills, fever, headache, muscle pains and rashes on chest, trunk and extremities (266). The mortality rate reported for epidemic typhus range from 10 to 60%. *R. prowazekii* damages the vascular endothelial cells lining the vital organs such as liver, kidney, and lungs resulting in compromised blood supply to those organs and death. In the event of early recognition, the disease can be effectively treated with antibiotics such as tetracycline or doxycyline (266). Although *R. prowazekii* associated infections are not commonly seen in clinics, this pathogen remains a serious concern because of its potential as a bioterrorism agent (13). In fact, *R. prowazekii* is classified as category A pathogen and the research related to this pathogen is carried out under very controlled laboratory conditions to avoid potential risk of infections to people.

Rocky Mountain spotted fever (RMSF):

RMSF is one of the oldest diseases known for more than a century, and is still reported in high numbers. Besides human beings, RMSF is also reported to occur in dogs (12). *R. rickettsii* is the causative agent for this disease and is transmitted by *Dermacentor* species of ticks (268). As with *R. prowazekii* associated infections, *R. rickettsii* infects the vascular endothelium and lethal outcome of the disease is mainly due to irreversible damage to endothelial cells lining the blood vessels of vital organs (237). The incidence of RMSF remains high in Southeastern and Midwestern regions of the United States (384). According to a recent report nearly 4,000 cases are reported during 1997-2002 in the United States alone (57). The mortality associated with RMSF range from 5-10% (57). Most of the mortalities are associated with this disease are primarily due a delay in its diagnosis (237).

Mediterranean spotted fever (MSF):

MSF is a tick-borne rickettsial disease of people caused by *R. conori* (12). MSF has the highest incidence in the Mediterranean countries including southern Europe, northern Africa, and part of Asia, where the disease is endemic (333). This disease has been reported from other regions of the world including central Europe and southern and central Africa (333). *R. conori* is transmitted to a vertebrate host by *Rhipicephalus* species of ticks (138). Like the other rickettsial diseases described above, the symptom for MSF are highly non-specific and they include fever, severe headache, and maculopapular rash (83,333). MSF is reported to have a mortality rate ranging from 1-3% and a higher mortality rate of ~30% is also reported for patients containing other health problems such as liver diseases, diabetes, and deficiency of certain metabolic enzymes (319). If recognized in the initial stages of infection, MSF can be treated effectively with antibiotics such as chloramphenicol, tetracycline or doxycyline (333).

Siberian tick typhus:

Siberian tick typhus is another well known tick-borne Rickettsial disease in people caused by *R. sibirica* (12). This pathogen was first reported in central Siberia and later it is also reported from other parts of the world including Pakistan and northern China (105,112,329). Several ticks, including *Dermacentor* species, *Hyalomma* species, and *Haemaphysalis* species serve as the biological vectors for this disease (186). The disease is manifested in a mild clinical illness with the exhibition of several non-specific symptoms, which include fever, headache, muscular pain, digestive disturbances and rash (318). Usually the onset of the symptoms occurs 4-7 days after the infected tick-bite, and they usually disappear in about a week even without treatment (318).

Scrub typhus:

Scrub typhus is also one of the oldest known rickettsial diseases of people caused by *Orientia tsutsugamushi* (310). This pathogen is primarily vectored by infected trombiculid mite larvae (310,332,411). *O. tsutsugamushi* primarily infects endothelial cells of a vertebrate host. Small mammals such as rodents act as reservoir hosts for this pathogen. This disease is mainly endemic to a region spanning Pakistan, Japan, and Australia, which is often referred to as the 'tsutsugamushi triangle' (41,59). Scrub typhus is not endemic to the United States; however, it is often reported in soldiers or travelers who have been to areas that are endemic to the disease (155,379). The most commonly reported symptoms of the scrub typhus are sudden onset of a high fever, severe headache, muscle ache, and generalized swelling of lymph nodes. At the site of mite bite, eschar is seen in about 50% of the infected people (175). An incidence rate of 23% is reported for this disease in endemic areas with mortality rate ranging from 1-50%, which depends on the immune status of a person and the pathogenic strain involved (59,186). Mortality resulting from scrub typhus is primarily due to multiple organ failure as a result of vascular damage. This disease can be treated effectively, if recognized early, with antibiotics such as Doxycyline (387).

Emerging tick-borne diseases by the organisms of the genus Rickettsia:

The genus *Rickettsia* also includes several pathogens causing a number of recently discovered emerging tick-borne infections in people. A few examples of emerging human infections include African tick bite fever, Japanese spotted fever, Flinders Island spotted fever and Astrakhan fever. A brief description about these diseases and their causative agents is provided below.

African tick-bite fever:

African tick-bite fever caused by *R. africae* was first identified in 1992 in Zimbabwe (187). *Amblyomma* species ticks are the vectors for this pathogen. African tick-bite fever is more prevalent in southern Africa and the acute form of this disease is primarily reported in European and American travelers to this region of Africa (155,173,249,316). For example, a recent study conducted in German travelers returning from Africa indicated that nearly 11% of them had a clinical disease associated with *R. africae* infection (173).

Japanese spotted fever:

Japanese spotted fever is another emerging human disease reported for the first time in 1984 in rural areas of Japan and its causative agent, *R. japonica*, was isolated in 1985 (230,388,389). *Ixodes* species of ticks serve as vectors for *R. japonica* and this disease is mostly limited to southwestern and central Japan. Commonly reported symptoms for this disease include sudden onset of fever, chills, rashes all over the body and occasionally leading to complications such as encephalopathy, respiratory distress and failure of functioning of multiple organs resulting form damage to vascular endothelium (194,195,389).

Flinders Island spotted fever:

Flinders Island spotted fever is reported from Flinders Island near Australia in 1991 (365). The etiological agent of this disease, *R. honei*, was isolated later in 1998 (363,365). Various species of *Aponomma* ticks, which usually parasitize reptiles, serve as vectors for *R. honei* (143). This disease has also been reported from Tasmania, Thailand, and southeast regions of Australia where the vector ticks population is prevalent (395). The disease is usually less severe and associated with symptoms such as headache, muscle ache, joint pains, rash, swelling

of lymph nodes, and cough (394). To date, no fatalities associated with this disease have been reported.

Astrakhan fever:

Astrakhan fever, discovered in 1991, is endemic to Astrakhan region in Russia (80,376). The causative agent of Astrakhan fever, *R. conori* sub species *ca*species*ia*, was identified in 2005 (446). *Rhipicephalus* species of *ticks* serve as transmitting vectors for this pathogen (318). A recent study demonstrated the presence of this disease in Kosova and Chad (Africa), suggesting a broader distribution of this pathogen and its vectors other than Russia (17,121,297). The disease manifestation ranges from asymptomatic to exhibition of symptoms including fever, rash, and conjunctivitis (297,376). To date, no mortalities associated with Astrakhan fevers have been reported.

Diseases caused by the organisms of the family Anaplasmataceae:

The family *Anaplasmataceae* initially included only pathogens of veterinary importance. The only exception has been *Neorickettsia* (*Ehrlichia*) *sennetsu*, the agent of sennetsu fever in people. *N. sennetsu* is endemic to Japan and was identified in 1954. Sennetsu fever is reported only from Japan, Malaysia and Thailand (178,254,296,320). Although not yet confirmed, similar to other *Neorickettsia* species, flukes are suspected to serve as vectors for this organism (42,97,162). Most of the reported cases of sennetsu fever are linked to consumption of raw fish, which is suspected to carry fluke containing *N. sennetsu* (126). The generalized symptoms of Sennetsu fever are fever, myalgia, lack of appetite, constipation, insomnia, swelling of lymph nodes and leucopenia (29,42). The disease occurs very rarely and is not associated with fatalities (29).

Several *Anaplasmataceae* organisms that are responsible for emerging infections in people have been identified over the past three decades (46,78,228,303). Recent human infections caused by *Ehrlichia* and *Anaplasma* are vectored by ticks. *Anaplasmataceae* pathogens diseases in people include human granulocytic anaplasmosis, human ewingii ehrlichiosis, and human monocytic ehrlichiosis caused by *Anaplasma phagocytophilum*, *Ehrlichia ewingii*, and *Ehrlichia chaffeensisEhrlichia chaffeensisEhrlichia chaffeensis*, respectively. These diseases are emerging as a major public health concern. The genera *Ehrlichia* and *Anaplasma* also include several species that are responsible for diseases in companion animals and livestock. Some important animal diseases include canine monocytic ehrlichiosis and canine granulocytic ehrlichiosis in dogs, heartwater fever in ruminants, and anaplasmosis in cattle. More detailed description of these animal and human infections with their associated pathogens is provided in the following sections.

Canine monocytic ehrlichiosis:

Canine monocytic ehrlichiosis (CME), a potentially life threatening tick-borne ehrlichial infection in dogs, is caused by *Ehrlichia canis*. This pathogen is initially identified in 1935 in Algeria and later reported from various regions in the world except Australia (145,150,184). In two isolated cases, *E. canis* infections have also been reported in people (300,301). These case reports are documented by only one group of scientists from Venezuela (300,301). *Rhipicephalus sanguineus* ticks serve as the primary vectors for this organism (145). Experimental studies in *Dermacentor* species of ticks also suggest its possible role as a vector for this organism (177). Manocytes and macrophages infections of a vertebrate host by *E. canis* have been documented (69). The most commonly reported symptoms for CME include fever, depression, lethargy, anorexia, swelling of lymph nodes and haematological abnormalities such

as leucopenia, anemia and pancytopenia (149). The CME is a serious disease and is associated with severe mortalities reported in military dogs. Active surveillance programs and chemoprophylaxis measures have lead to a considerable reduction in *E. canis* associated fatalities in dogs (77,416). CME is fatal in young and immunocompromised dogs (251). Early diagnosis followed by treatment with antibiotics such as doxycyline is proven to be very effective in curing the clinical disease (77). However, even after recovery, dogs remain infected with *E. canis* throughout their life (412).

Canine granulocytic ehrlichiosis:

Canine granulocytic ehrlichiosis is a disease of dogs caused by *E. ewingii*. Initially, this pathogen was thought to be a variant of *E. canis* and was referred to as canine granulocytic *Ehrlichia* (109,366). Based on 16s RNA sequence, this pathogen was later classified as *E. ewingii* in 1992 (7). This pathogen is also known to cause disease in people known as human ewingii ehrlichiosis (46). *Amblyomma americanum* serve as the vector for this pathogen (10). Canine granulocytic ehrlichiosis is generally a milder disease compared to CME and is usually characterized by fever, depression, anemia, thrombocytopenia, and polyarthritis (141). Doxycyline is widely used to treat the infection.

Heartwater fever:

Heartwater fever is an economically important tick-transmitted disease of domestic and wild ruminants including cattle, sheep and goats. This disease is caused by *Ehrlichia (Cowdria) ruminantium* (97,180). This pathogen is transmitted to a vertebrate host by exotic ticks of the genus *Amblyomma* (31,393). Although this disease is endemic to sub-Saharan Africa, it is widespread in several islands of the Caribbean (26,392,393). Due to the presence of indigenous

Amblyomma species ticks that support the growth of *E. ruminantium*, this pathogen poses a continuous threat to domestic and wild ruminants of the US mainland (229,391). This pathogen infects vascular endothelial cells of a vertebrate host. The disease is characterized by high fever up to 107°C, depression, anorexia, excessive salivation and various neurological symptoms including muscle twitching, seizers and circling (72). The fatalities associated with the heartwater disease in cattle are mainly due to severe encephalitis and hydropericardium resulting from destruction of vascular endothelium. Mortality rate up to 90% is reported in naïve animals and animals from non-endemic regions (180,392). Antibiotics such as tetracycline are shown to be effective the initial stages of the clinical disease. The recovered animals remain as carriers of the pathogen for a very long period of time (9). Isolated cases of human fatalities associated with *E. ruminantium* have been reported recently suggesting its zoonotic potential to people (3,223).

An experimental study in goats, involving *Amblyomma* species ticks collected from the Panola Mountain State Park in Georgia, identified a novel *Ehrlichia* species that is closely related to *E. ruminantium* (216,217). Later, this new *Ehrlichia* species, referred to as the Panola Mountain Ehrlichia was shown to cause natural infections in goats and white-tailed deer (216,217,426). Although, one case of human illness associated with Panola Mountain Ehrlichia species is reported recently, its zoonotic potential to people remains unknown (324).

Bovine anaplasmosis:

Bovine anaplasmosis is another economically important disease of cattle caused by *Anaplasmataceae* pathogen, *Anaplasma marginale*. In the beef cattle industry bovine anaplasmosis is estimated to cause an annual loss of nearly $300 millions and it is reported to be

even more in Latin American countries (193). *A. marginale* also infects wild ruminants such as elk, water buffalo, pronghorn, bighorn sheep, deer and antelopes (200). *Dermacentor* species ticks serve as vectors for this organism. Biting flies, fomites such as contaminated needles and dehorning equipment also aid in mechanical transmission of the *A. marginale* from an infected to naïve animal. A less virulent species, *A. centrale*, is also known to cause anaplasmosis in cattle. Because of its antigenic similarity and low virulence, *A. centrale* is used to premunize the cattle to prevent the severe disease caused by *A. marginale*, which is a more virulent species (200). Cattle are also immunized against bovine anaplasmosis using inactivated *A. marginale* antigens and infection and tetracycline treatment method. Erythrocytes are the target sites for *A. marginale* infection which leads to clinical symptoms and disease. Inside the erythrocytes these organisms reside in a host cell derived membrane bound vacuoles where they multiply by binary fission. *A. marginale* and *A. centrale* reside at the margin and centre of erythrocytes, respectively (201). The calves are less susceptible to the disease as they develop non sterile immunity, whereas an infection in adult cattle may result in a mild to a severe form of the disease (192). Clinical signs of bovine anaplasmosis typically include fever, depression, loss of body weight, abortion, anemia and haemoglobinuria. After a period of parasitemia, the number of erythrocytes reduces due to removal of infected RBC by phagocytosis. As a result of this, the infected cattle become persistently anemic (328). If recovered from an acute form of the disease, cattle remains infected for their life (123,189) and acquires lifelong immunity to further infection (193).

Human granulocytic anaplasmosis:

Human granulocytic anaplasmosis (HGA) is an emerging human infection caused by *Anaplasma phagocytophilum*. This disease was first reported in 1994 in the United States in a

human patient from Minnesota and later in Europe in 1997 (18,60,303). Subsequently, HGA cases are reported from many parts of the USA, Europe and Asia (98). *A. phagocytophilum* also infects horses, cattle, and dogs (69,383). This pathogen primarily infects neutrophils of a vertebrate host (97). *Ixodes* species of ticks and white footed mice serve as the vectors and reservoir hosts, respectively, for *A. phagocytophilum* (289,360,377). HGA cases are mostly reported from northeastern and upper midwestern regions of the United States where the *Ixodes* ticks are highly prevalent (85). Passive surveillance data suggests an incidence rate of 1.4 cases of HGA per million people each year with a mortality rate of about 1% (85). As the passive surveillance data usually tend to underestimate the disease, the incidence rate could be much higher than what is reported to date. The most commonly reported clinical and laboratory signs for HGA include fever, chills, vomitions, muscle pain, headache, confusion, respiratory distress, thrombocytopenia, leucopenia and elevated liver enzymes (18,95,100). The disease is more severe in old and immunocompromised people (85). If recognized in the initial stages, the disease can be treated effectively with antibiotics such as doxycyline.

Human ewingii ehrlichiosis:

Human ewingii ehrlichiosis (HEE) is another important emerging rickettsial disease caused by *E. ewingii*. This organism is initially known to cause granulocytic ehrlichiosis in dogs (109). In 1991, *E. ewingii* associated infections are reported in humans in Missouri (46). Transmission of *E. ewingii* to a vertebrate host occurs through a bite of an infected *A. americanum* tick (10). White-tailed deer serves as the reservoir host for this organism (207,428). The definitive host, dog, can also serve as a reservoir host for this pathogen. *E. ewingii* associated human infections are reported only in the United States (100). HEE cases are mostly documented in Arkansas, Missouri, Oklahoma, North Carolina and Virginia where *A.*

americanum ticks are prevalent (141,207,261). *E. ewingii* exhibits tropism for neutrophils of a vertebrate host (46). Commonly reported symptoms for HEE include fever, headache, thrombocytopenia and leucopenia (100). This disease can be life threatening in immune compromised people (278). HEE can be treated effectively with doxycyline. Often people can recover form the infection even without an antibiotic treatment (368).

Human Monocytic Ehrlichiosis:

Human monocytic ehrlichiosis caused by *E. chaffeensis* is one among the most important emerging tick-borne human infections in the United States and other parts of the world (78,228,281). Initial identification of *E. chaffeensis* is made over two decades ago in the blood smear of a patient who suffered with tick bites (228). This pathogen is transmitted by *A. americanum* tick to a vertebrate host. Human monocytic ehrlichiosis distribution in the United States and several regions of the world correlates well with the distribution of its vector ticks (96,277,414). Since molecular aspects of *E. chaffeensis* has been the primary focus of this thesis, a more detailed discussion about this organism and its associated disease is provided below.

Ehrlichia chaffeensis, an emerging human infectious disease agent

Epidemiology:

Ehrlichia chaffeensisEhrlichia chaffeensisEhrlichia chaffeensis is a Gram negative, obligate intracellular rickettsial pathogen responsible for an emerging infectious disease, human monocytic ehrlichiosis (HME) (78,228,281). This disease when first reported in a human patient from Arkansas (228) and was initially thought to be caused by *E. canis* (228). In 1991, molecular evidence has been presented, which distinguished the HME agent from *E. canis* and is

named as *E. chaffeensis* (6,78). This pathogen also infects dogs, coyotes, goats, white-tailed deer and raccoons (93,94,428,433). White-tailed deer and *A. americanum* tick are identified as the primary reservoir host and vector, respectively, for this pathogen (8,79,215,297). An increased incidence of human infections by this pathogen has been reported in recent years (248,277). According the most recent report, an average of 600 human cases of HME is reported each year in the United States alone (248). Figure 1.3 contains a graph showing the incidence of HME from 1999 to 2006, plotted based on the most recent information (248). Most of the reported HME cases are from south-central and south-eastern regions of the United States where the vector tick population is more prevalent (Figure 1.4) (19,20,96,168,248,275,277,361,414). HME is included in the list of nationally notifiable diseases of the United States in 1998 (288).

Clinical signs of HME:

Incubation period from the time of tick bite to onset of the clinical disease ranges from 1- 4 weeks (297). General manifestation of HME range from asymptomatic to mild flu-like symptoms, sometimes progressing to a severe life threatening disease. The most often reported symptoms for this disease include fever, headache, muscle aches, chills, nausea and lymphadinopathy (100,277). Complications such as septic shock-like syndrome, meningitis, organ damage are also reported in some patients (98,103,278,368). Most common laboratory reports indicate leucopenia, thrombocytopenia, and an increase in hepatic transaminase levels (100,277). Although people of all ages are susceptible, the most severe form of the disease and a fatal outcome is often reported in immunocompromised people, pregnant woman, young children and elderly people (134,277).

Diagnosis and treatment:

In most of the cases HME diagnosis is difficult as clinical symptoms are not very specific to this disease and are often confused with other bacterial and viral illnesses. History of the tick bites followed by some of the clinical signs or laboratory findings (described in the previous paragraph) help in presumptive diagnosis of this disease (100). Routine diagnostic tools used to identify the infection include examination of peripheral blood smear, IFA, and PCR. Culturing and identification of the organism is done sometimes for confirmatory diagnosis of *E. chaffeensis* infections (6,57,100,135). Soon after the presumptive diagnosis is made, treating the infection with tetracycline antibiotics is proven to be very effective in clearing the infection (277). Doxycyline, a derivative of tetracycline having a better efficacy and tolerated by most patients, is currently the drug of choice for treating HME (100).

Life cycle:

The completion of *E. chaffeensis* life cycle, like any other tick-borne pathogen, requires its propagation in its tick vector and a vertebrate host (Figure 1.5). Usually larval or nymphal ticks acquire infection while feeding on an infected vertebrate animal. *E. chaffeensis* is passed onto another vertebrate host from nymphal or adult tick carrying the pathogen. There is no evidence reported in the literature to suggest that the pathogen can be transovarially transmitted (220). Maintenance of *E. chaffeensis* in nature is primarily due to its transmission between its tick vector and reservoir host. Several small mammals and wild animals such as coyotes, raccoons and deers serve as vertebrate hosts for *E. chaffeensis* (93,94,428,433). White tailed deer is reported to be a natural reservoir host for this organism (8,79,215,297). Humans are considered as accidental hosts for this pathogen who usually acquire the infection form a tick-bite during their outdoor activities in a tick inhabited area.

Pathogenesis:

Initial infection of *E. chaffeensis* to a vertebrate host occurs when an infected tick takes a blood meal. After the inoculation into a vertebrate host through a tick-bite, the organisms are eventually internalized by monocytes in the blood or macrophages in various organs through phagocytosis (42). Though *E. chaffeensis* exhibits a special tropism towards the macrophages or monocytes, it has also been shown to infect lymphocytes, premyelocytes and metamyelocytes, and neutrophils (42,277). *E. chaffeensis* infection of phagocytic cells of various organs such as liver, spleen, bone marrow, lymph nodes and perivascular mononuclear infiltrates of brain and visceral organs results in damage to those organs (100). The histological findings associated with such infections include multiple focal necroses in liver, spleen, damage to alveolar tissue of lung, interstitial pneumonia, diffuse hemorrhages in various organs, and meningitis. Such multiple organ involvement is most often associated with fatal outcomes in infected patients (80,99,235,279). Pancytopenia and hypocellular bone marrow are rarely reported in patients with acute form of the disease (99,291). Fatal outcomes associated with high bacteremia are often reported in immunocompromised people and also in patients receiving prolonged sulfa drug therapy for other underlying disease conditions (259,278,291). Other pathological findings associated with HME include hyperplasia and megakaryocytosis of the bone marrow (2433). In an infected individual *E. chaffeensis* is predominantly seen in spleen, lymph nodes and bone marrow that have large proportion of mononuclear phagocytic cells (80,235). *E. chaffeensis* is also seen in perivascular infiltrates of brain, heart, kidney, pancreas and digestive tract (80,235).

Attachment, internalization and intracellular multiplication are critical steps in a path to establish infection by an intracellular bacterium, including *E. chaffeensis.* Bacterial attachment to host cells is usually achieved by specialized structures like pili, flagellum, and capsule

(139,334,442). *E. chaffeensis* lacks any of these specialized structures. Although there is no firm evidence, outer membrane proteins such as a 120 kDa protein appear to serve for adhesin (307). In a recent study, indirect evidence was presented about the 120 kDa protein of *E. chaffeensis* as adhesion. *E. coli* expressing this protein was able to attach and gain entry into HeLa cells (307). Nonetheless, it is unclear how *E. chaffeensis* is phagocytosed by the host monocytes or macrophages. Within the phagosome, *E. chaffeensis* undergo replication by binary fission to produce several organisms (morulae) (306). It is not clear how *E. chaffeensis* can overcome the phagosomal clearance in support of its survival and replication within the host cells. The reports from literature suggest that *E. chaffeensis* avoids the phagolysosomal fusion by yet unknown mechanisms (326).

Developmental cycle:

E. chaffeensis has two morphological forms, namely dense-core and reticulate bodies (306,441). Dense-core forms are characterized by highly condensed chromatin, whereas reticulate forms contain loosely arranged chromatin. Transformation of dense-core forms to reticulate forms usually occurs after 24 hours of infection (441). This type of transformation from dense-core to reticulate and back to dense-core forms is typical for the pathogens of the genera *Ehrlichia* and *Anaplasma*. *Chlamydia* species also have very similar developmental cycle, which progresses from dense-core to reticulate and back to dense-core forms (11,157,422,424). Both forms of *E. chaffeensis* are shown to divide by binary fission inside the host cells. Similar to *Chlamydia* organisms, the dense-core bodies of *E. chaffeensis* are the forms that infects naïve cells, whereas the reticulate forms are non infective, metabolically active and highly replicative in nature (199,422,441). The reticulate bodies will transform to dense-cored forms in the phagosome towards the end of their infectious cycle and released from

monocytes or macrophages to start a fresh infectious cycle (441). Transmission electron microscopic studies from our laboratory identified morphological differences in *E. chaffeensis* that are associated with its growth in tick cells and macrophages (DeDonder and Ganta, unpublished data). The striking differences include synchronized growth of reticulate and dense-core bodies of *E. chaffeensis* in macrophages compared to pleomorphic, larger dense-core and reticulate bodies in tick cell-grown organisms (DeDonder and Ganta, unpublished data).

Molecular biology of *E. chaffeensis*:

E. chaffeensis was discovered two decades ago and several advances have been made in understanding the molecular structure of this pathogen. They include sequencing of the complete genome and annotation and characterization of several gene products at the molecular level (162). The genome size of *E. chaffeensis* is 1,176 kbp and contains 1,115 predicted protein coding sequences (162). Considerably more information about the expressed genes of *E. chaffeensis* has been reported in our recent studies involving global proteome and transcriptome analyses methods (Sirigireddy and Ganta, 2007, unpublished data) (347). Comprehensive proteome analysis of *E. chaffeensis* identified 278 expressed proteins representing functional genes for metabolism, structure, transport, and immunogenicity (347). This analysis also aided in the identification of several proteins whose function is yet to be established (347).

Transcriptome analysis utilizing the open reading frame-based microarray also aided in the identification of nearly one third of predicted genes from this pathogen and they also included several differentially expressed genes by the pathogen in response to its growth in tick and macrophage cells (Sirigireddy and Ganta, 2007, unpublished data). The expressed genes identified though microarray analysis are clustered into several groups based on their function.

They include genes that are involved in various cellular processes such as cell division and chromosome partitioning. The list of expressed genes also include those that are implicated in the biosynthesis of amino acids, vitamins and co-factors, metabolism of amino acids, fatty acid metabolism, DNA metabolism, and energy metabolism. Several genes that encode hypothetical proteins were also identified in this analysis (Sirigireddy and Ganta, 2007, unpublished data). A detailed functional characterization of many of the expressed genes remains to be performed.

As the outer membrane proteins of *E. chaffeensis* can be a contact point for its interaction with the host, host immunity may primarily target to these proteins (206,274,421). In fact, the majority of the immunogenic proteins for which antibodies are made during *E. chaffeesnis* infection are against indeed for the outer membrane proteins (347). In this regard, the pursuit for surface expressed proteins of *E. chaffeensis* for diagnostic purpose over the past few years identified several immunoreactive outer membrane proteins. The list of immunogenic proteins recognized include a variable length PCR target gene (VLPT), a homolog of heat shock protein gene (groESL), three glycoproteins (gp47, 120 and 156) and an abundantly expressed p28 kDa outer membrane proteins (p28-Omp's) (135,352,370,371,425,436,437). The p28-Omp proteins are also glycosylated and, in addition, contain a second post-translational modification by phosphorylation (352). Other *E. chaffeensis* genes reported in the literature include a highly conserved 16s rRNA gene, DO/DeqQ family serine proteases, DsbA-like disulfide bond formation proteins, quinolinate synthetase A (NAD/A), VirB/D, a hypothetical protein named as Esp73, ftsZ gene, and several hypothetical proteins (135,203,246,347,438). A brief description of *E. chaffeensis* partially characterized genes is provided below.

16s rRNA: An identical 16s rRNA gene sequence has been reported from various *E. chaffeensis* isolates (412). Because of its highly conserved nature within the *E. chaffeensis* species, the 16s rRNA is used for species differentiation and phylogenetic classification of other related organisms (6,97).

GroESL: *E. chaffeensis* homologs of *E. coli* heat shock genes, groES and groEL (groESL operon), are first identified and characterized from a genomic library of *E. chaffeensis* in 1993 (372). The GroESL operon consists of two open reading frames that encode for a 10.3 kDa protein (GroES) and a 58 kDa protein (GroEL) comprising 94 and 550 amino acids, respectively (372). Both genes in the GroESL operon are separated by a 100 bp non coding sequence (372). In a recent study by Ge and Rikihisa (135), GroEL is identified as a surface exposed protein. In general, bacterial heat shock proteins play a critical role in cellular protection under stressful conditions. Their production increases under various types of environmental stresses including increase in temperature, nutrient deprivation (174,214). The precise role of the *E. chaffeensis* groESL genes remains to be evaluated.

Variable Length PCR Target gene (VLPT): VLPT is another immunoreactive protein reported in 1999 (370). VLPT of Arkansas strain of *E. chaffeensis* is 44 KDa in molecular weight and contains four non-identical, 90 bp, direct tandem repeats (370). The number of repeats is variable in different isolates of *E. chaffeensis*, and hence, it is used as a PCR target for strain differentiation (62,370). A homologue of VLPT that encodes for a 19 kDa protein has also been identified recently in *E. canis* (245). The exact role of VLPT in *E. chaffeensis* pathogenicity is not known.

FtsZ: An *E. chaffeensis* homolog of the ftsZ gene of *E. coli* was first identified in 2003 (203). *E. coli* ftsZ sequence was utilized for designing degenerate primers to amplify its homolog from *E. chaffeensis* genome. The length of ftsZ open reading frame in *E. chaffeensis* is 1263 bp and is predicted to encode for a 45.7 kDa protein (203). FtsZ gene of *E. coli* has been well characterized and was shown to play an important role in cell division (179,225). The functional role of ftsZ in *E. chaffeensis*, however, is remains to be characterized.

VirB/D: Whole genome sequence analysis of *E. chaffeensis* identified homologs of the genes that encode for type four secretion system (T4SS), virB/D (162). Typically, in most pathogenic organisms the genes that encode for T4SS apparatus are clustered in a single locus (66). In *E. chaffeensis*, the virB/D genes are present in two separate loci (63,162,273). The genes virB8-1, virB9-1, virB10, virB11, virD4 are present in one locus; whereas, the second locus contains the genes virB3, virB4-1, and four paralogous virB6 genes (63,162,273). All these genes are reported to be transcriptionally active *in vitro* in macrophage (DH82) cultures (63). A recent study by Cheng et al (63), demonstrated that expression of these genes is high in the exponential growth phase of the pathogen and drops down prior to their release from host cells. This study also identified a regulatory protein of *E. chaffeensis*, EcxR, that serves as a transcriptional activator for all virB/D genes (63). The expression of EcxR protein was also shown to be autoregualted to allow the stage specific expression of these genes. The precise role of these proteins in *E. chaffeensis* remains to be investigated.

Glycoproteins: Several immunoreactive glycoproteins (gp) of *E. chaffeensis* are identified and they include gp47, gp120, gp200, and p28-Omp proteins (91,92,244,437). Brief description of these proteins is provided in the following paragraphs.

Gp47: Gp47 is a 47 kDa immunoreactive glycoprotein of *E. chaffeensis* identified recently (91,92). This protein is characterized by several serine and threonine rich repeats that vary in number in various *E. chaffeensis* isolates. The glycosylation sites for this protein are present within these repeats and the carboxy-terminal repeats contain epitopes for antibodies (92). Gp47 protein is shown to be expressed only on the surface of infectious dense-core forms of *E. chaffeensis* (92). An ortholog of *E. chaffeensis*'s gp47, which is a serine rich 36 kDa glycoprotein (gp36), has also been identified in another closely related *Ehrlichia* species, *E. canis* (92). The precise role of this glycoprotein in *E. chaffeensis* pathogenicity remains to be studied.

Gp120: Gp120 is a glycoprotein of *E. chaffeensis*. Cloning and characterization of the gene that encodes for this glycoprotein was first reported in 1997 (437). The estimated molecular weight, based on amino acid sequence of gp120 is 67kDa (247). It is one of the highly immunoreactive membrane proteins and contains four identical, serine-rich, tandem repeat sequences. The length of each repeat is 240 bp and it contains highly hydrophilic domains (437). A considerable variation in the molecular weight for this protein resulting from variation in number of repeats was also reported for different *E. chaffeensis* isolates (63,361). This glycoprotein was reported to be preferentially expressed in dense-core forms of the *E. chaffeensis* (307). Analysis of glycosylation sites in gp120 revealed 91 O-linked and 2 N-linked glycosylation sites (gp120) (247). The sugars identified at the glycosylation sites of this protein include glucose, galactose and xylose (247). An ortholog of *E. chaffeensis* gp120, which is referred to as gp140, has also been identified in *E. canis* (247,436). The immunogenic gp140 of *E. canis* also contains several identical, serine rich tandem repeats, each 108 bp in length (247). These repeats in *E. canis* may

similarly vary in different isolates, but this has not been reported. *E. coli* expressing the recombinant *E. chaffeensis* 120 kDa protein acts as an adherent protein as evidenced by studies in HeLa cells under *in vitro* (307).

Gp200: Gp200 is another immunoreactive glycoprotein of *E. chaffeensis* with a predicted molecular weight of 156 kDa. The recombinant gp156 protein exhibited a larger molecular weight that is close to ~200 kDa. The increase in molecular mass is due to the contribution of glycan moieties that are post translationally added to this protein (244). To date, gp200 is the largest of all the immunoreactive glycoproteins identified in *E. chaffeensis* (244). An ortholog of *E. chaffeensis* gp200, which is also designated as gp200 has also been identified in *E. canis* (244). Gp200 of *E. canis* is shown to be highly reactive to the immune sera obtained from natural or experimentally infected dogs (244). Gp200 of *E. chaffeensis* and *E. canis* are homologous to AnkA protein of *A. phagocytophilum* and are also reported to contain several ankyrin repeats (53,264,294). AnkA protein of *A. phagocytophilum* is shown to be transported into the nuclei of vertebrate granulocytes and bind to host cell DNA and nuclear proteins (53,294). *A. phagocytophilum* AnkA protein is demonstrated to be secreted by T4SS and tyrosine-phosphorylation by the host cell tyrosine kinase during its initial stages of infection (167,210). Knockdown or inhibition of host cell tyrosine kinase, or cytoplasmic administration of anti-AnkA antibodies effectively inhibited the host cell infection by *A. phagocytophilum in vitro* (210). These findings demonstrate that tyrosine phosphorylation of *A. phagocytophilum* AnkA protein is critical for its infection of vertebrate host cells (210). The biological significance of AnkA homologues of *Ehrlichia* species remains to be established.

So far, the characterization of genes that encode for glycoprotein genes in several *Ehrlichia* species is limited to their initial analysis for their sequence analysis and presence of glycan moieties. No predictions with regards to importance of these proteins and presence of several tandem repeats in these genes have been made to date. Studies pertaining to other bacteria suggest that bacterial glycoproteins may be involve in several important functions such as protein stabilization, maintenance of cell structure, adhesion of pathogens to host cells, and altering the host immunity (52,250,292,309,343,399). Glycosylation of outer surface proteins is also reported for *Anaplasma* species pathogens (133,338,385). Removal of glycan moieties from outer surface proteins reduced the binding ability of *Anaplasma* organisms to host cells *in vitro*. These findings suggest that glycosylation of these outer surface proteins may play a role in pathogen adhesion and entry into its host cells (133,293). Reports from literature also suggest that post translational modifications of bacterial proteins alter the ability of T-cells to recognize the antigenic epitopes by altering the host immunity against the pathogenic organisms (131,171,330). In a recent study, non-glycosylated synthetic proteins of gp47 of *E. chaffeensis* and its homolog of *E. canis* (gp36) were shown to be less immunoreactive compared to their glycosylated forms (92). These findings suggest that glycosylation of the proteins plays an important role in bacterial pathogenicity. It remains unclear if the glycosylation pattern including the number of repeats in the glycoproteins in tick cell background remains the same. The precise role of various carbohydrate moieties added post translationally to the proteins of several *Anaplasmataceae* pathogens is yet to be revealed.

28 kDa outer membrane proteins (p28-Omp): Several studies reported 28 kDa outer membrane proteins encoded by a multigene locus (p28-Omp locus) that contains 22 tandemly arranged paralogous genes (62,274,321,322,347,352,436). Open reading frames of these

paralogous genes are separated from each other with non-coding sequences ranging from 9 to 603 bp (162). These genes differ mostly from each other by containing three highly variable regions that are hydrophilic in nature (322). These hyper variable regions also included the immunogenic B-cell epitopes (204,206,421). Considerable variation in the p28-Omp locus genes of several *E. chaffeensis* isolates has also been reported (62,255). The differences include variations resulting from insertion mutations, several nucleotide sequence differences within each gene and also included gene deletions (62,255).

E. chaffeensis isolates are organized into three groups (I, II, and III) based on the sequence variations within the p28-Omp locus as judged by restriction digestion analysis (62). The analysis was performed using 10 different isolates of *E. chaffeensis* recovered from human patients from various regions with in the United States. Group I included three isolates; Arkansas, Osceola, and Lithonia. Group II was comprised of St. Vincent, Chattanooga, West Paces, Heartland, and Wakulla isolates. Liberty and Jax isolates were included in Group III (62). The p28-Omp gene 18 is present only in Group I isolates. Duplication of the p28-Omp gene 15 is observed in isolates belonging to Group III. Loss of gene 18 is also noted in *E. chaffeensis* isolates belonging to Groups II and III (62). Analysis of *E. chaffeensis* genome spanning two outer membrane protein genes, gp120 and VLPT also revealed differences within the pathogen isolates. They include variation in the length of these genes resulting from the loss or gain of 240 and 90 bp long repeats with in the gp120 and VLPT genes, respectively. Grouping of isolates based on these variations were not correlated with the p28-Omp based grouping (62). Based on these data the authors suggested that variations exist within genomes of different *E. chaffeensis* isolates. These variations may have resulted from the pathogengrowth in a vertebrate

host, particularly to overcome host immune responses for its continued persistence. This hypothesis remains to be tested.

Homologues of the p28-Omp locus genes were also identified in other *Ehrlichia* species (75,147,272,402,439). The homologues of the p28-Omp locus were referred to as p30-Omp in *E. canis* and Map1 in *E. ruminantium* (272,402). In *E. ewingii* and *E. muris,* the loci were referred with the same name as that for *E. chaffeensis* (75,147,439). Similar to *E. chaffeensis* p28-locus, the p-30 locus of *E. canis* includes 22 tandemly arranged paralogous genes (272). The p28-Omp homologous loci of *E. ruminantium*, *E. ewingii*, and *E. muris* were reported to contain 16, 19, and 21 paralogous genes, respectively (75,402,439). The gene numbers in the p28-Omp locus may also be variable for *Ehrlichia* species isolates, including *E. chaffeensis* resulting from the loss or gain of genes as evidenced from the sequencing of a subset of genes spanning p28-Omp 14 through 19 (62).

One of the novel observations of the gene structure of the p28-Omp loci of different *Ehrlichia* species is the presence of a gene that encodes for a putative transcriptional regulator and a secA gene positioned at the 5′ and 3′ ends of the loci, respectively (75,272,402,439). The hypothetical transcriptional regulator of *E. ruminantium* was shown to be polycistronically transcribed along with p28-Omp homologues of this pathogen (402). Recent studies from our laboratory also reported the expression of this putative regulatory protein in *E. chaffeensis* (347) (Sirigireddy and Ganta, 2007, unpublished data). The secA protein is reported to a play major role in translocation of bacterial outer membrane proteins to outer membrane (30). The regulatory role of this transcriptional regulator and the significance of secA in *E. chaffeensis* gene expression and transport, respectively, remain to be studied.

The p28-Omp antigens are highly immunoreactive and are recognized by sera from the *E. chaffeensis* infected people (60,274,421). These antigens are also similarly recognized by the murine host, assayed in experimental infection studies (204,206,347,352,353,421). The p28-Omp antigens may also serve as protective antigens for use in vaccine development. For example, Map1 DNA based vaccine is protective against *E. ruminantium* challenge offered a partial protection in both natural and experimental hosts (mice and sheep) (270,271). Similarly, monoclonal antibodies against p28-Omp of *E. chaffeensis* conferred protection against lethal disease in SCID mice as long as the antibodies are supplemented (421). Ohasi et al. (274), reported rapid clearance of *E. chaffeensis* in mice immunized with recombinant p2-Omp 19 protein compared to control mice. Similar protective role of p28-Omp 19 proteins has also been reported in other *Ehrlichia* species. Mice immunized with the *Ixodes ovatus* ehrlichia-specific p28-Omp 19 monoclonal antibodies protected against fatal *Ixodes ovatus* ehrlichia infection (262).

The immunogenic regions of the p28-Omps are located with in their hypervariable regions (204,206,322,421). The major differences in the p28-Omp paralogues of *Ehrlichia* species are also located with in the hypervariable regions. The presence of multiple genes having hypervariable regions where the immunogenic epitopes are located led to considerable interest in understanding their possible role in immune evasion by the pathogen and pathogenicity. Several studies have been performed to determine gene expression profile and proteins made from this locus (62,221,274,321,322,436). Initial transcriptional analysis reported in the literature is mostly based on non-quantitative RT-PCR assays. Although there are differences in the reported data by different research groups, the conclusions are consistent that multiple genes of the p28-

Omp locus for the bacteria are transcriptionally active in *E. chaffeensis* originating from macrophages (62,221,272,398,436). Similar analysis for tick cell-derived *E. chaffeensis* both *in vitro* and *in vivo* identified the expression from one gene, the p28-Omp 14 (397,398). Similar expression pattern from the p28-Omp homologues has also been reported for other *Ehrlichia* species, *E. canis* and *E. ruminantium* (28,272,396,402). It is not clear why multiple genes of the p28-Omp locus are transcriptionally active in macrophages infected with *E. chaffeensis* under *in vitro*. One hypothesis is that one or more of these are expressed at higher levels while others are not. Alternatively, not all the transcripts may be translated into mature proteins. These hypotheses require the evaluation of RNA using quantitative methods and identification of expressed proteins.

Studies by Ohasi et al. (274) and Long et al. (221) identified one major expressed protein from vertebrate macrophages, i.e., the p28-Omp 19. Similarly, the p28-Omp 14 is the only protein expressed from this locus in tick cell grown *E. chaffeensis* in both *in vitro* and *in vivo* (221,397). Recent proteomic analysis of individually picked immunodominant *E. chaffeensis* proteins selected from 2D gels from our laboratory further confirmed the major protein expression in macrophages from the p28-Omp 19 and in tick cells from the p28-Omp 14 genes (347,352,353). These differentially expressed p28-Omp proteins of *E. chaffeensis* are expressed in multiple forms as a result of post translational modifications such as glycosylation and phosphorylation (352,353). These findings are also supported by global proteome and open reading frame based microarray analysis from our laboratory (Sirigireddy and Ganta, 2007, unpublished data) (347). Tick cell and macrophage-specific expression predominantly from the p28-Omp gene 14 and 19 homologs of *E. canis*, p30-10 and p30, respectively, has also been reported in the literature (113,353,396). The host-specifically expressed p28-Omp homologues

of *E. canis* proteins are also post translationally modified by glycosylation and phosphorylation (352,353). The predominant expression form the p-28 gene 14 homolog of tick cell-grown of *E. ruminantium*, Map 1-1 is also reported both *in vitro* and *in vivo* (28). A cartoon representing the p28-Omp multigene locus including the host-specifically expressed genes is shown inFigure 1.6.

The p28-Omp proteins of *E. chaffeensis* are also homologous to a polymorphic, outer membrane protein multigene family, referred to as the major surface protein 2 (msp2/p44) in *A. marginale* and *A. phagocytophilum*, two closely related tick transmitted rickettsiales (440). The msp2/p44 genes encode for 42-49 kDa, highly immunoreactive proteins that are recognized by immune sera from infected animals or human patients (23,102,123,169,261,287). Several p44 paralogous genes scattered throughout the genome of *Anaplasma* species (162,443,444). The msp2 genes differ from the p28-Omp of *Ehrlichia* species by their size and number of variable repeats. Msp2/p44 genes contain only one variable region, which is flanked by highly conserved C- and N-terminal sequences (114,162,212). T-cell epitopes are located within the variable and conserved regions; whereas B-cell epitopes are predominantly found in the hypervariable regions of the msp2 antigens (1,43). Immunization of a vertebrate host with msp2/p44-specific monoclonal antibodies is also shown to offer protection against *Anaplasma* species infections (44,45,190). Generation of antigenic variants from the msp2/p44 genes is documented in persistently animals infected with *A. marginale* (98,102,211,286). The antigenic variants generated from this multigene locus may be one of the important mechanisms of immune evasion.

Tick and vertebrate host-specific differential expression from the msp2 locus of *Anaplasma* species, similar to the p28-Omp proteins, has also been reported

(123,170,213,219,408,445). The msp2/p44 proteins of *A. pahgocytophilum* are also recently reported to undergo post-translational modifications such as glycosylation (338,385). *A. phagocytophilum* msp2 has also been demonstrated to have porin activity and aids in acquisition of sugars and metabolic intermediates from the host cell (164). Adhesin role of a porin-like outer surface protein of has also been reported for other bacterial pathogens (159). For example, in a Gram negative human respiratory tract pathogen, *Moraxella catarrhalis*, the porin-like outer surface protein is reported to have adhesin role (159). In a recent study by Kumagai et al., (198), porin structure and activity has been reported for the p28-Omp gene 19 protein of *E. chaffeensis*. However, the functional importance of this property in these pathogens is yet to be determined.

Based on the above discussion it is clear that *Ehrlichia* and *Anaplasma* species pathogens contain several common features. They include the presence of orthologous genes in their genomes that encode for immunodominant outer membrane proteins. The commonalities also include the presence of multiple homologous outer membrane protein genes that contain hyper variable regions which make these paralogous genes differ from each other. The hyper variable regions of the outer membrane proteins contain dominant immunogenic epitopes recognized by vertebrate host immune cells. Loss or gain of genes or number of variable regions within the genes from the multigene locus may aid in pathogen adaptation and persistence in its hosts. In fact, the evidence from *Anaplasma* species, suggest that antigenic variation is possibly contributing to pathogen persistence in a vertebrate host. Other features shared by this pathogen with regards to these outer membrane proteins include porin structure and host-specific expression. Presence of multiple outer membrane protein genes in their genome with predominant expression from one gene, suggest their possible role in evasion of host immune

responses by the pathogen. Together, all these data suggest that *Ehrlichia* and *Anaplasma* pathogens alter their gene expression in support of their survival and possibly adapting to its tick and vertebrate host environments. It is entirely unknown how these pathogens are able to sense the host environment to regulate their gene expression. It also remains unclear what are the molecular strategies employed by these tick-borne pathogens to achieve their host-specific gene expression. A significant gap of knowledge also exists with regards to the importance of existing multiple forms and functions of p28-Omp proteins. The biological significance of host-specific expression, post translational modification and their relevance to *Ehrlichia* infection remains to be understood to gain important insightabout the biology of host pathogen interactions.

Host immune responses against *E. chaffeensis* infection:

Immune response of an infected animal against *E. chaffeensis* infection depends on several factors including host species, age, an underlying disease condition, and immune status of an infected host. For example, the HME is reported to be more severe in children, elderly and immunocompromised people (134,277,335). Presence of antibody titers in people from HME endemic areas with no apparent clinical signs suggests variation in host responses to the pathogen. The host responses may vary from manifestation of a clinical disease, clearing of clinical signs and possibly even clearing the pathogen (233,432). Similarly, in reservoir hosts, pathogen infection does not appear to cause clinical disease, but the persistence of the pathogen and antibody titers are documented (76).

Several studies have been carried out since the discovery of *E. chaffeensis* in 1987, for a better understanding of host immunity against this pathogen (24,55,128-130,202,419-421). In recent years, use of murine models has led to a significant progress towards understanding of

host immune mechanisms against *E. chaffeensis* infection (36,128-130,419-421). Experimental infection studies with *E. chaffeensis* demonstrated that immunocompetent mice are able to clear the infection within 10-17 days post infection (129,130,420). Contrary to these findings, severe combined immune deficient (SCID) mice which lack T- and B-cells fail to clear the pathogen and exhibit severe illness after 24 days of infection resulting in fatal outcomes (420). *E. chaffeensis* infection to major histocompatibility complex-II (MHC-II) deficient mice also demonstrated the persistence of the pathogen as long as 92 days (the longest time evaluated in these studies) (129,130). Similar studies in toll-like receptor 4 (tlr4) deficient mice resulted in a delay of *E. chaffeensis* clearance of up to 30 days (129,130). Together, these findings suggest that T-cells, B-cells, and macrophages are the major contributors of host response in clearing the *E. chaffeensis* infection. It is also evident that antibodies (made by activated B-cells) alone or T-cells or macrophage activation independently are not sufficient for complete clearance of the pathogen. Effective clearance of this intracellular bacterium, therefore, appears to be needing contributions of all these components of immunity, including T-cells, B-cells and macrophages. The contributions of these three components of the immune system are described in the following paragraphs.

The significance of antibodies in clearing the severity of infection is well described by Winslow et al. using a SCID mouse model (419,421). The studies involving these mice clearly illustrate the inability of antibodies to completely cure the pathogen from a vertebrate host. SCID mice are protected from a severe disease and fatal outcome when adaptively transferred an immune serum from *E. chaffeensis* infected immunocompetent mice, prior to or after establishment of active infection with *E. chaffeensis* (421). Prolonged protection, up to 70 days, from fatal infection is observed in SCID mice as long as they receive repeated immune serum

(206,421). *E. chaffeensis* infected immunocompetent mice are also able to clear the infection three days after administration of immune serum (421). These studies clearly demonstrate that antibodies play a critical role in clearing *E. chaffeensis* infection. Presence of bacteremia at low levels in SCID mice, even after 70 days of antibody administration and complete pathogen clearance by BLAB/c mice suggest that antibodies alone are not sufficient and require T-cells and possibly macrophage activation for effective clearance of *E. chaffeensis* infection (206,421).

The antisera against *E. chaffeensis* are predominantly made against outer membrane expressed proteins (347). Importantly, very few *E. chaffeensis* cytoplasmic proteins are recognizable by the pathogen immune sera (347). This is also consistent with the recognition and elimination of severity of infection by the polyclonal serum that predominantly reacted with the outer membrane proteins (421). Further detailed analysis of antigens reacted with the immune sera aided in the identification of 28 kDa outer membrane proteins (p28-Omps) as the predominant immunogens (421). In fact, studies utilizing a 28 kDa outer membrane protein (P28-Omp 19)-specific monoclonal antibodies also offered similar protection to SCID mice from *E. chaffeensis* infection (206,419). Repeated administration of P28-Omp 19 specific monoclonal antibodies at weekly intervals starting 10 days after infection prolonged the protection of SCID mice from the severity of *E. chaffeensis* infection up to 70 days (206). The 28 kDa proteins are also shown to be dominant immunogens recognized by immune serum from several human patients infected with *E. chaffeensis* (60,274,421). Together all these findings suggest that outer membrane proteins, particularly the p28-Omp proteins are primary targets for the host immune system (204,206). To date, no evidence is available for antibody- mediated intracellular killing of *E. chaffeensis*. A recent study by Li and Winslow (205) suggests that the *Ehrlichia* that are released extracellularly from the infected and lysed host cells during its

infectious cycle in a vertebrate host are susceptible to antibody-mediated killing (205,419). This may be the most effective way antibodies work against *E. chaffeensis*. The precise mechanism of action of antibodies in clearing this intracellular pathogen remains to be understood.

Utilization of several laboratory knockout mouse strains with targeted disruption to immune system genes led to a considerable understanding of the role of various subsets of T-cells in host defense against *Ehrlichia* infection (32-34,55,129,130,172,262). In a study by Ganta *et al*. (129), it has been demonstrated that MHC II knockout mice, which are deficient in helper T-cells, are unable to clear the infection for several months. $CD4^+$ helper T-cell deficient mice are able to clear the *E. chaffeensis* organisms but clearance is delayed by about two weeks compared to immune competent mice (129,130). These findings suggest that $CD4^+$ helper T-cells are critical for pathogen clearance, but $CD4^+$ T-cells alone may not necessarily be the only cells required for clearing the infection (55,129). These observations are consistent with those made for clearing infection of monocytes with other intracellular bacteria such as *Mycobacterium tuberculosis* and *Francisella tularensis* (73,74). Previous studies from the literature suggest that a vertebrate host that lacks $CD4^+$ T-cells may contain other T-cells including $CD4^-$ and $CD8^-$ T-cells and natural killer T-cells, which may compliment for the $CD4^+$ T-cell deficiency, aiding pathogen clearance (73,74,242,314).

The role of cytotoxic lymphocytes ($CD8^+$ T-cells) in host immunity against *E. chaffeensis* has also been investigated in our laboratory (55,129). *E. chaffeensis* infected MHCII knockout and/or $CD4^+$ T-cells deficient mice were utilized to evaluate the contributions of $CD8^+$ T-cells in clearing the infection. Both types of mice exhibited a minimal $CD8^+$ T-cells activity after a single dose of *E. chaffeensis* infection; whereas a detectable $CD8^+$ T-cells activity was observed

after a second dose of infection with this pathogen (129). In a separate study, experimental infection of *E. chaffeensis* to β2 microglobulin (β2M) (a structural component of MHC I)-knockout mice resulted in complete clearance of the infection with a slight delay compared to $CD4^+$ positive mice (55). Together, these findings suggest that $CD8^+$ T-cells are minor contributors in pathogen's clearance. Similarly, the function of the NKT cells also appears not to be critical for *E. chaffeensis* clearance. Indirect evidence supports this hypothesis; for example, in MHC II deficient mice the NKT cells are functionally normal, yet they are persistently infected (56,129,130). Moreover, the SCID mice that are deficient for T- and B-cells but contain functionally normal NKT cells exhibited persistent infection by *E. chaffeensis* (420). These findings suggest that NKT cells can't override the deficiency of T-cells in clearing the infection. Other subtype of T-cells known as γδ T-cells are present in peripheral blood of *E. chaffeensis*-infected human patients indicating that γδ T-cells may be essential components of host immunity against this pathogen (49,50). The precise role of these T-cell subtypes needs further investigation.

The importance of cytokines in mediating *E. chaffeensis* clearance by a vertebrate host has also been investigated by several researchers (24,32,442). In a study by Zhang *et al*. (442), microarray analysis of total RNA isolated from *E. chaffeensis* infected human monocytic cell lines (THP1 cells) revealed suppression of several proinflammatory cytokine expression including IL-1α, IL-4, IL-6, IL-12, IL-15, and IL-18 compared to uninfected monocytic cells. These cytokines are responsible for early inflammatory responses towards an infection (88). Suppression of these cytokine expression during initial stages of *E. chaffeensis* infection may help the pathogen to escape from being killed by NKT cells and cytotoxic T-cells and colonize inside the macrophages or monocytes (88). IL-15 and IL-18 are also known to play role in

activation of $CD4^+$ T-cells that produce IFN-γ to activate macrophage-mediated killing of infectious agents (24,32,208). This is primarily achieved by IFN-γ medicated limiting of iron availability that is critical for the growth of *Ehrlichia* organisms (24). Therefore, suppression of these cytokines may in turn help *E. chaffeensis* to survive and replicate inside the infected macrophages (234,263). Although cytokines appear to mediate host immunity against *E. chaffeensis* organisms, further studies are required to identify the precise role of these cytokines in host defense mechanisms against this intracellular bacterium.

Together all the above discussed findings from various studies utilizing a mouse model suggest that a coordinated effort of various components of the immune system may be necessary for effective pathogen clearance. Importantly, components of the immune system including antibodies, macrophage activation, involvement of several T-cell sub types and cytokines may play a critical role in *E. chaffeensis* clearance by a vertebrate host (24,129,130,202,420,421). This coordinated effort of multiple components of the immune system may be critical for the effective clearance of the pathogen during its extracellular stage while infecting the naïve macrophages or monocytes and during its survival within them.

Studies utilizing the mouse model clearly show that an immunocompetent vertebrate host can clear *E. chaffeensis* infection in a short period of time. However, this model fails to explain how the bacteria are able to persist in a natural vertebrate host during its life cycle. The studies did not provide any clues about the strategies employed by this pathogen to persist in its immunocompetent vertebrate hosts, for example in a white-tailed deer. It remains to be known what really happens in a vertebrate host that aids *E. chaffeensis* to overcome host immunity to successfully complete its lifecycle. There may be several reasons why we do not see the

persistence of the pathogen in an immunocompetent mouse model. Firstly, most of the infection studies up to now are conducted using *E. chaffeensis* grown in vertebrate macrophage cells (24,55,129,130,202,420,421). Secondly, the infection experiments are always carried out by needle inoculation intraperitoneally or subcutaneously but not by tick transmission. It is possible that *E. chaffeensis* persistence may require its growth in tick cell background, as in the natural setting, for persistence in a vertebrate host. Other possibility may be that the mouse may need a natural route of inoculation, which is infection from a tick bite. These are the two important components that may be required to demonstrate theof the pathogen in a mouse model. They include infection of mice through an infected tick-bite or using tick cell-grown *E. chaffeensis* as inoculum. Recent studies from our laboratory using the mouse model but with the *E. chaffeensis* inoculum originating from the tick cells demonstrated an altered host response (128). Comparisons are made between mice that are infected with tick cell or macrophage-grown bacteria and the findings from this study are discussed in the following few paragraphs.

Several interesting observations are made from our recent study including delayed clearance of tick cell-grown *E. chaffeensis* by the murine host (128). An increased bacterial load is observed in the livers and spleens of mice infected with tick cell-derived bacteria compared to those infected with macrophage-grown *E. chaffeensis.* Moreover, although it appeared that bacteria are cleared as assessed by the real-time RT-PCR assays, mice infected with *E. chaffeensis* originating from tick cells showed a significant increase in pathogen-specific IgG response. The antibody levels also increased steadily and are more prolonged for tick cell-derived bacteria compared to macrophage-derived organisms (128). It is possible that bacteria may be persisting but at a lower level and are undetectable.

The predominant IgG response noted in mice infected with macrophage- or tick cell-derived *E. chaffeensis* is from IgG2 and IgG3. However, the concentrations of these IgG subtypes is 2-3 times more in mice infected with tick-cell grown *E. chaffeensis* compared to those infected with macrophage-grown bacteria. Altered cytokine induction by the host in response to the bacterial growth in tick or macrophage cells is also reported (128). The concentrations of IL-1α, IL-4, IL-6, and IL-10 cytokines are considerably low for the mice infected with tick cell-originated bacteria compared to those infected with macrophage-grown *E. chaffeensis* (128). This is another indication suggesting that when the bacteria is originating from the tick cells it is suppressing the cytokine-mediated arm of the immune response, which may in turn contribute to delayed clearance by the host. Other interesting observation has been that antigens recognized by the host immunoglobulins for the tick cell- and macrophage-grown bacteria are significantly different (128).

The above discussed findings clearly suggest that host cell backgrounds in which *E. chaffeensis* is grown may be one of the contributing factors for whether or not the pathogen is cleared or persists. The natural infectious cycle of *E. chaffeensis* involves transmission of the pathogen to a vertebrate host though the bite of an infected tick. Ticks typically take a blood meal on a host for a long period of time ranging from 2-14 days, which depends on the life cycle stage of a tick (358). To prevent rejection by a vertebrate host, it may be necessary for ticks to alter the host immune responses. Several studies described the impact of tick feeding on host immunity (116,117,137). For example, tick saliva is shown to reduce the proliferation of salivary antigen-specific T-cells and also reduce macrophage activation (116). Tick saliva is also shown to increase the production IL-10 and reduction of the IFN-γ by splenocytes. Successive tick feeding is also shown to influence T-cell response changing from T-helper type 1(Th1) to T-

helper type 2 (Th2). This shift in T-cell response is characterized by an increased cytokine profile for IL4, IL-10, and transforming growth factor (TGF)-β and is accompanied with a reduction in IL-2, IL-12 and IFN-γ (116). The Th2 response in turn reduces the IFN-γ mediated macrophage activity against the pathogens. This selective promotion of Th2 cell development may help a tick for its continued feeding on a host. TGF-β was previously shown to suppress the host immunity by reducing the Th1 cytokine profile (54).

There are two aspects that may influence *E. chaffeensis* persistence in a vertebrate host. Based on the published evidence, discussed above, it is clear that tick feeding influences the host immune responses (116,117,137). The tick induced changes in host immunity may provide a favorable environment for tick transmitted pathogenic organisms, such as *E. chaffeensis*, in support of their adaptation and persistence in a vertebrate host (137). The contributions of *Amblyomma americanum* tick feeding in altering host immunity and *E. chaffeensis* persistence, however, are yet to be determined. Secondly, the pathogen may also employ strategies to adapt to its tick vector. They may include altering protein expression in support of its survival in the invertebrate host cell environment and to overcome tick defenses. The altered tick-specific differences in pathogen may also aid the pathogen to overcome vertebrate host defenses and to persist after its transmission from a tick. This hypothesis is supported by recent studies from our group demonstrating that *E. chaffeensis* originating from the tick cell environment alters host immune response (including delayed clearance) by a vertebrate host (assessed in the murine host model, which was discussed in detail above) (128). It is not clear how *E. chaffeensis* differs in its molecular structure during its survival in its vertebrate and in vertebrate host cell environments. To address this gap of knowledge, our research group also has undertaken several approaches to find out what are the differences between the *E. chaffeensis* organisms originating

from tick and vertebrate host cells. The approaches include the evaluation of *E. chaffeensis* by proteomic and transcriptomic methods to identify differences in the gene expression profiles. Electron microscopic studies are also performed to assess the morphological differences in the pathogen. These studies revealed considerable tick cell- and macrophage-specific variations.

Proteomic analysis of *E. chaffeensis* originating from tick and macrophage cells revealed numerous differences in protein expression patterns of the pathogen (347,352). Two-dimensional gel electrophoresis (2DE) analyses revealed several differences in the *E. chaffeensis* proteomes originating from macrophage and tick cell backgrounds. Many tick cell-specific *E. chaffeensis* proteins are identified on 2DE gels, most of them are resolved on the pH gradient 7-9. Similarly, many macrophage-specific proteins are identified in the 2DE gels on a pH gradient of 5-7 (352). Comparisons of the 2DE gels also aided in identification of nearly 50% proteins being differentially expressed by *E. chaffeensis* organisms (352). A more comprehensive analysis to identify proteins of *E. chaffeensis* proteome is reported recently from our research team, utilizing mass spectrometry methods (347). The analysis aided in identifying 278 *E. chaffeensis* expressed proteins. These proteins represent nearly one fourth of the predicted genes of the *E. chaffeensis* genome (162,347). The identified proteins include those involved in metabolic pathways, DNA synthesis, protein and energy synthesis, transport functions, and also included many hypothetical proteins whose function is unknown (347). Host cell specifically expressed proteins of *E. chaffeensis* included several outer membrane proteins, cofactor and vitamin biosynthesis proteins, and many hypothetical proteins (347). The mass spectrometry analysis also confirmed the differential expression of the major outer membrane proteins of the p28-Omp locus. The P28-Omp gene 14 is the major expressed protein when the pathogen is grown in tick cells *in vitro* and *in vivo* (347,352,353,397,398).

Whole genome microarray analysis of *E. chaffeensis* total RNA also aided in identification of several host specifically expressed genes (Sirigireddy and Ganta, 2007, unpublished data). The analysis aided in the identification of about 350 genes each in *E. chaffeensis* originating from tick and macrophage cells. These represent nearly one third of the total predicted genes for this pathogen (162). Among these, 263 genes are common for *E. chaffeensis* coming from either host cell background. Tick cell- and macrophage-grown *E. chaffeensis* uniquely expressed 78 and 118 genes, respectively (Sirigireddy and Ganta, 2007, unpublished data). The host specifically expressed genes by the pathogen predominantly included several hypothetical and membrane proteins. These observations are further confirmed by RT-PCR analysis for a subset of genes randomly chosen from this analysis. Among the membrane proteins, the predominant expression is also noted to be the p28-Omp genes 14 and 19 in *E. chaffeensis* cultured in tick and macrophage cells, respectively (128,353) (Sirigireddy and Ganta, 2007, unpublished data).

Electron microscopic analysis of *E. chaffeensis* organisms originating from macrophage and tick cells backgrounds also identified several host-specific morphological differences (DeDonder and Ganta, unpublished data). They include synchronized growth of reticulate and dense-core forms of *E. chaffeensis* in macrophage environment, whereas both forms are observed in the phagosome of tick cells infected with the organisms are highly irregular in their shape during their growth in a tick cell. Moreover, the reticulate forms of *E. chaffeensis* are larger in size compared to the same in macrophage-grown bacteria (DeDonder and Ganta, unpublished data) (Figure 1.7). Based on all these studies, it is clear that *E. chaffeensis* organisms originating from the tick cells and macrophages are considerably different in their morphology, which is

consistent with numerous differences observed in the expressed transcripts and proteins in this pathogen. The host cell-specific proteome and morphological differences may be contributing to the observed variation in the murine host immune response for these organisms coming from two different host backgrounds.

Previous reports also demonstrate the dominant immunogenic nature of the p28-Omp proteins of *E. chaffeensis* (204,206,419). The p28-Omp19-specific monoclonal antibodies also offer a protective response in SCID mice against *E. chaffeensis* even after a well established infection (204,206,419). Immunogenic epitopes of the p28-Omp proteins are located within highly variable, hydrophilic domains of the proteins referred to as variable regions I to III (204,206,419). Extensive studies from several research groups including from our team demonstrated the predominant expression of outer membrane proteins and most notably the p28-Omp genes 14 and 19 in the tick cell- and macrophage-derived *E. chaffeensis*, respectively (347,352,353). Host response and clearance is faster when the bacteria are originating from the macrophages where the p28-Omp 19 is the major expressed membrane protein (128). This suggests that *E. chaffeensis* infection from macrophages into a vertebrate host is cleared faster, possibly because of the host induced antibody response against the p28-Omp 19 protein. In fact, these data are consistent with a published report where SCID mice with an established infection can clear the severity of infection when the p28-Omp-specific antibodies are injected (204,206,419). Given all these data it is evident that the host response can be protective if the bacteria is coming from vertebrate macrophages. It remains to be known whether a vertebrate host infected with tick cell-derived bacteria can similarly be protected, if primed with tick- or macrophage-specific immunodominant antigens such as the p28-Omp 14 or 19, respectively.

Together all the above discussed findings including evidence from a murine host for its differential clearance pattern, suggest that the differences in gene expression alone seem to be making a difference in the pathogen's persistence or clearance in a vertebrate host. Based on the intriguing data, our group (128) hypothesized that delayed clearance by mice against *E. chaffeensis* originating from tick cells is the result of a failure of the host to adequately generate immune repose to the changing antigen composition of the organism within its macrophages. This hypothesis has been supported by indirect evidence that a steady increase in antibody response, delayed clearance and a suppressed cytokine responses despite the effort of the host to generate equal levels of immune cell activations (128). More direct experimental evidence for this hypothesis, however, remains to be provided. It is clear that the origin of bacteria from tick cells is an important contributor for pathogen's adaptation to a vertebrate host environment. Importantly, the tick cell-specific differentially expressed pathogen proteins may aid in overcoming the host response and support its persistence. It is, however, not clear how the pathogen is able to achieve its host-specific differential expression by sensing the host environments. It is also not known what are molecular strategies employed by this pathogen to achieve its host-specific differential gene expression. The knowledge about differential expression, i.e., molecular events leading to altered gene expression, will lead to novel strategies to block transmission of the pathogen.

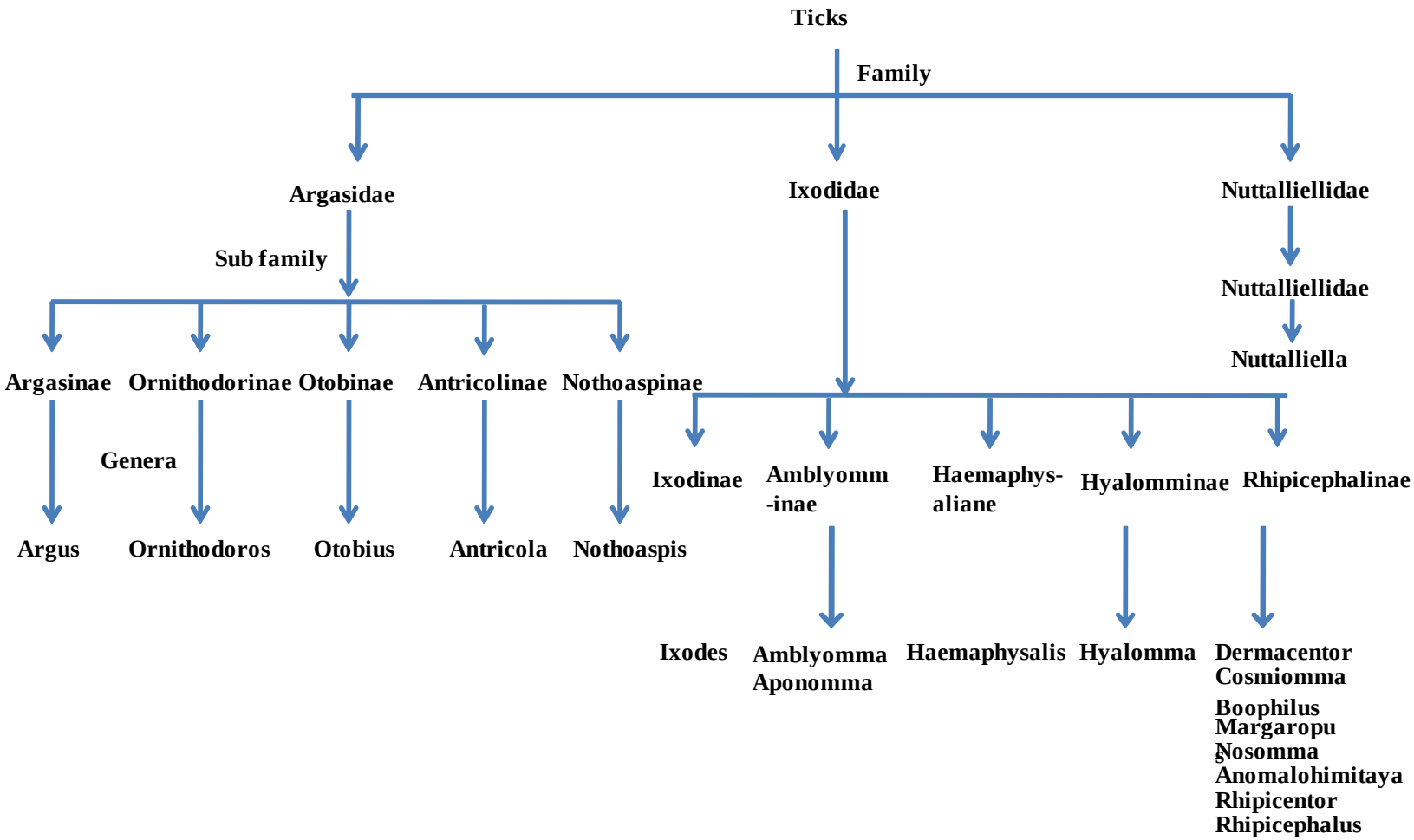
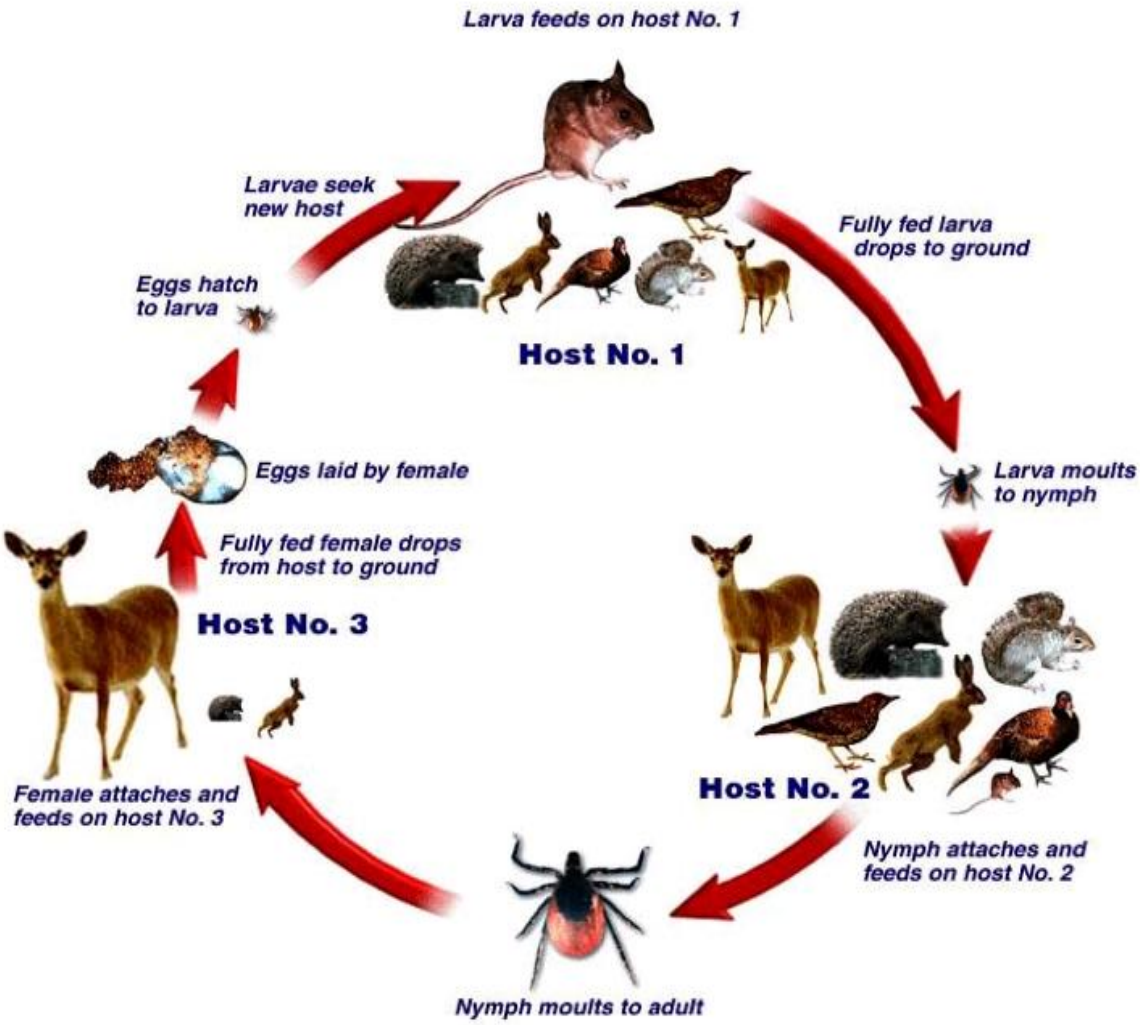
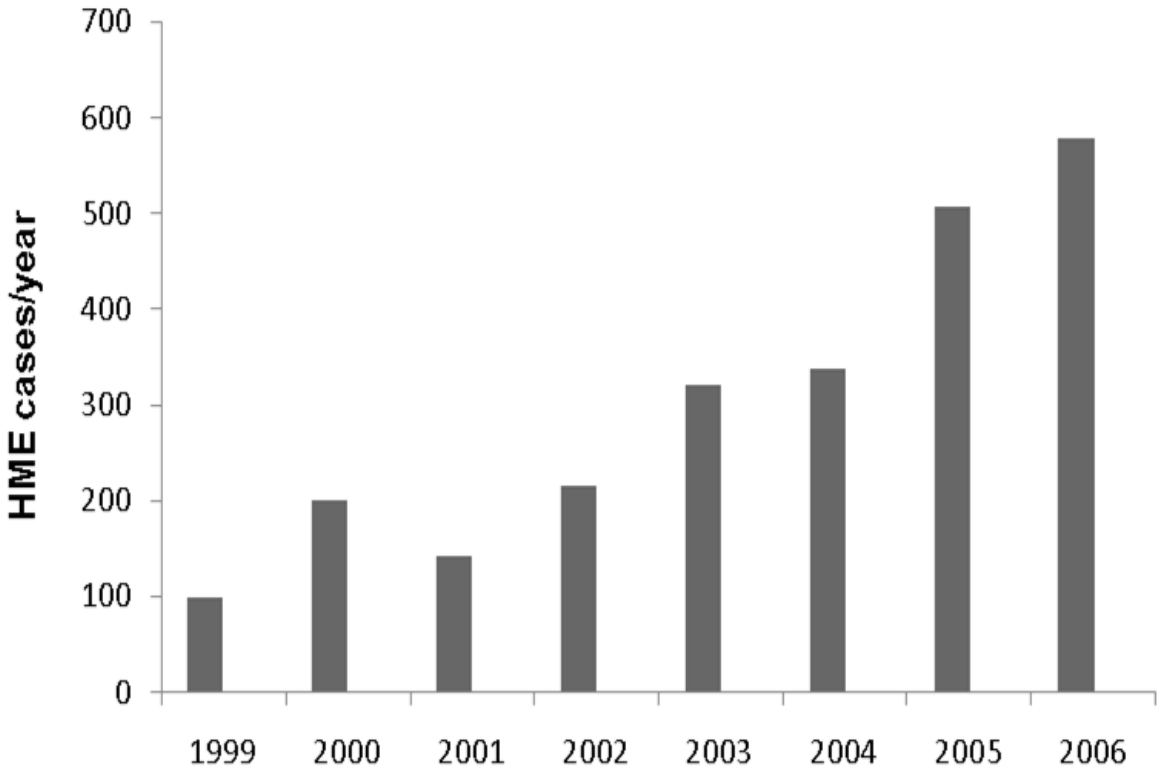


Figure 1.1. Classification of ticks



Source: http://www.iassistdata.org/images/ticklifecycle.jpg

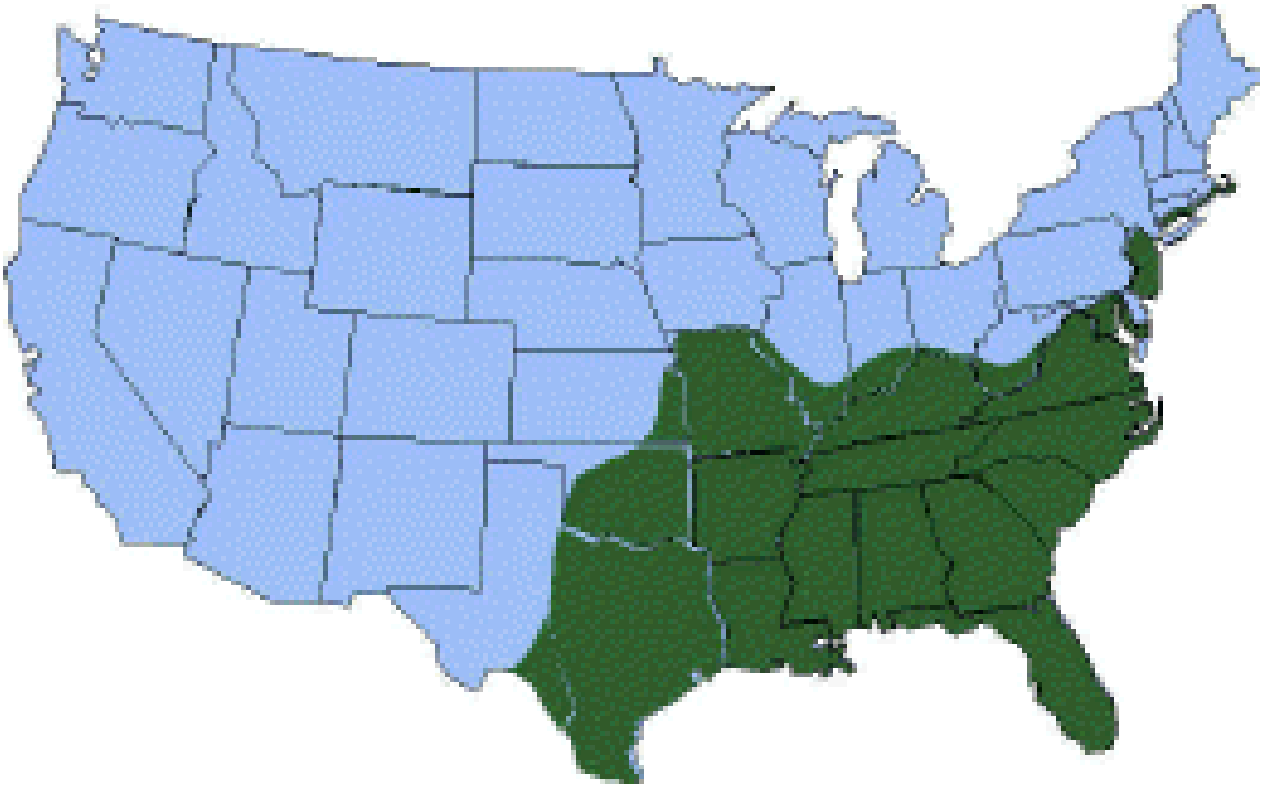
Figure 1.2. A typical three-host life cycle of an Ixodid tick.



Source: McNabb et al., 2008

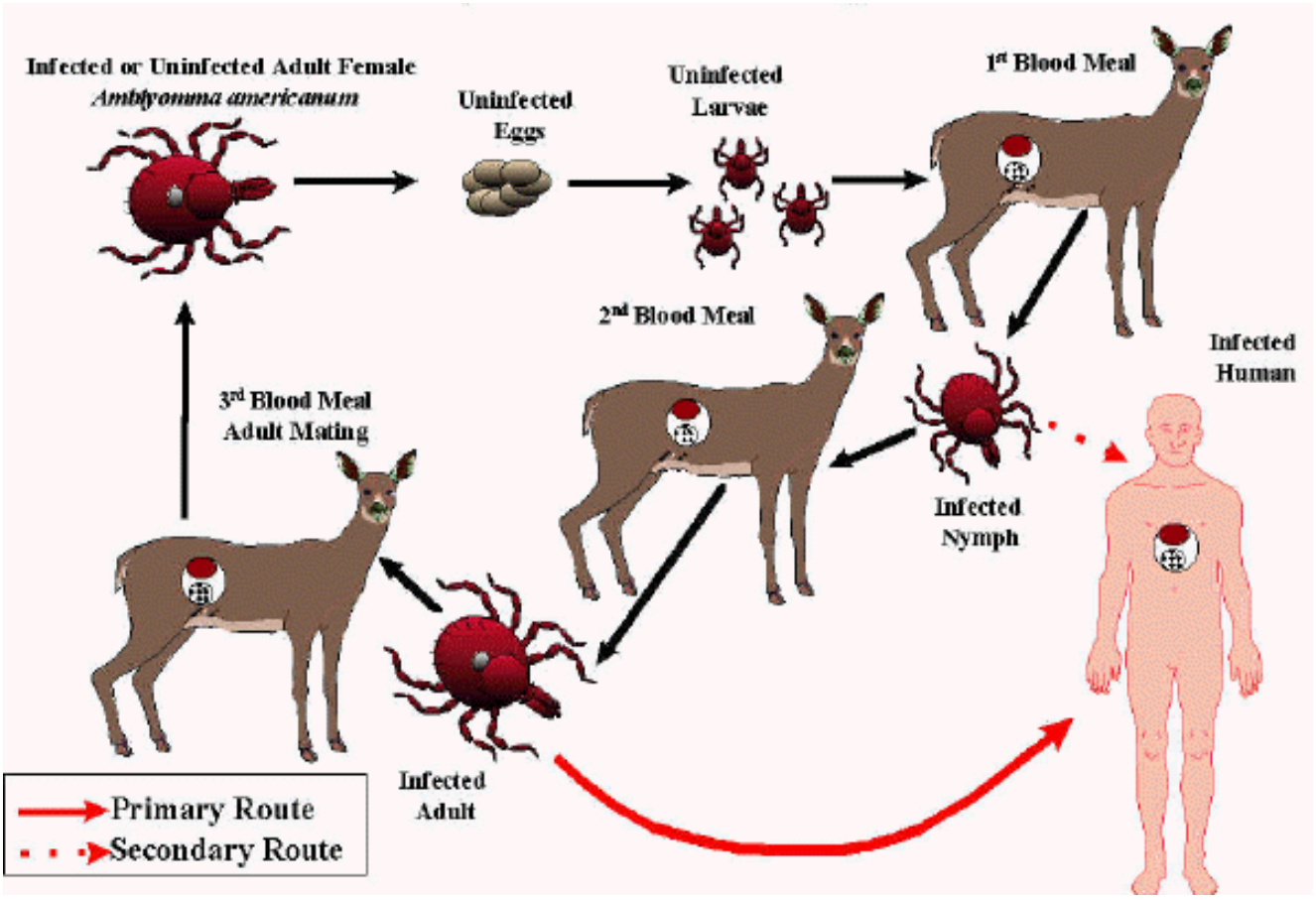
Figure 1.3. Number of HME cases reported from 1999 to 2006

***Amblyomma americanum* (Lone star tick)**



Source: http://www.cdc.gov/ncidod/dvrd/Ehrlichia/Natural_Hx/Natural_Hx.htm

Figure 1.4. Distribution of *Amblyomma americanum* tick (green colored regions in the map)



Source: http://www.cdc.gov/Ncidod/dvrd/ehrlichia/Natural_Hx/nathx1.htm

Figure 1.5. Proposed life cycle for *E. chaffeensis*

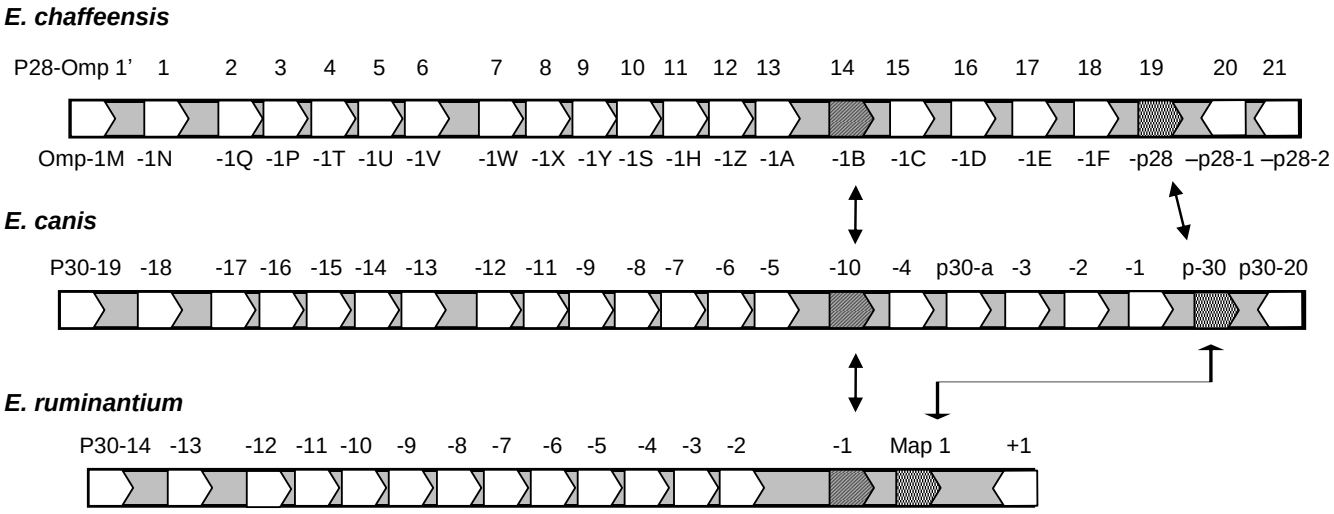


Figure 1.6. A cartoon representing the p28-Omp loci of *E. chaffeensis*, *E. canis* and *E. ruminantium* with identified expressed proteins from the p28-Omp genes in vertebrate macrophages (hatched boxes) and tick cells (checker board boxes) are presented (Reproduced with permission from *Frontiers In Bioscinces*. 14: 3259-73).

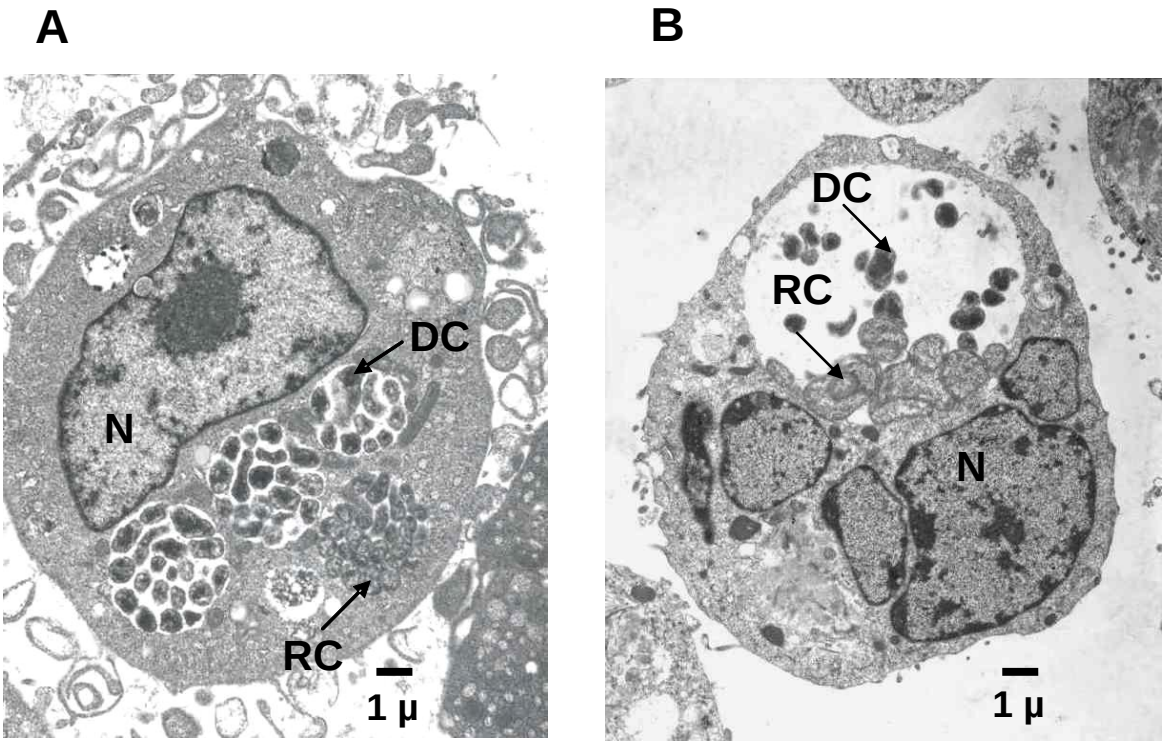


Figure 1.7. Transmission electron microscopy analysis was performed on *E. chaffeensis*-infected macrophages (A) and tick cells (ISE6) (B). (N, nucleus; DC, dense-cored bodies of *Ehrlichia* in phagosomes; RC, reticulate bodies of *Ehrlichia* in phagosome) (Reproduced with permission from *Frontiers In Bioscinces*. 14: 3259-73).

Arthropod vector	Disease causative agent	Host	Disease
Mosquitoes			
Anopheles species	Protozoa		
	Plasmodium species	Humans	Malaria
	Nematodes		
	Brugia malayi	Humans	brugian filariasis
	Wuchereria brancrofti	Humans	Bancroftian filariasis
Aedes species,			
Culex species	*Dirofilaria immitis*	Dogs, cats	Heart worm disease
	Virus		
Aedes species	*Dengue fever virus*	Humans	Dengue fever
Aedes aegypti	*Yellow fever virus*	Humans	Yellow fever
Aedes species, *Culiseta* species	*Eastern equine encephalitis virus*	Humans, horses, birds	Eastern Equine encephalitis
Culex species	*Western equine encephalitis virus*	Humans, birds	Western Equine encephalitis

	Venezuelan equine encephalitis virus	Humans, horses	Venezuelan equine encephalitis
Culex tritaeniorhynchus	*Japanese encephalitis virus*	Humans, pigs, birds	Japanese encephalitis
Culex species	*St. Louis encephalitis virus*	Humans, birds	St. Louis encephalitis
Aedes triseriatus	*La Crosse virus*	Humans, chipmunks, squirrels	La Crosse Encephalitis
Culex species	*West Nile virus*	Humans, horses, birds	West Nile fever
Aedes or Culex genera	*Rift valley fever virus*	Cattle, sheep, goat, camels	Rift Valley fever
Spilopsyllus cuniculi	*myxomatosis virus*	Rabbits	Myxomatosis
Culicoides paraensis	*Oropouche virus*	Humans	Oropouche fever

Flies

Chrysops species	Bacteria		
	Francisella tularensis	Humans, Rabbits, Cats	Tularemia
Glossina species	Protozoa		

	Trypanosoma brucei	Humans	African trypanosomiasis
Phlebotomus species	*Leishmania* species	Humans	Leishmaniasis
	Virus		
	Vesicular stomatitis virus	Cattle	Vesicular stomatitis
	Sand fly fever virus	Humans	Sand fly fever
	Helminth		
	Onchocerca volvulus	Humans, Cattle	Onchocerciasis
Fleas			
Xenopsylla cheopis	Bacteria		
	Rickettsia typhi	Humans	Murine typhus
	Yersinia pestis	Humans	Bubonic plague
	Virus		
Spilopsyllus cuniculi	*Myxomatosis virus*	Rabbits	Myxomatosis

Mites

Leptotrombidium species	Bacteria		
	Orientia tsutsugamushi	Humans	Tsutsugamushi/ Scrub typhus
	Rickettsia akari	Humans	Rickettsial pox

Lice

Pediculus humanus humanus	Bacteria		
	Rickettsia prowazekii	Humans	Epidemic typhus
	Bartonella Quintana	Humans	Trench fever
	Borrelia recurrentis	Humans	Louse-borne relapsing fever
	Virus		
Cattle-sucking lice	*Pox virus*	Swine	Swine pox

	Fungal		
Cattle lice	*Trichophyton verrucosum*	Cattle	Bovine dermatomycosis
	Helminth		
Pseudomenopon pilosum	*Pelecitus fulicacatrae*	Aquatic birds	Avian filariasis
Trichodectes canis	*Dipylidium caninum*	People, dogs	Double-pored tapeworm
Bugs			
Triatoma infestans	Protozoa		
	Trypanosoma cruzii	Humans	American sleeping sickness/ Chagas disease
Beetle			
Tribolium species (Flour beetle)	Cestode		
	Hymenolepsis nana	Humans	Hymenolepsis

Biting Midges

Culicoides species	Virus		
	Blue tongue virus (orbiviruses)	Sheep, Cattle	Blue toungue disease
	BEF virus	Cattle	Bovine ephemeral fever
	Epizootic hemorrhagic virus (orbiviruses)	Deer	Epizootic hemorrhagic disease
	African horse sickness virus (orbiviruses)	Horse	African horse sickness
	Protozoan		
	Leucocytozoon species	Birds	Leukocytozoonosis
	Nematodes		
	onchocerca cervicalis	Horses	Equine onchocerciasis
	Mansonella ozzardi	Humans	Mansonellosis

Table 1.1. Arthropod vector-borne infectious diseases of humans and animals (excluding tick-borne infections)

Source: G. Mullen and L. Durden (Eds.). 2002. Medical and Veterinary Entomology. Academic Press, London.

Tick Vector	Agent	Disease	Host
	Protozoa		
Ixodes and *Boophilus* species	*Babesia microti*, *B. divergens*, *B. major*, and *B. bigemina*	Babesiosis	Human, mice, cattle
Boophilus species	*B. equi*	Equine babesiosis	Horses
	Virus		
Ixodes species	*Flavivirus*	Tick-borne encephalitis	Rodents, Insectivores, carnivores, Humans etc
Ixodes, *Dermacentor*, and *Haemaphysalis* species	*Flavivirus*	Powassan encephalitis	Rodents, hares, carnivores
Dermacentor andersoni	*Coltivirus*	Colorado tick fever	Rodents, carnivores, humans,

			domestic animals
Hyalomma species	*Nairovirus*	Crimean-Congo hemorrhagic fever	Hares, hedgehogs, small mammals, humans
	Bacteria		
Ixodes species	*Borrelia* species	Lyme disease	Small mammals, birds, humans
Dermacentor species	*Rickettsia rickettsii*	Rocky Mountain spotted fever	small mammals, carnivores, rabbits, humans
Rhipicephalus and *Dermacentor* species	*Rickettsia conorii*	Boutonneuse fever	Small mammals, hedge hogs, dogs
Many tick species	*Coxiella burnetii*	Q-fever	Large domestic livestock, humans
	Borrelia species	Tick-borne relapsing fever	Various mammals

Ornithodoros species			
Haemaphysalis species	*Francisella tularensis*	Tularemia	Lagomorphs, rodents, carnivores
Argas persicus	*Borrelia anserina*	Avian spirochetosis	Birds
Rhipicephalus, *Dermacentor*, *Amblyomma* species	*Theileria* species	Theileriosis/East Coast fever	Cattle, yak, buffaloes
Dermacentor species	*Anaplasma marginale*, *A. centale*	Bovine anaplasmosis	Cattle
Dermacentor species	*A. ovis*	Ovine and Caprine anaplasmosis	Sheep, goats
Ixodes species	*Ehrlichia ruminnatium*	Heartwater fever	Ruminants
Ixodes scapularis	*A. phagocytophilum*	Human granulocytic ehrlichiosis	Humans, rodents, dogs

	Ehrlichia		
	chaffeensisEhrlichia		
	chaffeensisEhrlichia	Human monocytic	
Amblyomma americanum	*chaffeensis*	ehrlichiosis	Humans, deer, dogs, coyotes

Table 1.2. List of tick-borne animal and human infectious diseases

Source: Sonenshine, D. E., Lane, R. S., and Nicholson, W. L 2002. Ticks (Ixodida). Medical and Veterinary Entomology G. Mullen and L. Durden (Eds.), pp. 518–556. Academic Press, London.

Chapter 2

Scope of the Dissertation

In the previous chapter, I have reviewed the current knowledge on rickettsial pathogens and the importance of rickettsial diseases to animal and human health. I have also described the emergence of several newly identified tick-borne diseases, includeding human monocytic ehrlichiosis (HME) caused by *E. chaffeensis.* Moreover, I presented a detailed review about the current knowledge of *E. chaffeensis* epidemiology, pathogenesis, molecular biology and immunology. From the detailed overview presented, it is evident that there are several areas where a significant gap of knowledge exists. The primary focus of my research has been addressing some of the important missing gaps of knowledge about *E. chaffeensis.*

Several immunological studies reported in the literature, including the research from our laboratory, aided in understanding of various aspects of host immunity needed for clearing *E. chaffeensis* infection. These studies suggested that *E. chaffeensis* clearance by a host requires several components of the immune system. They include the activation of macrophages, responses of T- and B-cells and cytokines. However, to date how *E. chaffeensis* is able to persist despite host efforts to clear infection remains largely unknown. It is also not clear how *E. chaffeensis* is able to adapt to its tick vector and vertebrate hosts.

Recent studies from our laboratory demonstrated significant differences in protein expression for *E. chaffeensis* coming from its tick and vertebrate host cells environments. The differentially expressed proteins include several outer membrane proteins such as those made from the p28-Omp multigene locus. Proteins made from genes 14 and 19 of the p28-Omp locus are the major expressed membrane proteins when the pathogen is

grown in tick cells and vertebrate macrophages, respectively. *In vivo* studies also confirm the differential expression from these two genes in tick and vertebrate host-specific manner. It is not clear how the pathogen is able to sense differences in the tick and vertebrate host environments to alter its gene expression. In a more recent study, using the murine host model our group reported that a vertebrate host clears the infection at a faster rate for *E. chaffeensis* originating from macrophage cultures compared to that from tick cells. Taken together, these data suggest that host-specific differences in gene expression in *E. chaffeensis* could be one of the major contributing factors for variations in the host immune response against the pathogen. However, it remains entirely unknown how the pathogen is able to sense changes in the host cell environment and what molecular mechanisms are employed to alter its gene expression. It is also not clear how the differences in gene expression resulting from vertebrate and tick cell environments are impacting the host immune responses. There is a significant gap of knowledge in these fronts which can be better addressed by mapping molecular mechanisms associated with differences in gene expression by *E. chaffeensis*.

Much of the fundamental knowledge about the biology of this tick-borne pathogen can be gained by assessing the contributions of host-specifically expressed proteins. The importance of differentially expressed outer membrane proteins and several other gene products during *E. chaffeensis* infectious cycle can be better addressed if we have tools that aid in creating mutations targeted to a gene of interest. Currently, these approaches are more complicated to undertake in *E. chaffeensis* due to lack of an established genetic manipulation system. Research on other members of the *Anaplasmataceae* family in creating mutations is also in progress and is encouraging.

For example, a recent report by Felsheim *et al*. (115) describes an insertion mutation in *A. marginale.* Similarly, in distantly related rickettsiales of the genus *Rickettsia*, several investigators reported mutations which included the integration of foreign DNA into the genome of a *Rickettsia* species (312,386). Thus, methods for creating mutations in *E. chaffeensis* may be established soon. Availability of such tools in the future may be valuable in studies exploring the functional significance of a gene of interest and also to map the molecular events leading to host-specific gene expression differences. However, as these methods are yet to be developed, alternative approaches to map the molecular mechanisms of gene regulation are necessary. One such approach could be to develop methods useful in assessing differences in gene expression under *in vitro* conditions.

A significant gap of knowledge in understanding the biology and pathogenicity of *E. chaffeensis* can also be bridged by mapping transcriptional differences in the genes of the pathogen resulting from its growth in different host backgrounds, characterizing the promoters of differentially regulated genes and identifying the regulators that are essential to accomplish regulation of gene expression. To advance these areas of *E. chaffeensis* research, I undertook several research projects as part of my PhD program. I performed experiments to map the transcription of two differentially expressed genes from the p28-Omp locus using several independent RNA analysis methods (Chapter 3). Molecular tools were also used to define differences in the prompter regions of host-specifically expressed genes (Chapter 4). These studies are intended to shed insights on what regions of the genome spanning the p28-Omp genes are involved in influencing RNA polymerase activity in driving the expression or lack of expression in a host cell-specific manner. In an effort to develop methods useful in evaluating host-specific differences in gene expression, *in vitro* transcription and transcription coupled translation

systems have also been developed (Chapter 5). Finally, to learn about what regulatory proteins may contribute to differences in gene expression, gene 14 and 19 promoters were evaluated for their interactions with *E. chaffeensis* proteins. In addition, five putative *E. chaffeensis* regulators were cloned and evaluated for their contributions in altering RNA polymerase activity (Chapter 6). These investigations are important in opening the path for continued research for furthering our knowledge about *E. chaffeensis* biology and pathogenesis.

Chapter 3

Transcriptional Analysis of The p28-Omp Genes 14 and 19

Abstract

Ehrlichia chaffeensisEhrlichia chaffeensisEhrlichia chaffeensis is a Gram negative intracellular rickettsial pathogen that causes an emerging infectious disease in people, human monocytic ehrlichiosis. This tick-transmitted pathogen establishes persistent infection in its invertebrate and vertebrate hosts, which may be critical for the successful completion of its lifecycle. One of the possible mechanisms by which the pathogen may persist is by altering its gene expression in accordance to its host background. In our recent studies, evidence was presented for differential host cell-specific protein expression. The host cell-specific protein expression by *E. chaffeensis* includes those from the multigene locus encoding 28 kDa, immunodominant, outer membrane proteins (p28-Omps). The p28-Omp antigens expressed in infected macrophage and tick cells are predominantly the products of p28-Omp genes 19 and 14, respectively. Our central hypothesis is that *E. chaffeensis* exploits host environments in achieving unique host cell-specific protein expression via modulating its transcription. In support of this hypothesis, transcriptional activity of a subset of genes from the p28-Omp multigene locus was analyzed in vertebrate and tick cell environments. Specifically, RNA isolated from *E. chaffeensis* grown in macrophage and tick cells was analyzed by Northern blot, primer extension, ribonuclease protection assay, and real-time RT-PCR methods. All these experiments identified the major transcripts from the p28-Omp locus in macrophage and tick cells to be genes 19 and 14, respectively. The molecular experiments also aided in locating the transcriptional start sites and putative promoters for *E. chaffeensis* p28-Omp 14 and 19 genes. In addition, *E. chaffeensis* RNA isolated from infected tick and macrophage cells at different times post-infection was also analyzed by real-time RT-PCR. This analysis identified that, independent of time after

infection, the p28-Omp gene 14 is the predominantly expressed transcript in *E. chaffeensis* grown in tick cells; whereas the major transcript is from the p28-Omp gene 14 in macrophage grown pathogen.

Introduction

Ehrlichia chaffeensisEhrlichia chaffeensisEhrlichia chaffeensis, a tick-borne pathogen, infects a wide range of vertebrate animals including humans and establishes persistent infections in its hosts. The successful completion of *E. chaffeensis* life cycle requires its adaptation and persistence in its vector and vertebrate hosts. The molecular strategies employed by this pathogen to overcome host immune responses and to achieve its dual host adaptation and persistence remain largely unknown. Several strategies employed by intracellular bacteria to overcome host defense mechanisms are reported in the literature. They include prevention of lysosomal fusion with the phagosome in which the pathogen resides (25), escape from phagolysosomal fusion (407), export of virulent proteins and toxins through specialized secretion system (51), and altering the expressed antigens to evade the host immune responses (339,346,400).

Differential gene expression by a pathogen in response to the host environment has also been shown to be an important strategy employed by several pathogenic bacteria (39,71,106,418). For example, *Borrelia burgdorferi*, the agent of Lyme disease, differentially expresses its outer membrane proteins in its tick vector and vertebrate hosts (81,82,345,369). This pathogen up-regulates the expression of its outer surface proteins, Osp A and Osp C in its tick vector and vertebrate hosts, respectively (81,345,369). This host-specific differential expression of outer surface proteins by *B. burgdorferi* is

essential for the pathogen's colonization and persistence in its vertebrate and invertebrate hosts (81,163,282,284,285). Similarly, in more closely related pathogens of *E. chaffeensis*, *Anaplasma marginale* and *A. phagocytophilum*, differential expression of outer surface proteins have been reported in their vertebrate host and tick vector, respectively (132,170,219,269,408,445). Differential expression and antigenic variation of the outer membrane proteins in these *Anaplasma* species pathogens is observed in persistently infected vertebrate hosts (124,170,258,269). Persistence in both arthropod and vertebrate hosts may be necessary for tick-borne pathogens as it improves the chances of a vector to acquire the organism and transmit to another suitable host, thus maintaining the infectious cycle of the pathogen.

Previous studies from the literature and recent studies from our laboratory demonstrated that *E. chaffeensis* p28-Omp outer membrane proteins are expressed in a host cell-specific manner (221,274,347,352,353,397). The major expressed protein in macrophage grown *E. chaffeensis* organisms is the product of p28-Omp19 gene; where p28-Omp14 is predominantly made in *E. chaffeensis* originating from tick cells (221,274,347,352,353,397). To date, it is not clear whether the pathogen's regulation of its p28-Omp genes is achieved at transcriptional or post transcriptional level. Transcriptional analysis performed using non-quantitative RT-PCR assays of the the p28-Omp locus genes demonstrated the expression of multiple transcripts from this multigene locus (108,221,272,398). Considerable discrepancy was also reported for the transcripts identified from the p28-Omp locus (108,221,398). For example, a study by Long et al. (221) reported 16 transcripts of 22 genes from the p28-Omp locus for macrophage-derived *E. chaffeensis* and they include all genes but 2, 6, 13, 14, 17, and 21 (221).

Unver et al. (398) also identified same number of the p28-Omp transcripts but the detected transcripts for the p28-Omp genes are different from those reported by Long et al (221). Similarly, RT-PCR analysis of a subset of genes, spanning the p28-Omp 14 though 19 in macrophage-derived *E. chaffeensis*, identified transcripts only for four genes (14, 15, 18 and 19) (62). The transcriptionally active genes identified by Cheng et al (62) are consistent with those identified by Unver et al (398) but differ from the transcripts reported by Long et al (221). The findings from all these studies are inconsistent from the protein expression data reported for this locus (274,347,352,353).

Despite the presence of multiple transcripts, only a limited number of proteins are expressed from this locus in host cell-specific manner (347,352,353). These differentially expressed proteins are present as multiple forms due to post-translational modifications including glycosylation and phosphorylation (352,353). The presence of transcripts as assessed by non-quantitative RT-PCR methods for 16 of 22 genes in macrophage derived *E. chaffeensis* RNA is contradictory to protein expression data, where only major protein was detected (p28-Omp 19) (108,221,274,347,352,353,398). Non-quantitative RNA analysis, however, for tick or tick cell derived *E. chaffeensis* detected only one major transcript, i.e., for the p28-Omp gene 14 (397,398). This data is also consistent with protein expression identified only for gene 14 in tick-cell grown *E. chaffeensis* organisms (347,352). The differences in RNA data and protein data suggest that the pathogen may be employing two levels of regulation; transcriptional and translational. Importantly, not all transcripts made are used by the protein synthesis assembly to make proteins. Alternatively, there may be quantitative differences in

transcription and translation, where only a subset of genes are transcribed and translated in higher levels.

In this study, we carefully evaluated the transcripts for a subset of genes by several quantitative RNA analyses methods. Specifically, we evaluated expression for the p28-Omp genes 14 and 19 in *E. chaffeensis* in infected macrophages and tick cells. The reason for our selection of these two genes is that the protein expression data for the p28-Omp locus genes demonstrated host cell-specific differential protein expression of the p28-Omp genes 14 and 19 in tick and vertebrate host cells, respectively. Several independent quantitative RNA analyses methods such as Northern blot, primer extension, ribonuclease protection assay, and real-time RT-PCR are utilized for this analysis. We also extended our analysis to RNA isolated from infected tick cell and macrophage cultures at various time intervals after infection. To further evaluate if the transcription is influenced by the host environment, we infected the macrophage cultures with the *E. chaffeensis* organisms that are previously grown in tick cell or macrophage cultures. Following the final infection, total RNA from the infected cultures is evaluated by real-time RT-PCR method. Similarly, RNA extracted from mice that were infected with *E. chaffeensis* organisms grown in tick cells or macrophages, was also analyzed by real-time RT-PCR analysis. Overall, this study identified that the p28-Omp genes 14 and 19 are regulated at transcription level and their expression levels remained the same independent of time after infection. Expression of these outer membrane protein genes is influenced by the host environment.

Materials and methods

***In vitro* cultivation of *E. chaffeensis*:** The Arkansas isolate of *E. chaffeensis* was cultivated *in vitro* in the canine macrophage cell line (DH82) at 37°C and the tick cell lines (ISE6 or AAE2) at 34°C, by following the protocols established previously (61,260).

Isolation of total RNA: About 20 ml of confluent monolayers of DH82 or ISE6 cell cultures with about 80-100% infection of *E. chaffeensis* were recovered from a T-150 flask and used for isolation of total RNA. Total RNA was isolated from the infected cultures using Tri-reagent method by following the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO) (A more detailed description about this method is provided in the Chapter 8). RNA was also isolated from 5 ml of the infected tick and macrophage cultures and 5 ml of peritoneal wash cells (peritoneal macrophages) from *E. chaffeensis* infected mice, at various time points post-inoculation. The RNA pellets recovered were resuspended in 100 µl of nuclease-free water containing 40 units of RNase inhibitor, RNasin, (Ambion Inc., Austin, TX) and stored at -70°C until use. Quality and concentration of RNA was assessed by spectrophotometry using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and by calculating the ratio between the optical densities at 260 nm and 280 nm. RNA quality was further confirmed by resolving samples on 1.5% formaldehyde agarose gels.

Northern blot analysis: *E. chaffeensis* total RNA was assayed by Northern blot method by following the standard procedures described in Sambrook and Russell (336). About 10 µg of total RNA of *E. chaffeensis* isolated from infected macrophage or tick cell lines

was resolved on a 1.5% denaturing formaldehyde agarose gel. The RNA from the gel was then transferred to a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by the capillary transfer method (336). The nylon membrane containing transferred RNA was hybridized with ^{32}p-labelled probes specific for genes p28-Omp 14 or 19. To generate specific probes for the genes 14 and 19, a 0.86 kb and 0.77 kb segments of the genes, respectively, were amplified from *E. chaffeensis* genomic DNA using the primers listed in Table 3.1. PCR condition for amplification included initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 50°C (for gene 14 probe) or 55°C (for gene 19 probe) for 30 s, and 72°C for 50 s, and one final extension at 72°C for 2 min. Positive controls for this experiment included genomic DNA hybridized with the above synthesized probes. Labeling of the probes with α-^{32}p [dATP] was done by random primer labeling of gene-specific PCR amplicons utilizing random primer labeling kit (Stratagene, La Jolla, CA) and by following manufacturer's protocol. Hybridization of the labeled probes to the RNA was performed overnight at 68°C in a buffer containing 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 10 mM sodium phosphate buffer (pH 6.8), 0.5% SDS, 1 mM EDTA, 10× Denhart's solution and 100 μg ml^{-1} sheared and denatured salmon sperm DNA. The blots were washed once each for 30 min at 68°C with 6× SSC containing 0.1% SDS, 2× SSC containing 0.1% SDS, 1× SSC containing 0.1% SDS and 0.2× SSC containing 0.1% SDS. After the final stringent wash, the membranes were exposed for about 24 h or longer (to obtain signals with desired intensity) to an X-ray film at −80°C with an intensifying screen, and the film was developed using a Konica film processor (Wayne, NJ). The experiment was repeated for three independent times using independently isolated RNA samples.

Primer extension: Primer extension analysis was performed by utilizing a Primer Extension System AMV Reverse Transcriptase kit (Promega, Madison, WI). Briefly, oligonucleotides complementary the p28-Omp genes 14 and 19 transcripts (Table 3.1) were end labeled with γ-^{32}p [ATP] using T4 polynucleotide kinase (Promega, Madison, WI) at 37°C for 10 min. The kinase reaction was stopped by heat inactivation at 90°C for 2 min. The end labeled primers (one ρ mole each) were annealed to *E. chaffeensis* RNA (~10 μg) by incubating at 58°C for 20 min in 11 μl reactions containing AMV primer extension buffer that was provided in the kit. To reverse transcribe RNA into cDNA, 1 μl of AMV reverse transcriptase (1 unit) was added and the reaction was incubated at 42^{o}C for 30 min. The reaction products were then concentrated by ethanol precipitation and resolved a 6% polyacrylamide gel containing 7 M urea. The gel containing the primer extended products was then transferred to a Whatman paper, dried and exposed to an X-ray film. The primer extended products were detected after developing the film using a Konica film processor (Wayne, NJ).

Ribonuclease protection assay (RPA): RPA was performed for macrophage culture-derived *E. chaffeensis* total RNA using the riboprobes specific for the 5' and 3' ends p28-Omp gene 19 sequences. Gene-specific complementary DIG-labeled RNA probes were generated by *in vitro* transcription strategies. To prepare *in vitro* transcripts that are complementary to the predicted gene 19 transcripts, initially gene-specific segments were prepared by PCR (primers used for this experiment were listed in the Table 3.1). The amplicons (a fragment each spanning the 5' and 3' ends of the p28-Omp genes 19) included a T7 promoter sequence at the 3' end. The DIG labeled *in vitro* transcripts (riboprobes) were then synthesized for the complementary strands of genes 19 amplicons

using a DIG RNA labeling kit and T7 RNA polymerase as per the kit protocol (Cat # 1175025, Roche Applied Sciences, Indianapolis, IN).

RPA was performed to detect the presence of transcripts by following the manufactures protocol (Cat# 1427580, Roche Applied Sciences, Indiana police, IN). Briefly, 300 pico grams of DIG labeled riboprobes were allowed to hybridize with 10 µg of macrophage derived *E. chaffeensis* total RNA at 45°C overnight. The hybridization reactions were then subjected to RNase T1 digestion for 30 min at 30°C to remove all unbound, single stranded RNA with the exception of protected parts of the mRNA. Following this reaction, proteinase K digestion was performed for 15 min at 37°C. The protected products were then purified by standard phenol: chloroform: isoamylalcohol (25:24:1) method, followed by ethanol precipitation. One µg of yeast transfer RNA was added as a carrier to support RNA precipitation (336). The products were resuspended in 5 µl of sample loading buffer (provided in the kit), denatured by heating at 95°C for 5 min and then resolved on a 6% polyacrylamide gel containing 7M urea. Subsequently, the resolved products were transferred to a nylon membrane and incubated with antidigoxigenin alkaline phosphatase conjugate and chemiluminescent substrate, CSPD, as specified by the kit protocol (Roche Applied Sciences, Indiana police, IN). Chemiluminescent signals from protected fragments were then detected by exposing an X-ray film for one hour developing it using a Konica film processor (Wayne, NJ).

Real-time RT-PCR analysis: Quantitative differences in the transcripts for the p28-Omp genes 14 and 19 were assessed by a TaqMan-based diplex real-time RT-PCR assay using the Smart Cycler system (Cepheid, Sunnyvale, CA). The TaqMan probes and primer sets for

genes 14 and 19 utilized in this assay were designed from the variable regions of their coding sequences (Table 3.1). For analyzing 16S rRNA expression, previously reported primers and probe were utilized (354). The specificity of the primers and probes was confirmed in initial control experiments prior to their use in a diplex real-time RT-PCR assay. Initial RT-PCR analysis was performed on *E. chaffeensis* total RNA isolated form approximately 90% infected macrophage or tick cell cultures. Subsequently, to evaluate the influence of host background on transcription from genes 14 and 19 at different time intervals after infection, several T25 flasks containing uninfected cultures of macrophages (DH82) or tick cells (ISE6) were infected with *E. chaffeensis* and total RNA was isolated at various time intervals. They included 12h, 24h, 48h, 96h, and 120h post infection. The quantitative gene expression differences for the genes 14 and 19 obtained by real-time RT-PCR for post-infection samples are presented per 10^6 *E. chaffeensis* organisms.

For the post-inoculation experiments *E. chaffeensis* grown in ISE6 cells are purified and cell-free *Ehrlichia* are then used as inoculum to infect ISE6 cells or DH82 cells. Similarly, DH82-derived cell free *Ehrlichia* were used to infect DH82 cultures. In a separate study, *E. chaffeensis* infected tick cell and macrophage cultures are used to infect two mouse strains (B6 and C2D) (128). Total RNA samples isolated form the peritoneal macrophages, collected from normal and *E. chaffeensis* infected mice at various points post-infection, were utilized to determine p28-Omp genes 14 and 19 expression.

The RNA samples described above were assessed for the presence of transcripts and genome copies for genes 14 and 19. To assess the true presence of transcripts, the

contaminating genomic DNA of *E. chaffeensis* in RNA preparations were analyzed by diplex real-time RT-PCR in the presence or absence of reversetranscriptase. Typically, the reaction was carried out in a 25 µl volume which included 2 µl of RNA, 5 n mol of dNTP's, 125 n mol of $MgCl_2$, 5 p mol each of the TaqMan forward and reverse primers for both the genes, 3.75 pmol of each of gene-specific TaqMan probe for both the genes, and 1 µl of SS-III and *Taq* mix (SuperScript-III, One-Step RT-PCR system with platinum *Taq* DNA polymerase; Invitrogen Technologies, Carlsbad, CA). The thermal cycling conditions used in this assay included one cycle each at 48°C for 30 min and 95°C for 3 min, followed by 45 cycles of 95°C for 15 s, 50°C for 30 s, and 60°C for 60 s. Quantitative data relative to the number of *E. chaffeensis* organisms was calculated by following procedures described in (354).

Statistical analysis: Statistical analysis of real-time RT-PCR data for measuring the quantitative differences in the p28-Omp genes14 and 19 gene expression was performed by unpaired Student *t*-test. The values presented represent an average of three independent experiments. GraphPad InStat Software (La Jolla, CA) was used in performing these analyses. *P* values less than 0.05 were considered as significant.

Results

Northern blot analysis: To evaluate the gene expression at transcriptional level, Northern blot analysis was performed for the p28-Omp genes 14 and 19 utilizing macrophage and tick cell-derived *E. chaffeensis* total RNA. Approximately 0.9 kb transcript for the p28-Omp 19 gene was detected in macrophage-grown *E. chaffeensis* RNA. In contrast, RNA from tick cell-grown *E. chaffeensis* contained similar sized

transcript for the p28-Omp gene 14 (Figure 3.1). Similar results were obtained from all three independent experiments.

Primer extension analysis: Host cell-specific gene expression from the p28-Omp genes 14 and 19 identified by Northern blot analysis was verified by primer extension analysis (Figure 3.2, A and B). Consistent with the Northern blot data, the primer extended products for the p28-Omp gene 14 were detected only in tick cell-derived *E. chaffeensis*, whereas macrophage cells-derived *E. chaffeensis* RNA contained primer extended products only for the p28-Omp gene 19 (Figure 3.2, A and B). Transcription start sites for the p28-Omp genes 14 and 19 were also identified in this experiment. They are located at 34 and 26 nucleotides upstream to ATG for genes 14 and 19, respectively (Figure 3.2). For both the genes the nucleotide identified at the transcription start sites is adenosine.

Ribonuclease protection assy (RPA): Transcription start sites of gene 19 for macrophage-derived *E. chaffeensis* RNA was further validated by ribo nuclease protection assay. A riboprobe, targeted to 5' end of gene 19 was utilized in this analysis. Protected fragments was evident and the transcription start site estimated form the 5' end protected fragment migrated at a similar location as predicted from the primer extension analysis (Figure 3.3, B)

RNase protection assay was also performed to map the transcription termination site for the p28-Omp gene 19 using a 3' end gene specific riboprobe. The 3' end probe included sequence downstream to stop codon of the gene 19. The entire riboprobe used in this experiment was protected suggesting that the transcription termination site is further downstream to the gene 19 stop codon (Figure 3.3, B).

Real-time RT-PCR analysis: Transcriptional analysis by direct RNA analysis methods (discussed above) revealed the expression of gene 14 in tick cells and gene 19 in macrophage-grown *E. chaffeensis*. Previous studies by three independent research groups, by non-quantitative RT-PCR analysis of *E. chaffeensis* RNA from macrophage grown organisms, identified expression from multiple genes from the p28-Omp locus (108,221,398). The RNA expression for gene 14 in tick cells by previous reports, however, is consistent with the results in this study (397,398). The presence of multiple transcripts as assessed by non-quantitative RT-PCR methods is inconsistent to our present observations by direct RNA analysis methods for macrophage-derived pathogen's RNA in this study. The Northern blot and primer extension data, however, supports the protein expression data from this locus for gene 14 in tick cells and gene 19 in macrophages for both *in vitro* and *in vivo* analysis (274,347,352,398). To resolve the conflicting observations, we developed diplex real-time RT-PCR assay for use in mapping expression for the p28-Omp genes 14 and 19. Transcripts for both the genes 14 and 19 were detected in *E. chaffeensis* RNA isolated from both macrophage and tick cell derived *E. chaffeensis*, but expression levels were different (Figure 3.4). The diplex assay detected predominant expression for the p28-Omp gene 19 in macrophage-derived *E. chaffeensis* (as assessed by low Ct values) and for gene 19 in tick cell grown *E. chaffeensis* (Figure 3.4).

The diplex real-time RT-PCR analysis was also carried out to assess influence of bacterial growth on the p28-Omp genes 14 and 19 expression at different times post infection including its progression from dense-cored cells to reticulate cells and reverting back to dense-cored cells for their subsequent infection to naïve cells. *E. chaffeensis* RNA

harvested at different times post infection from macrophages and tick cells was investigated. At all times post-infection, transcripts for both the genes 14 and 19 were detected in RNA isolated from both the host cell backgrounds (Figure 3.5, A and B). Independent of the time post infection the major expression from the tick cell derived *E. chaffeensis* remained the same i.e., from the gene 14 ($P \leq 0.05$) (Figure 3.5, A). Similarly, major expression from the gene 19 was steadily observed at all time points after infection in macrophage-derived *E. chaffeensis* RNA ($P \leq 0.05$) (Figure 3.5, B).

To verify if the differences in gene expression are truly the result of host cell environment *E. chaffeensis* organisms were purified from tick cell cultures and used to infect several macrophage or tick cell cultures. Similarly, macrophage culture-derived *E. chaffeensis* was purified and used to infect macrophage cultures. Total RNA isolated from the infected cell cultures at several times post-inoculation was evaluated for the p28-Omp genes 14- and 19-specific real-time RT-PCR assay. For RNA samples isolated form macrophage cells infected with *E. chaffeensis* from tick cells, the expression levels of the gene 14 was high for the initial harvest time points. The expression was slowly switched to gene 19 by day 5 (Figure 3.6). Infection of macrophage cell line with *E. chaffeensis* organisms that were previously grown in macrophage cells maintained predominant expression form the gene 19. Similarly, *E. chaffeensis* maintained consecutively in tick cell lines retained high levels of expression from the p28-Omp gene 14 (Figure 3.6).

To determine if the similar shift in gene expression occurs in *E. chaffeensis in vivo*, transcriptional analysis of the p28-Omp 14 and 19 was also performed in mice

experimentally infected with pathogens originating from tick cells or macrophages. Two mouse stains, Immunocompetent C57BL6 (B6) mice and MHC II deficient (C2D) mice were used for this analysis. The bacteria are cleared in about 14 days in B6 mice, whereas they persist in C2D mice (129,130,420). *E. chaffeensis* total RNA isolated form the peritoneal macrophages of the infected mice was observed for different post infection days. Predominant expression of gene 14 transcripts was observed for days 1 and 3 post infections in B6 mice infected with *E. chaffeensis* organisms from tick cells. The expression was then reversed to gene 19 from day 7 and remained unchanged till 17 post infection. B6 mice infected with macrophage-grown *E. chaffeensis* organisms showed major expression for gene 19 from day 1 onwards and remained unchanged until the pathogen was cleared by the host (day 7) (Figure 3.7). C2D mice infected with *E. chaffeensis* from tick cells revealed predominant expression from the gene 14 up to 3 days after infection. The expression was then slowly switched back predominantly to gene 19 from day 5 until day 25 after infection (Figure 3.8).

Discussion

It may be necessary for arthropod transmitted pathogens to use novel adaptation strategies in support of their growth in vector and vertebrate host backgrounds, each representing a diverse environment, for their successful maintenance in nature. Very little is known about the vector-borne pathogens adaption strategies. Differential gene expression may be one of the important strategies employed by pathogenic organisms in support of their adaptation and persistence in their vectors and vertebrate hosts (81,113,163,218,269,282-285,396). Studies from ours and other research teams demonstrated host-specific protein expression from several *E. chaffeensis* pathogen genes

including from the p28-Omp locus. The host-specific expression in both *in vivo* and *in vitro* included the predominant protein expression from the p28-Omp gene 14 in tick cells grown pathogens and p28-Omp gene 19 in macrophages (221,274,347,352,353,397). Immunological studies utilizing mouse model demonstrated that the host clearance is slower for *E. chaffeensis* originating form tick cells (128). Secondly, considerable differences in host responses against tick cell-derived pathogens have been documented, particularly at B-cell, T-cell, macrophage and cytokine responses (128). These findings suggest that host environment influences the pathogen's protein expression and the host-specific protein expression may be an important contributor for the pathogen's extended survival in a vertebrate host.

In the current study, we have utilized several independent RNA analysis methods; Northern blot, primer extension, RPA and real-time RT-PCR for assessing the transcription from the p28-Omp genes 14 and 19. The analysis was performed to determine if the differences in protein expression reported earlier (274,347,352,397,398) are the result of regulation of gene expression at the level of transcription or due to differences in the protein stability influenced by host cell environments. The results from these experiments support the host cell-specific differences in protein expression paralleled differences in the transcripts made from the p28-Omp genes. Importantly, the major expression of p28-omp gene 19 protein in *E. chaffeensis* originating from macrophages (347,352) is consistent with the abundance of its transcripts. Similarly, the major transcription seen for gene 14 in tick cells is consistent with the observation of protein expression reported earlier (397,398).

The Northern blot, primer extension and ribonuclease protection analyses also suggested that the genes 14 and 19 are expressed as monocistronic messages. Primer extension experiments were valuable in identifying the transcription start sites and for locating the putative promoter regions for these genes. The base identified at the transcription start site for both the genes 14 and 19 is adenine. Previous studies from several other rickettsial organisms belonging to the genera *Rickettsia* and *Anaplasma* also identified adenine as the most common base at the transcription start sites (22,290,313,349).

Real-time diplex RT-PCR assays were useful in identifying quantitative differences in the transcripts and also to examine if the p28-Omp genes 14 and 19 are transcriptionally active. The analysis confirmed that both the p28-Omp genes are transcriptionally active independent of the host cells in which the organism is replicating. These observations are consistent with the previous reports of non-quantitative RT-PCR analysis (108,398). However, the current study demonstrated that the host cell environments influence the gene expression leading to altered transcription levels. The real-time diplex RT-PCR assay is effective in identifying quantitative differences in gene expression. This is the first study which resolved the confusion existed due to differences in the gene expression assessed by non-quantitative RT-PCR methods and protein analysis data. In particular, the current study demonstrated that the major expression of gene 19 protein in macrophages and gene 14 protein in tick cells is the results of quantitative differences in their transcription.

In vivo experiments using the murine host model strengthen our *in vitro* observations that the host cell environment is indeed influencing the gene expression in *E. chaffeensis*. It is well documented that *E. chaffeensis* progresses from its infectious dense-core form to non-infectious reticulate form during its replication and reverts back to dense-core form in a phagosome of infected tick cells and macrophages (306,441). Similar dense-core and reticulate forms are also reported for *Chlamydia* species (11,158,424). The gene expression in *Chlamydia trachomatis* is driven by three sigma (σ) factors, σ66 (homolog of σ70), σ54 and σ28, of the RNA polymerase (196,364,435). σ66 and σ54 subunits are constitutively expressed in both the morphological forms of *C. trachomatis*, whereas the expression of σ28 is dependent on the morphological forms (241,348). Stress response genes and late stage dense-core from-specific gene expression appears to be regulated by σ28 of RNA polymerase (40,110,350,435).

To assess if the genes 14 and 19 expression is altered during *E. chaffeensis* progression through the dense-core and reticulate forms, real-time RT-PCR analysis was performed on pathogen's RNA harvested at different times post infection. The analysis was also carried out using RNA recovered from both the tick cells and macrophages. These data suggest that the p28-Omp gene expression is not influenced by the morphological changes in the bacterium form dense-core to reticulate forms. These data, together with the *in vivo* analysis from mouse infection studies suggest that the differences in gene expression are primarily the result of the impact of host cell environment. The constitutive higher expression for the p28-Omp gene 14 in *E. chaffeensis* grown in tick cells and gene 19 in macrophages may have resulted from the RNA polymerase containing σ70 subunit. This is based on the assumption that sigma

factors in *E. chaffeensis* are functionally similar to those of *C. trachomatis*. The differences in *E. chaffeensis* gene expression, therefore, may have resulted alterations in RNA polymerase binding by sensing the host cell environments. This exciting hypothesis, however, requires considerable understanding of *E. chaffeensis* promoters and their detailed characterization.

In summary, the current study identified that host cell-specific differential expression of the p28-Omp genes 14 and 19 in *E. chaffeensis* is occurring primarily at transcriptional level. All the experiments undertaken in this study clearly demonstrate that transcriptional regulation of these genes is influenced by the host cell environments. The host-specific differential gene expression may be an important step in the process of *E. chaffeensis* immune evasion and adaption to its diverse host backgrounds. Identification of the precise mechanism of gene regulation and the molecular events involved in this process are important for a better understanding of strategies employed by this and other tick transmitted organisms. The data reported in this study leads to the investigation of characterizing promoters of the p28-Omp genes 14 and 19.

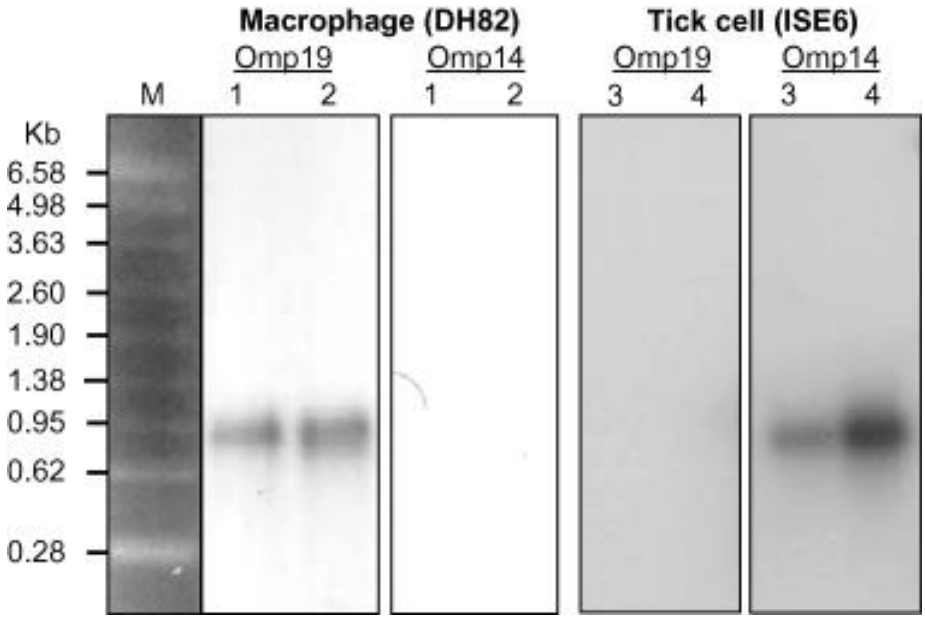


Figure 3.1. Northern blot analysis of the p28-Omp genes 14 and 19. About 10 µg each of two independent batches of *E. chaffeensis* RNA isolated from cultures grown in macrophage cell line, DH82 (lanes 1 and 2), and tick cell line, ISE6 (lanes 3 and 4), were resolved in duplicate sets on a denaturing RNA gel and assessed by Northern blot analysis using p28-Omp 14 or 19 gene-specific ^{32}p-labelled probes. RNA molecular weight markers were resolved in lane M (Reproduced with permission from *Cell Microbiol.* 8, 1475-87, 2006, Wiley-Blackwell Publishing).

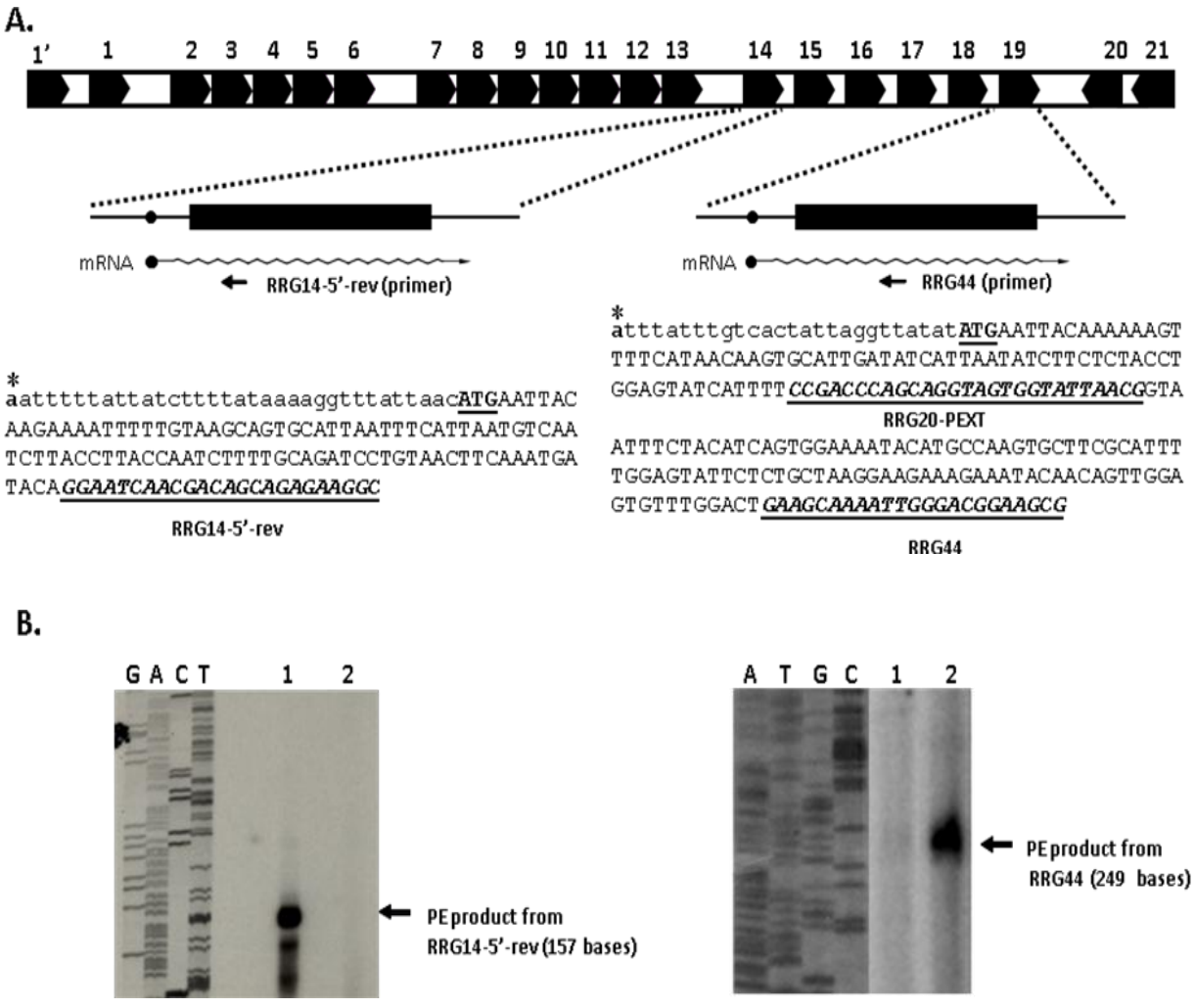


Figure 3.2. Primer extension analysis (PE) of the p28-Omp genes 14 and 19. Panel A has a cartoon spanning all 22 genes (221). This panel also has an expansion of genes 14 and 19 cartoons with predicted transcripts, the primers used for the PE analysis and sequences of the primer extended products with transcription start sites identified with asterisks. PE analysis products resolved on a sequencing gel are shown in panel B. Blots on the left and right represent the data for the genes 14 and 19 transcripts, respectively. Sequence ladder for the gene 14 analysis was created using the same primer used for the PE analysis, but using a DNA template spanning the gene 14 sequence. For the gene 19,

PE analysis was performed using RRG 44 primer and the sequencing ladder was generated using RRG20-PEXT primer with a gene 19 DNA template. (Lane 1, *E. chaffeensis* RNA from tick cells; lane 2, *E. chaffeensis* RNA from macrophages) (Reproduced with permission from *BMC Microbiology*, *In press*).

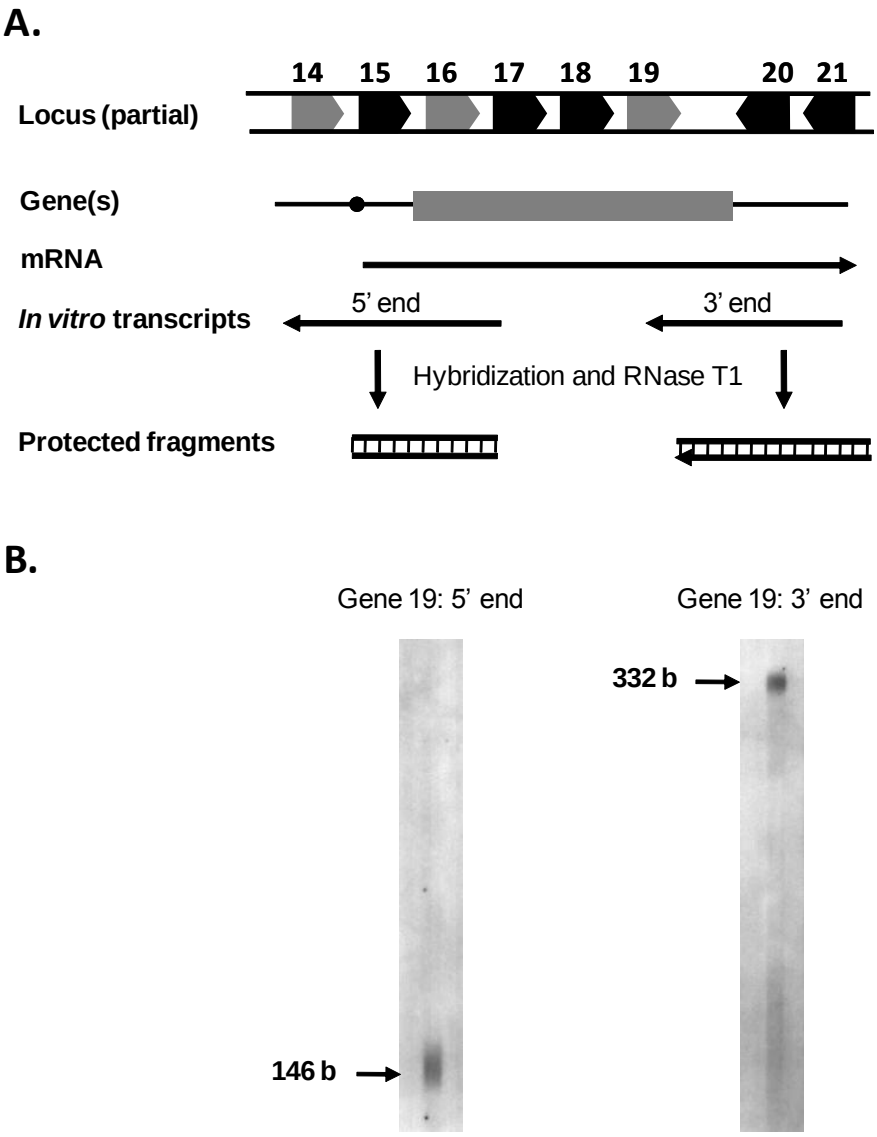


Figure 3.3. Ribonuclease Protection Assay. RPA was performed on macrophage-derived *E. chaffeensis* RNA using 3′ and 5′ end gene-specific riboprobes for the p28-Omp gene 19. The p28-Omp locus cartoon spanning the genes 14 -21 and a schematic representation of the experiment were shown in panel A. The 3′ and 5′ protected fragments for gene 19 resolved on a sequencing gel was shown in panel B.

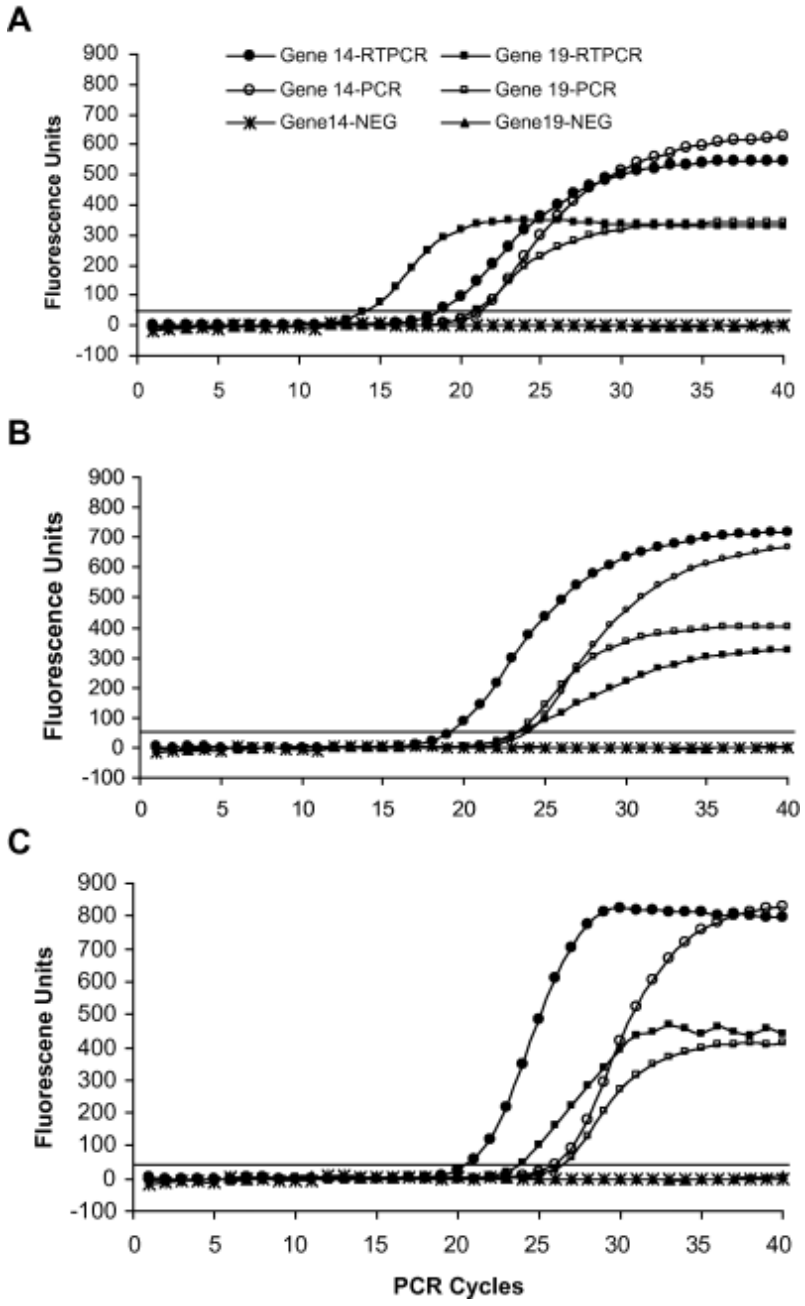


Figure 3.4. TaqMan-based diplex real-time RT-PCR analysis was performed three independent times using total RNA isolated from infected macrophages (DH82) (A) or tick cells; ISE6 (B) and AAE2 (C). The amplification cycles are plotted against fluorescence emission for the analysis performed in the presence or absence of reverse transcriptase. A horizontal solid line in panel on the *x*-axis depicts the line for threshold

fluorescence. Fluorescence emission crossing this line at an amplification cycle is regarded as the Ct value. Gene 14-NEG and Gene 19-NEG represent data derived for reaction negative control diplex assay that included all assay components but no template; Gene 14-RTPCR and Gene 19-RTPCR represent data derived for a reaction containing RNA in the presence of reverse transcriptase; Gene 14-PCR and Gene 19-PCR represent data generated for RNA from the diplex assay that did not include reverse transcriptase (Reproduced with permission from *Cell Microbiol*. 8, 1475-87, 2006, Wiley-Blackwell Publishing).

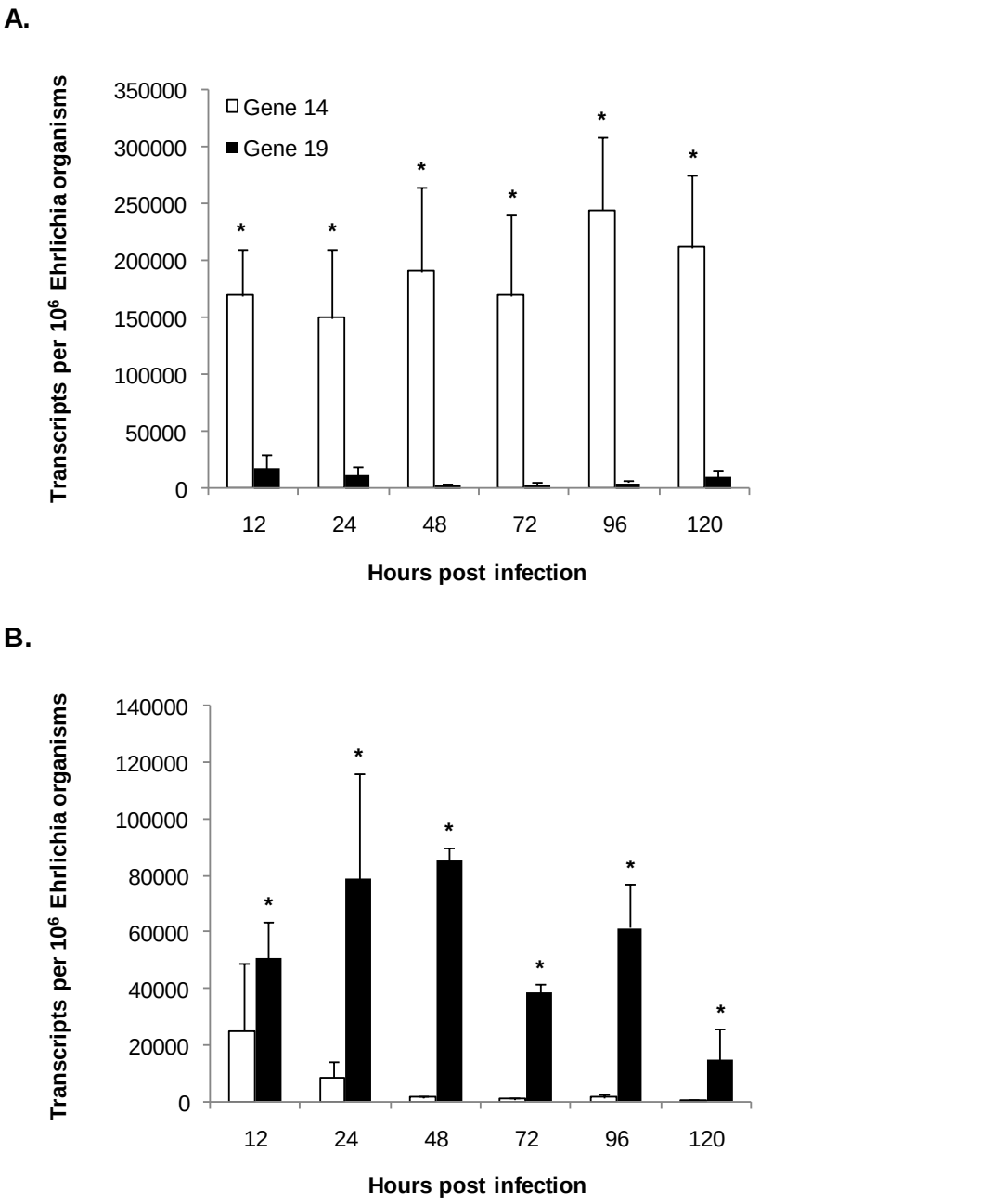


Figure 3.5. A TaqMan-based diplex real-time RT-PCR analysis is performed using RNA isolated from tick-cell (A) and macrophage (B) cultures harvested at different times post infection. Transcript numbers are estimated and presented per million *E. chaffeensis* organisms. Data are presented with SE values calculated from three independent experiments ($P \leq 0.05$) (Reproduced with permission from *BMC Microbiology*, *In press*).

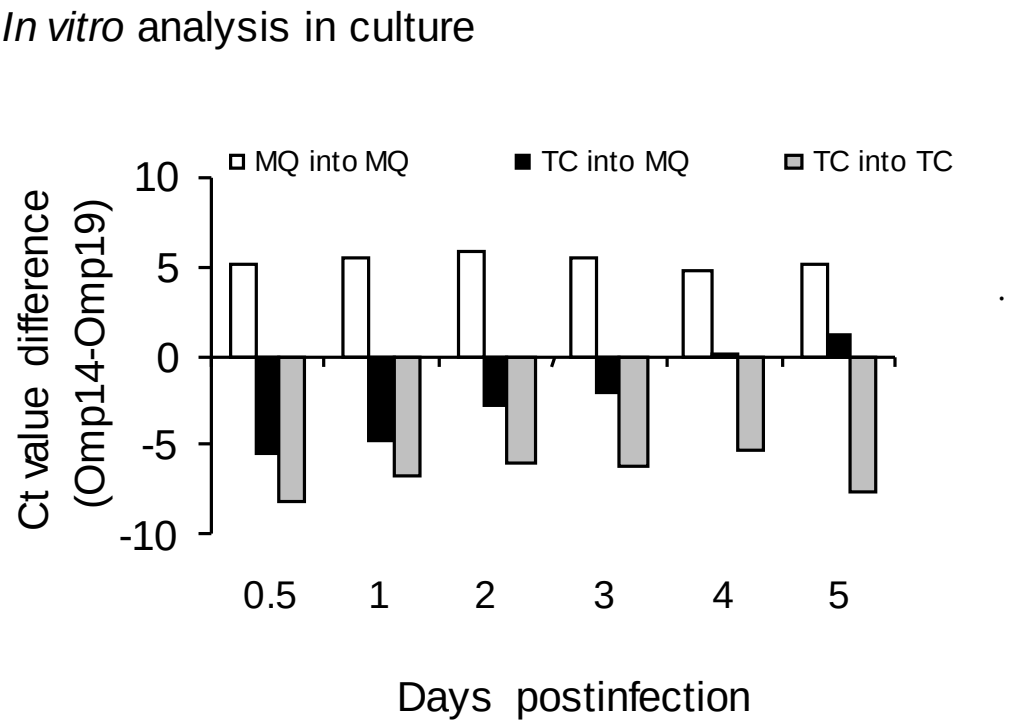


Figure 3.6. Real-time RT-PCR analysis of *in vitro* gene expression of p28-Omp locus genes 14 and 19. A TaqMan-based, diplex real-time RT-PCR analysis was performed to assess the expression for p28-Omp multigene locus genes 14 and 19 in RNA isolated from *in vitro* cultures infected with tick cell- or macrophage culture-derived *E. chaffeensis*. CT value differences (i.e., the gene 14 C_T value – the gene 19 C_T value) in the amplification cycles were plotted for analyzed RNA samples collected at different times after infection. The negative values refer to high-level transcription of gene 14 relative to gene 19, whereas the positive values indicate that the transcription of gene 19 was higher. MQ into MQ refers to *E. chaffeensis* organisms grown in macrophages that were used to infect macrophages; TC into MQ indicates that tick cell-grown bacteria were used to infect macrophage cells, whereas TC into TC represents tick cell culture-derived *E. chaffeensis* that was used to infect tick cells (Reproduced with permission from *Infect Immun*.75, 135-45, 2007, ASM).

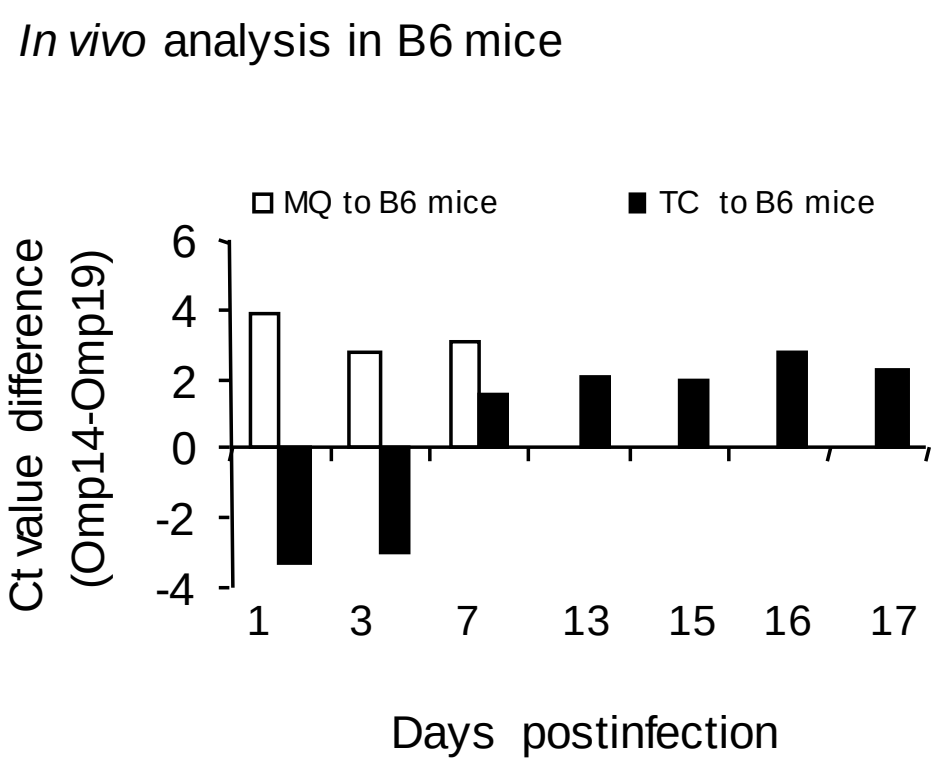


Figure 3.7. TaqMan-based, diplex real-time RT-PCR analysis of *in vivo* gene expression of p28-Omp locus genes 14 and 19 in B6 mice. The analysis was performed on the RNAs isolated from the B6 mice infected with macrophage- or tick cell-derived *E. chaffeensis*. CT value differences (i.e., the gene 14 C_T value – the gene 19 C_T value) in the amplification cycles were plotted for analyzed RNA samples collected at different times after infection. The negative values refer to high-level transcription of gene 14 relative to gene 19, whereas the positive values indicate a higher transcription of gene 19. MQ into B6 mice indicates that macrophage-grown bacteria were used as the inoculum to infect B6 mice; TC into B6 represents B6 mice infected with tick cell culture-derived *E. chaffeensis* (Reproduced with permission from *Infect Immun*.75, 135-45, 2007, ASM).

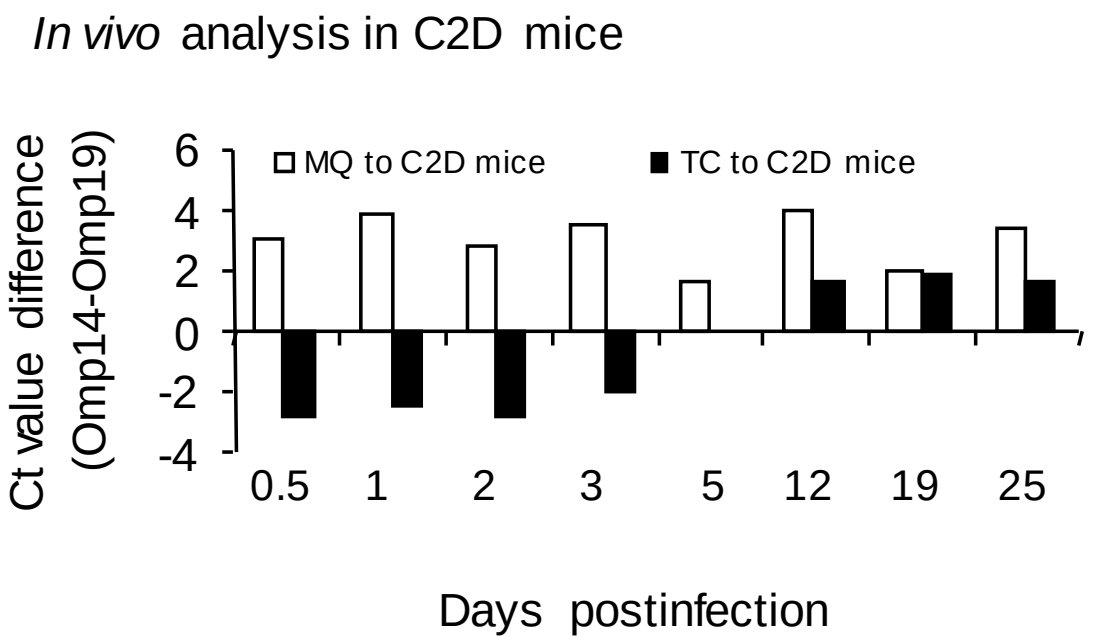


Figure 3.8. TaqMan-based, diplex real-time RT-PCR analysis of *in vivo* gene expression of p28-Omp locus genes 14 and 19 in C2D mice. RNA was isolated from C2D mice infected with macrophage- or tick cell-derived *E. chaffeensis*. CT value differences (i.e., the gene 14 C_T value – the gene 19 C_T value) in the amplification cycles were plotted for analyzed RNA samples collected at different times after infection. The negative values refer to high-level transcription of gene 14 relative to gene 19, whereas the positive values indicate that the transcription of gene 19 was higher. MQ into C2D mice indicates that macrophage-derived bacteria were used as the inoculum to infect C2D mice; TC into C2D represents C2D mice infected with tick cell culture-grown *E. chaffeensis*(Reproduced with permission from *Infect Immun*.75, 135-45, 2007, ASM).

Primer name		Sequence	Orientation	Annealing temperature (°C)
NORTHERN BLOT				
Gene 14 probe				
RRG28	5’	gaaagaaattttatattctagacttgc	Forward	50
RRG71	5’	gagctccttctaatactac	Reverse	
Gene 19 probe				
VSA 5EF	5’	gacccagcaggtagtggtattaacgg	Forward	55
VSA 5ER	5’	caatctttcgtttggaaggagg	Reverse	
PRIMER EXTENSION ANALYSIS				
Gene 14				
RRG 14- 5’ rev	5’	gccttctctgctgtcgttgattcc	Reverse	52
Gene 19				
RRG 20-PEXT	5’	cgttaataccactacctgctgggtcg	Reverse	58
RRG 44	5’	cgcttccgtcccaattttgcttc	Reverse	
RIBONUCLEASE PROTECTION ASSAY				
Gene 19-3'				
ROR 73	5’	gtttgaagctacaaatcc	Forward	58
RRG18-T7	5’	gtaatacgactcactataggaactaataattacaatgtgt	Reverse	
Gene 19- 5’				
RRG185	5’	gactctagacttttaattttattattgccacatg	Forward	55
Gene20-PEXT-T7	5’	gtaatacgactcactatagggcgttaataccactacctgctgggtcg	Reverse	

Real-Time RT-PCR

Gene 14			
RRG215	5’ gacactaatagcggtgac	Forward	48
RRG216	5’ atgccttcatttttaaggac	Reverse	
RRG217	5’ FAM/tactttggactatctcgtgaagac/BHQ	TaqManProbe	
Gene 19			
RRG220	5’ aaattgggacggaagcg	Forward	48
RRG221	5’ gcaaaac ctaaaaacggg	Reverse	
RRG222	5’ TET/tcctccccaaacgatgtattca/BHQ	TaqManProbe	

Table 3.1. List of primers used in this study

Chapter 4

Promoter Characterization of Host-specifically Expressed p28-Omp Genes 14 and 19 of *Ehrlichia chaffeensis*

Abstract

Ehrlichia chaffeensisEhrlichia chaffeensisEhrlichia chaffeensis, the agent of human monocytic ehrlichiosis, requires its adaptation and persistence within its tick vector and vertebrate hosts for the successful completion of its lifecycle and maintenance in nature. In the previous Chapter, I described studies demonstrating the host-specific differences in gene expression from the p28-Omp genes 14 and 19 of *E. chaffeensis*. It is not clear how gene regulation by *E. chaffeensis* is accomplished in response to its host cell environments. Also, very little is known about the promoters of differentially expressed genes and their role in regulating gene expression. In this study, we evaluated the sequences upstream to the transcription start sites of the p28-Omp genes 14 and 19 for their promoter activity in *E. coli* and identified their functional promoters. Promoter deletion analysis was also performed, which aided in mapping several DNA sequences that influenced promoter activity. Putative RNA polymerase binding sites that are similar to those of *E. coli* consensus sequences were also identified for the p28-Omp genes 14 and 19. Furthermore, deletion analysis experiments were performed to confirm the location of the RNA polymerase binging regions, -10 and -35. This is the first study to identify and characterize functional promoters for *E. chaffeensis* genes. The data reported in this study provide information about sequences that are important for gene expression. The strategies utilized in this study to assess the promoter activity are novel and have broader implications to perform similar studies for other *E. chaffeensis* genes and in other *Anaplasmataceae* pathogens.

Introduction

Tick-borne pathogenic organisms belong to the family *Anaplasmataceae* including *E. chaffeensis* can't be transovarially transmitted by ticks (220). Persistent infection in their vertebrate and tick hosts is reported for all known *Anaplasmataceae* pathogens including *E. chaffeensis*, which may be essential for their successful completion of life cycle. Molecular mechanisms that aid in pathogens adaptation and persistence in its tick vector and vertebrate hosts are not clear. Studies in other tick-borne pathogenic bacteria revealed host-specific differential gene expression as one of the strategies employed by the organisms for their colonization in tick hosts and dissemination into a vertebrate host during tick feeding (81,82,136,344,345,431). Our recent proteomic and transcriptomic data and reports form other research laboratories, for a subset of genes, demonstrated tick and vertebrate cell-specific expression of *E. chaffeensis* genes including from two outer membrane protein genes (discussed in Chapter 3; Sirigireddy and Ganta, 2007, unpublished data) (128,353). Control of gene expression at transcription level appears to be a common regulatory strategy employed by several pathogenic bacteria (Discussed in Chapter 3) (113,218,231,283,349,408,409). As described in the previous Chapter, we also demonstrated the first and convincing evidence that *E. chaffeensis* gene expression is also regulated at the transcription level.

Differential gene expression of outer surface protein (OspC) of *B. burgdorferi* is shown to be regulated by a two component regulatory system involving two alternative sigma factors, RpoN and RpoS (136,165,429). Regulation of OspA gene of *B. burgdorferi* is influenced by interaction of a repressor protein with its promoter (232). Environmental signals such as temperature and pH also influence the expression of these

genes (4). For example, relaxation of super coiled DNA in the promoter regions of OspA and OspC in response to an increase in temperature from 25°C to 34°C also alters the promoter activity and influence the gene expression in *B. burgdorferi* (4). In a closely related rickettsial pathogen, *Rickettsia rickettsii*, differential expression of two outer membrane proteins is demonstrated to be the result of variations in their promoters' activities (305).

To date very little is known about transcriptional regulation of *E. chaffeensis* genes. The primer extension analysis of host cell-specific differentially expressed the p28 Omp genes 14 and 19 identified the transcription start sites and also aided in location of their putative promoter regions (discussed in Chapter 3). Understanding the molecular basis for the differential gene expression requires a detailed characterization of the promoters of the differentially expressed genes (22,63,90,408,409).

Genetic manipulation in *E. chaffeensis* has been a difficult task because of its strict host cell requirement and intraphagosomal growth inside the host cells (306,441). Genetic manipulation system for transforming the *E. chaffeensis* organisms remains to be established. Therefore, promoter studies of the differentially expressed genes *in vivo* in *E. chaffeensis* are not feasible at this time. In the current study, we utilized *E. coli* as a surrogate host to evaluate the promoter activity of the p28-Omp genes 14 and 19. We also described deletion analysis to map various regulatory elements and to locate RNA polymerase binding sites of the p28-Omp genes 14 and 19 promoters.

Materials and Methods

Cell cultures: Arkansas isolate of *E. chaffeensis* is cultivated *in vitro* in canine macrophage (DH82) cell line at 37°C by following earlier established protocols (61). *E. coli* strains DH5α (Stratagene, La Jolla, CA) and Top 10 (Invitrogen Technologies, Carlsbad, CA) are cultured in Luria-Bertani (LB) liquid medium or agar plates.

Standard molecular methods: Most of the standard molecular protocols utilized in this study such as cloning, transformation, sequencing and restriction enzyme digestion were described in detail under General molecular methods chapter (Chapter 8).

DNA isolation: Genomic DNA is isolated from 1.5 ml of 90-100% infected *E. chaffeensis* confluent monolayer cultures by the sodium dodecyl sulfate (SDS), proteinase K, phenol, chloroform, isoamyl alcohol method (336). Final purified DNA is resuspended in 100 µl of TE buffer (pH 8.0); concentration is assessed by spectrophotometry using an ND-1000 spectrophotometer and stored at -20°C. Quality of DNA is further confirmed by resolving about 5 µl (~50 ng) on a 0.9% agarose gel (336). Plasmid DNA from the *E. coli* strains is isolated by boiling preparation method by following the protocol described in Sambrook and Russell (336) (discussed in Chapter 8).

Bioinformatics analysis: Sequences upstream from the protein coding regions of *E. chaffeensis* p28-Omp 14 and 19 are obtained from the GenBank data base **(**GenBank accession #CP000236) and aligned using the GCG programs, PileUp and Pretty (87). Direct repeats and palindromic sequences in the upstream sequences were identified using the GCG programs, Repeat and StemLoop, respectively. *E. coli* σ70 promoter consensus sequences (-

10 and -35) (342) are utilized to locate similar elements in the p28-Omp genes 14 and 19 sequences upstream to the transcription start sites.

Oligonucleotides: Oligonucleotides used for the experiments described in this study are custom synthesized from Integrated DNA Technologies (Coralville, IA) and are listed in Table 4.1.

Promoter constructs: The promoter activities of complete non-coding sequences located upstream to the start codon of the genes 14 and 19 were evaluated utilizing two independent promoterless reporter vectors, pPROBE-NT (253) and pBlue-TOPO (Invitrogen Technologies, Carlsbad, CA). The pPROBE-NT vector contains a green fluorescent protein (GFP) gene as the reporter gene. This vector was the kind gift from Dr. Steven E. Lindow, Department of Plant and Microbial Biology, University of California, Berkeley. The use of pPROBE-NT vector in evaluating the promoter activity for *Anaplasma marginale* is recently reported by Barbet et al., (22). This promoterless reporter vector contains a cassette with multiple cloning sites and GFP as a reporter gene. It also contains four transcriptional terminator sequences upstream to multiple cloning sites to prevent transcription of GFP in a non specific manner (253). The reporter gene in pBlue-TOPO vector is a lacZ gene, which encodes for β-galactosidase enzyme (cat #K4831-01; Invitrogen Technologies, Carlsbad, CA).

***Promoter constructs using GFP as a reporter gene*:** Full length putative promoter region of gene 14, which includes sequences downstream to termination codon of gene 13 to upstream of gene 14 initiation codon, was amplified using sequence

specific forward and reverse primers (listed in Table 4.1). Similarly, the sequence upstream to gene 19 start codon to sequence immediately downstream to gene 18 termination codon, (representing full length putative gene 19 promoter), was amplified using a primer pair listed in Table 4.1. To aid in directional cloning into pPROBE-NT vector, the forward and reverse primers were designed to contain XbaI and SacI restriction enzyme sites at the 5' end, respectively. The PCR products and pPROBE-NT vector were digested with XbaI and SacI restriction enzymes and ligated (the cloning strategy is outlined in Figure 4.1, A) and transformed into *E. coli* DH5α strain (Stratagene, La Jolla, CA) by following standard molecular cloning methods (336). The transformed *E. coli* were then plated onto LB agar plates and transformants were selected using kanamycin antibiotic. The clones were verified for the presence of the inserts and their sequence accuracy by restriction enzyme digestion and sequence analysis.

***Promoter constructs using lacZ as a reporter gene*:** The putative promoter regions of the p28-Omp gene 14 were similarly amplified for generating recombinants in pBlue-TOPO vectors. Here, the primers did not include the restriction sites. This is to select the clones with promoter insert in both the orientations. The primer pairs utilized for generating these constructs were listed in Table 4.1. The PCR products were ligated in pBlue-TOPO vector and recombinant clones were selected in Top10 strain of *E. coli* (Invitrogen Technologies, Carlsbad, CA) by following the standard procedures (336). One clone each in forward and reverse orientations were selected for the gene14 (pBlue-TOPO14F or R) and 19 (pBlue-TOPO19F or R). In addition, a promoterless self ligated plasmid was selected to serve as a negative control (the cloning strategy is outlined in Figure 4.2, A). The presence

of inserts and their orientations were verified by performing restriction enzyme digestion and sequencing analyses.

Generation of deletion constructs of theP28-Omp genes 14 and 19 promoters: To identify the regions containing the regulatory sequences, various deletion fragments lacking parts of the 5' or 3' end promoter segments of the genes 14 and 19 were generated by PCR. The 5' deletions fragments were made by utilizing different 5' primers and same 3' primer specific to each promoter (listed in Table 4.1). The length of the 5'deletion fragments for the p28-Omp gene 14 and 19 promoter ranged from 80 to 410 bp and 70 to 180 bp, respectively. For making deletion constructs lacking part of the 3' end sequence for the p28-Omp genes 14 and 19 promoters were also generated utilizing the primer pairs listed in Table 4.1. The PCR amplicons were then cloned into pBlue-TOPO plasmid and transformed into TOP 10 strain of *E. coli* as described above. The presence of inserts, their orientation and accuracy in the recombinants were verified by restriction digestion and sequencing reactions. Table 4.2 contained the list of all promoter clones prepared and utilized in this study.

Generation of promoter deletion constructs lacking -10, -35, or both sequences: Deletion constructs of gene 14 and 19 promoters lacking the predicted -35 or -10 alone or the regions spanning from -35 to -10 were generated using Phusion site-directed mutagenesis kit and by following the manufacturers recommendations (New England Biolabs, MA). Primers used for these deletion experiments are also listed in Table 4.1. Presence of correct inserts for the clones was verified by sequence analysis.

Assessing the promoter activity:

Western Blot analysis to measure GFP expression: The GFP expression resulting from the p28-Omp genes 14 and 19 promoter activity was verified by Western blot analysis. The assay was performed on total cell lysates prepared from the green color colonies of *E. coli* transformed with pPROBE-NT recombinant plasmids containing either gene 14 or 19 promoter. About 10 µg of proteins from cell lysates were resolved on a 12% polyacrylamide gel and transformed on to a nitrocellulose membrane and the GFP protein was detected using polyclonal antibody raised against this protein (Cat# 600-101-215, Rockland Immunochemicals, Inc., Gilbertsville, PA).

β-galactosidase Assay: The *E. coli* colonies transformed with pBlue-TOPO constructs containing full length promoters and various deletions of the p28-Omp genes 14 and 19 promoters and no insert controls were evaluated for promoter activity by measuring β-galactosidase activity. To accomplish this, *E. coli* colonies containing the recombinant plasmids were grown to an optical density of 0.4 assessed by spectrophotometer set at 600 nm. Cells were lysed using the cell lysis buffer, centrifuged at 12000 g and supernatant containing soluble proteins were collected. Protein concentrations were estimated by BCA method (Cat # #23225, Pierce, IL). About 2.5 and 5 µg of protein preparations were utilized to assay the β-galactosidase activity using Ortho-Nitrophenyl-β-D-Galactopyranoside (ONPG) as the substrate and by following the manufacturer's instructions (Cat # K1455-01, Invitrogen Technologies, Carlsbad, CA). The experiments were repeated four independent times using independently isolated protein preparations. Each sample was assayed three times and an average value obtained from three measures was utilized in the data analysis.

Specific activity of β-galactosidase was calculated as described in β -gal assay kit protocol (Cat # K1455-01, Invitrogen Technologies, Carlsbad, CA).

Specific activity = nmoles of ONPG hydrolyzed/t/mg of protein

nmoles of ONPG hydrolyzed = (OD_{420}) (8x 10^5 nanoliters)/(4500nl/nmoles-cm)(1cm)

Where t = the time of incubation in minutes at 37^oC

Statistical Analysis: Statistical analysis for the experimental data was performed using repeated measures of ANOVA method. Bonferroni method was used to adjust for multiple comparisons. GraphPad InStat Software (La Jolla, CA) is used in performing these analyses. *P* values of less than 0.05 were considered significant.

Results

Evaluation of promoter activities of the p28-Omp genes 14 and 19 upstream sequences: Transcription analysis assessed by direct RNA mapping and real-time RT-PCR methods revealed quantitative differences in gene expression for the p28-Omp 14 and 19 genes influenced by invertebrate and vertebrate host cell environments. The promoter activities of the 5' non-coding sequences of these geneses were evaluated in *E. coli*. Sequences upstream to coding regions of the p28-Omp genes 14 or 19 cloned in front of the GFP reporter gene in pPROBE-NT were positive for green fluorescence as visualized by the presence of green colonies (Figure 4.1B, a and d). *E. coli* transformed with pPROBE-NT plasmids alone are negative for the green fluorescence (Figure 4.1B, b and c). The GFP protein expression was further verified by Western blot analysis (Figure 4.2).

Promoter activity for the genes 14 and 19 was further confirmed by another independent method using lacZ gene as the reporter in pBlue-TOPO plasmid and by assessing the β-galactosidase activity. The *E. coli* transformants with recombinant plasmids having gene 14 or 19 putative promoter sequences in correct orientation had significantly more β-galactosidase activity ($P \leq 0.001$) than the baseline activity observed for constructs with no promoter sequences or when the sequences inserted in reverse orientation (Figure 4.3B).

Bioinformatics analysis: The entire non-coding sequences upstream to genes 14 and 19 were evaluated to identify sequences similar to the consensus *E. coli* RNA polymerase binding site sequences, -10 and -35, and for the presence of ribosome binding sites (RBS) (Figure 4.4). Consensus -10 and -35 elements are identified upstream to the transcription start sites mapped by primer extension analysis (Figure 4.4). Relative distances of the consensus -10 and -35 sequences from transcription start sites also remained the same for both the genes (Figure 4.4C). Similarly, putative RBS (362) were identified 7 and 4 nucleotides upstream to the initiation codon of genes 14 and 19, respectively. Genes 14 and 19 sequences upstream to the predicted -10 and -35 sequences differed considerably in their lengths and homology (Figure 4.4 A and B). The gene 14 sequence is 581 bp in length which is 273 bp longer than the gene 19 upstream sequence (308 bp). The sequences included several gene-specific direct repeats and palindromic sequences. In addition, a unique 14 nucleotide-long 'G' rich sequence was detected in the gene 19 sequence. The consensus -35 sequences were identical for both the genes, while the -10 and RBS sequences differed by one nucleotide each (Figure 4.4C).

Promoter deletion analysis: To assess if the promoter activities are influenced by the sequences upstream to the transcription start sites of genes 14 and 19, deletion constructs were prepared in pBlue-TOPO plasmid and analyzed. β-galactosidase activity for several pBlue-TOPO plasmid constructs with segments deleted from 5' end for both the genes were evaluated (Figure 4.5). Deletions to the sequences ranged from 60 to 476 bp for the p28-Omp gene 14 and 69 to 183 bp for gene 19. All deletion constructs for the gene 14, except for deletions having 461 and 350 bp segments, had significantly higher β-galactosidase activity compared with negative controls lacking no insert and the insert in the reverse orientation. The first 60 bp deletion from the 5' end resulted in no significant change ($P \geq 0.05$) in the β-galactosidase activity compared with that observed for the full length insert, whereas a deletion of an additional 60 bp caused a decline of about 90% of the enzyme activity ($P \leq 0.001$). The β-galactosidase was restored completely by an additional 61 bp deletion. Further deletion of another 50 bp also resulted in another near complete loss of activity. Subsequent deletions by 64 bp each caused a stepwise restoration of the enzyme activity to 54% and 91%, respectively. Deletion of another 53 bp caused another drop in β-galactosidase activity to 24% which remained unaffected with an additional deletion of 64 bp fragment (Figure 4.5, panels A and B). Similar deletion analysis performed for the gene 19 upstream sequence also resulted in altered β-galactosidase activity compared with the full length sequence. The 5' end deletions of 69 and 120 bp resulted in 20 and 30% decline in enzyme activity. These declines, however, were not statistically significant ($P \geq 0.05$). Deletion of an additional 63 bp for gene 19 sequence caused an increase of about 60% more β-galactosidase activity. The increase in the β-galactosidase activity was unique to the gene 19 deletions. The deletion analysis data for both the genes promoters also confirm that the minimal promoter for RNA polymerase binding was unaffected by the step-wise deletions

performed in this study. To confirm that the RNA polymerase binding regions are located within the sequences spanning up to the consensus -35 sequences, 3' end deletion constructs lacking sequences up to the -35 region for genes 14 and 19 (65 bp and 57 bp, respectively) were prepared and assessed for β-galactosidase activity. These deletions led to the complete loss of β-galactosidase activity ($P \leq 0.001$) (Figure 4.5 A-B lane 11 and C-D lane 6).

Location of -10 and -35 regions: To determine if the consensus -35 and -10 represented true RNA polymerase binding site regions, constructs lacking either predicted -35 or -10 alone or the regions spanning from -35 to -10 were generated and the impact of the loss of these sequences on promoter activity was evaluated by measuring β-galactosidase activity. Deletion of the predicted -35 regions alone or in combination with the -10 for both the genes 14 and 19 resulted in decline of β-galactosidase activity to the background levels observed for negative controls. Deletion of the consensus -10 regions alone for both the genes had no significant impact on the promoter activity (Figure 4.6).

Discussion

Proteome and transcriptome analysis of *E. chaffeensis* demonstrated host-specific differential expression of several *E. chaffeensis* genes including those form the p28-Omp outer membrane protein multigene locus (183,274,347,352,398) (Sirigireddy and Ganta, 2007, unpublished data; Discussed in Chapter 3). Our detailed transcriptional analysis of the p28-Omp genes 14 and 19 further confirmed the host cell-specific differences in gene expression and also suggested that that *E. chaffeensis* regulates expression of these genes by sensing the host cell signals (discussed in Chapter 3). To date, very little is known about the molecular basis of differences in gene expression in *E. chaffeensis*. A better

understanding of the molecular mechanisms leading the alterations in gene expression by *E. chaffeensis* can be achieved by characterizing the sequences located to upstream to the transcription start sites of these genes. This is the first and detailed investigation, which evaluated the promoters of two differentially expressed *E. chaffeensis* genes, the p28-Omp genes 14 and 19.

Initial analysis of complete 5' non-coding sequences of the genes 14 or 19, ligated in front of a reporter gene (GFP or lacZ) of two promoters less reporter vectors, demonstrated that *E. chaffeensis* promoters are functional in *E. coli*. In addition, comparisons of genes 14 and 19 promoters with those of *E. coli* σ70 RNA polymerase binding sequences aided in identification of consensus -10 and -35 and ribosomal binding sites in these sequences. The consensus -10 and -35 sequences of the p28-Omp genes 14 and 19 promoter contained similar sequences. This observation suggests that RNA polymerase may have similar binding preference for these sequences and differences in transcription may be influenced by the sequences upstream to the -10 and -35. Bioinformatics analyses of the genes 14 and 19 promoters identified considerable differences between them. The differences included variations in the length of the upstream sequences, presence of several gene-specific direct repeats, inverted repeats, palindromic sequences and the presence of a 14 base-long ‘G’ rich region that is found only in the gene 19 sequence. Regulatory role of direct and inverted repeats in *E. chaffeensis* is unknown but are reported to play a role in transcriptional regulation for several bacterial pathogens (16,48,68,84,226,265). For example, in a closely related rickettsial pathogen, *Rickettsia prowazekii*, the presence of a palindrome sequence in the citrate synthase and its possible role in transcriptional regulation has been reported (48).

Similarly, transcription factors, such as Zinc finger proteins that influence the gene expression via interacting with G-rich sequences is well-established in the literature for both prokaryotes and eukaryotes (65,86,182,209,299,367,401). *E. chaffeensis* genome contains homologs of zinc finger proteins (Genbank #s ECH_0057 and ECH_0746) (162). It is of interest to investigate if the *E. chaffeensis* zinc finger proteins act as a transcription regulators for the p28-Omp gene 19.

This is the first study for an *Anaplasmataceae* pathogen, *E. chaffeensis*, where the importance of various putative regulatory sequences including direct repeats and inverted repeats of two promoters were assessed. Sequential deletions in the gene 14 upstream sequences from the 5' end, whereby some of the direct repeats and palindrome sequences were deleted, resulted in variations in the promoter activity. The alteration in promoters' activity fluctuated from complete or partial loss of activity compared with that observed for the full length upstream sequences. Additional deletions caused the restoration of 100% activity and subsequent additional deletions again led to decline in promoter activity. Similarly, deletion analysis in the gene 19 promoter region caused loss or gain of promoter activities relative to the inclusion of full length upstream sequence as a promoter. These data suggest that genes 14 and 19 promoter regions contain sequence domains that influence binding affinity of RNA polymerase to the respective promoters. Altered promoter activities observed in deletion analysis experiments may have resulted from the deletions in upstream sequences involved in altering DNA topology making RNA polymerase less or more accessible to its binding domains. Influence of 5' sequences altering the DNA topology for RNA polymerase binding has been well-established for promoters of several bacterial organisms, such as *Bacillus subtilis*, *C.*

tracomatis, *E. coli*, *Klebsiella pneumonia* (107,240,243,256,267,302,304). Previous reports also suggest that the inverted and direct repeats contribute to the DNA curvatures thus impacting RNA polymerase binding to the -35 and -10 regions (240,265). Although less likely, the presence of *E. coli* regulators that are homologues of *E. chaffeensis* may also bind and influence the promoter activity. Homologues of *R. prowazekii* repressors/enhancers in *E. coli* have been reported for the 16S rRNA gene (290).

Previous studies in other bacteria led to the identification of RNAP binding sites for several genes, which are located at -10 and -35 regions. To evaluate the consensus RNAP binding sequences identified in the p28-Omp genes 14 and 19 promoter regions of *E. chaffeensis*, deletion constructs spanning putative -10 and -35 were prepared and evaluated in *E. coli*. Deletion of the consensus -35 region alone or in combination of -10 region, but not -10 region alone, reduced the promoter activity to background levels. These data suggest that the -35 region identified indeed contributes to the RNA polymerase binding. It is unclear why deletion of the predicted -10 regions for both the genes had little effect in altering the promoter functions. Greater tolerance to changes or deletions of to the -10 regions compared to -35 regions has been reported for other prokaryotes as well (151,161,356,381). It is, however, possible that the -10 regions we predicted are not accurate and may be present at a different location. Alternatively, the -10 regions may be less important in *E. chaffeensis*. This hypothesis is too premature at this time as more detailed mapping of the -10 region is needed.

The novel strategies utilized in this study to assess the promoter activity opens up similar line of research in other tick-transmitted bacteria that belong to the family

Anaplasmataceae and other intracellular bacteria that are difficult to manipulate genetically. Although, this study provide information about contribution of several sequence elements to variations in promoter activity, it is important to note the fact that these observed variations could be limited to *E. coli* environment. It is possible that the variations in the promoters' activities in *E. coli* may be due to involvement of regulatory proteins that are specific to *E. coli* and may or may not be present in *E. chaffeensis*.

In conclusion, we have reported a detailed characterization of two *E. chaffeensis* promoters utilizing novel molecular methods. Experimental evidence has been provided that sequences located upstream to the transcription start sites influences the activities of the p28-Omp genes 14 and 19 promoters. The deletion analysis data reported for *E. chaffeensis* genes in this study demonstrate that the molecular evaluation in *E. coli* serves as a good system to map the regulatory regions of *E. chaffeensis* genes. This is particularly important as it is not possible, at this time, to carryout transformational analysis within *E. chaffeensis* in support of characterizing the transcription apparatuses of its genes. Presence of several promoter-specific putative regulatory sequences in the promoter regions of these genes suggests that *E. chaffeensis* regulatory proteins, which may be made by the pathogen in response to host environment, bind to these regions and influence the gene expression. More detailed investigations are needed to map the mechanisms controlling gene expression in *E. chaffeensis* and how host cell environments influence the gene regulation by this pathogen.

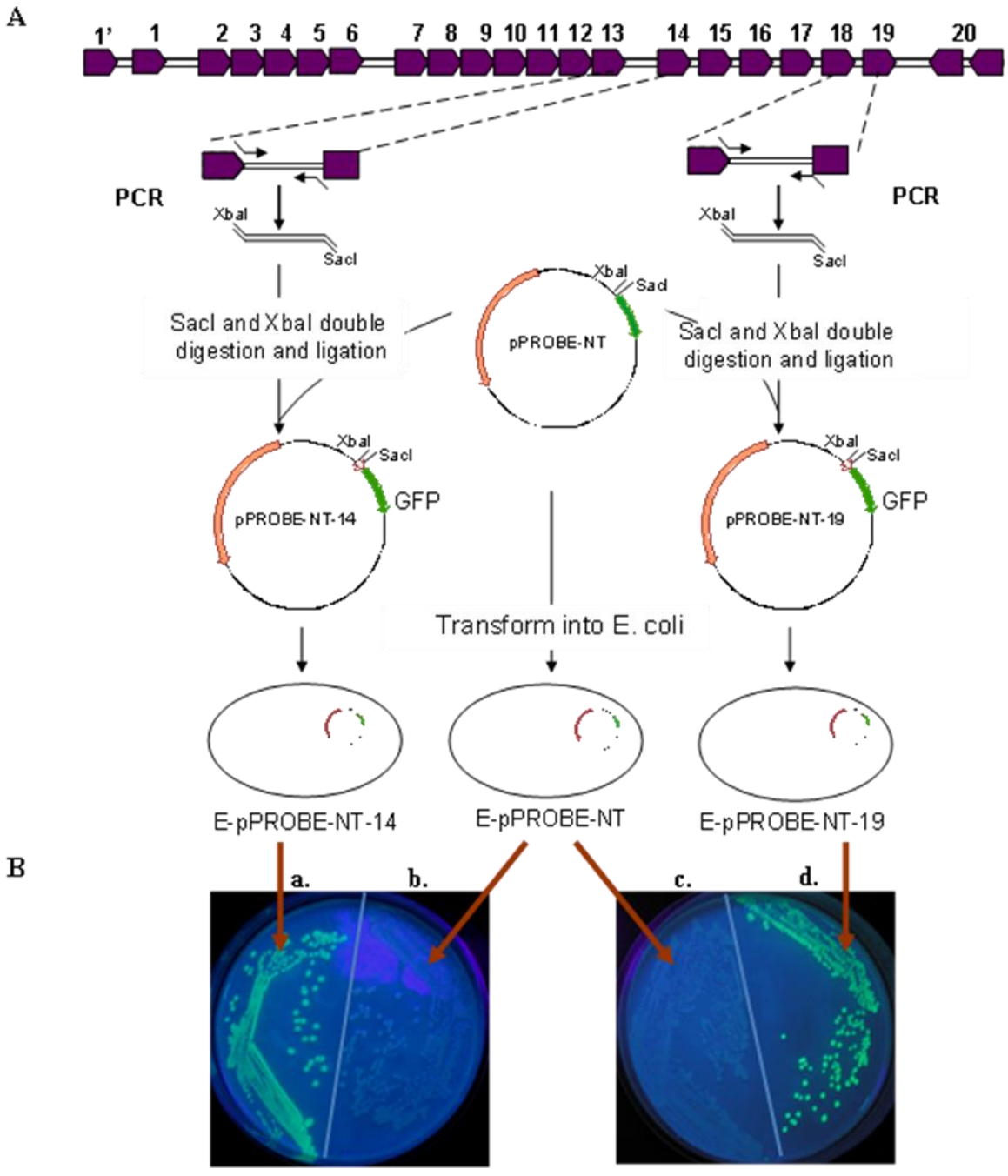

A
1' 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
PCR
PCR
XbaI
SacI
XbaI
SacI
SacI and XbaI double digestion and ligation
pPROBE-NT
XbaI
SacI
SacI and XbaI double digestion and ligation
XbaI
SacI
GFP
pPROBE-NT-14
XbaI
SacI
GFP
pPROBE-NT-19
Transform into E. coli
E-pPROBE-NT-14
E-pPROBE-NT
E-pPROBE-NT-19
B
a.
b.
c.
d.

Figure 4.1. Construction of GFP expressing plasmids containing the genes 14 and 19 promoters (A). Two sets of primers are designed for amplifying the putative promoter regions of genes 14 and 19. Restriction sites for XbaI and SacI are engineered into the primers to aid in the directional cloning. The primers are used to amplify the promoter regions using *E. chaffeensis* genomic DNA as the template. The amplicons are double digested with SacI and XbaI and ligated into the SacI and XbaI double digested pPROBE- NT plasmid. The plasmids containing inserts are selected after transformation into *E. coli* strain, XL1Blue (Stratagene, La Jolla, CA). GFP positives are detected for recombinants in *E. coli* for the promoter constructs **(B).** Green fluorescent protein (GFP) constructs evaluated for the promoter activity of the p28-Omp genes 14 and 19. The pPROBE-NT plasmid containing promoterless GFP gene (b and c) and genes 14 and 19 upstream sequences cloned in front of the GFP coding sequence (a and d, respectively) are evaluated for the GFP expression in *E. coli* (Reproduced with permission from *BMC Microbiology, In press*).

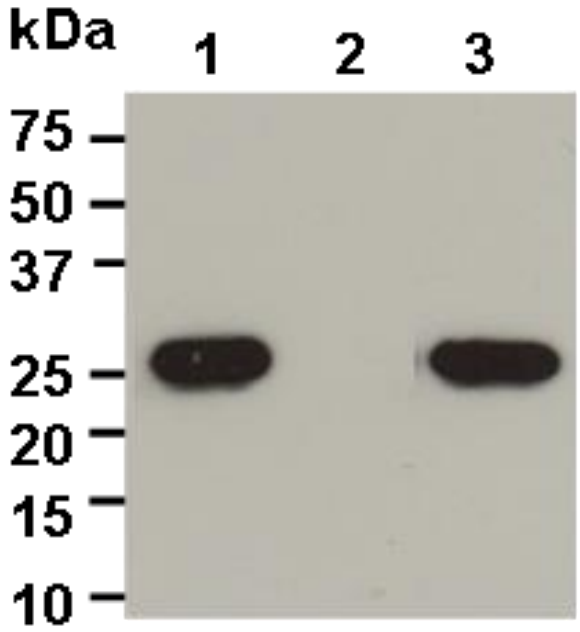
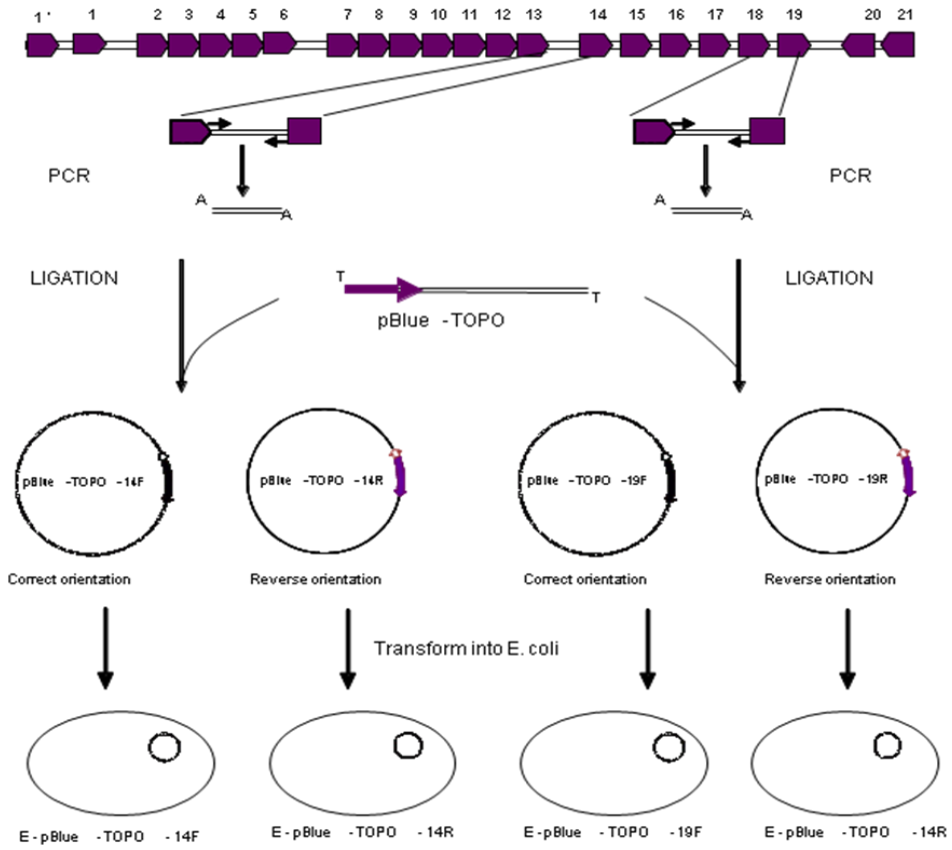


Figure 4.2. Western blot analysis to determine the GFP expression in *E. coli* containing genes 14 and 19 promoters constructs. *E. coli* lysates are prepared to examine the GFP expression in *E. coli* containing gene 14 promoter containing plasmid (pPROBE-NT14) (lane 1), promoterless GFP plasmid (pPROBE-NT) (lane 2), and gene 19 promoter containing plasmid (pPROB-NT19) (lane 3). Approximately 25 µg of protein is resolved on a 12 % polyacrylamide gel, transferred to a nitrocellulose membrane and assessed for the presence of GFP using polyclonal sera against the GFP protein (Rockland, Gilbertsville, PA). A 25 kDa GFP protein is detectable only in the *E. coli* lysates containing genes 14 and 19 promoter plasmids. Molecular weight markers are shown to identify the size.

A.



B.

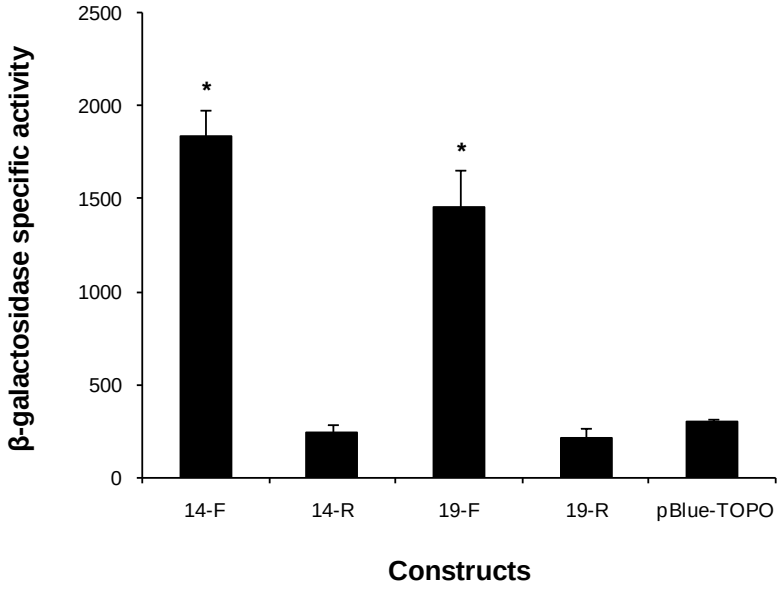
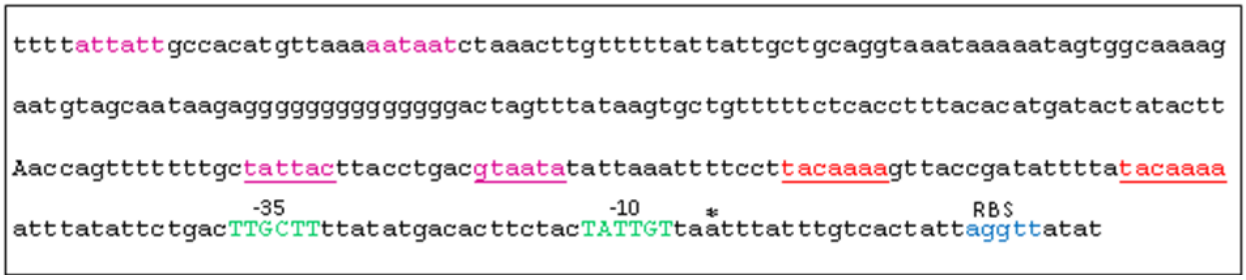


Figure 4.3. A. Construction of lacZ plasmids containing gene 14 and 19 putative promoters. Two primers are designed and used to amplify the promoter regions of genes

14 and 19. The amplicons are ligated into pBlue-TOPO plasmid having T over hang. Plasmid containing inserts are selected after transforming into *E. coli* strain, TOP10. As the PCR products contained over hand we expect to get the inserts ligated into both orientations. Transformants in *E. coli* containing recombinant plasmids with both orientations are selected and sequenced. LacZ plasmids containing correct orientation inserts for gene 14 and 19 can serve as the promoters to drive the expression of β-galactosidase protein if *E. coli* RNA polymerase recognizes *Ehrlichia* promoter and the sequences represent promoters. Promoter region ligated into the opposite orientation are not expected to serve the promoters to drive β-galactosidase expression. B, LacZ constructs evaluated for the promoter activity of the p28-Omp genes 14 and 19. The pBlue-TOPO vector containing promoterless lacZ gene (pBlue-TOPO), genes 14 and 19 upstream sequences inserted in forward (14-F and 19-F) and reverse orientations (14-R and 19-R) are evaluated for the β-galactosidase activity in *E. coli*. Data are presented with SD values calculated from four independent experiments ($P \leq 0.001$) (Reproduced with permission from *BMC Microbiology, In press*).

A.

B.



C.

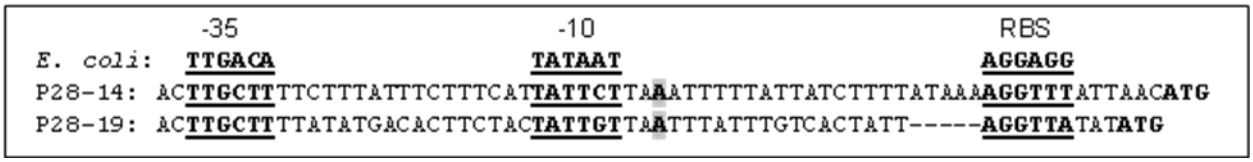


Figure 4.4. P28-Omp genes 14 and 19 promoter region sequence analysis. Genes 14 (panel A) and 19 (panel B) upstream sequences were evaluated for the presence of direct repeats (red text), palindromic sequences (pink text) and for the presence of unique sequences (G-rich region), consensus -35 and -10 regions (green text) and ribosome binding sites (blue text). Panel C has the comparison of -10, -35 and ribosome binding

sites of the genes 14 and 19 with the *E. coli* consensus sequences. Transcription start sites for the genes mapped by primer extension analysis are identified with bold and grey color highlighted text. Dashes were introduced in the p28-Omp gene 19 sequence to create alignment with the gene 14 sequence (Reproduced with permission from *BMC Microbiology*, *In press*).

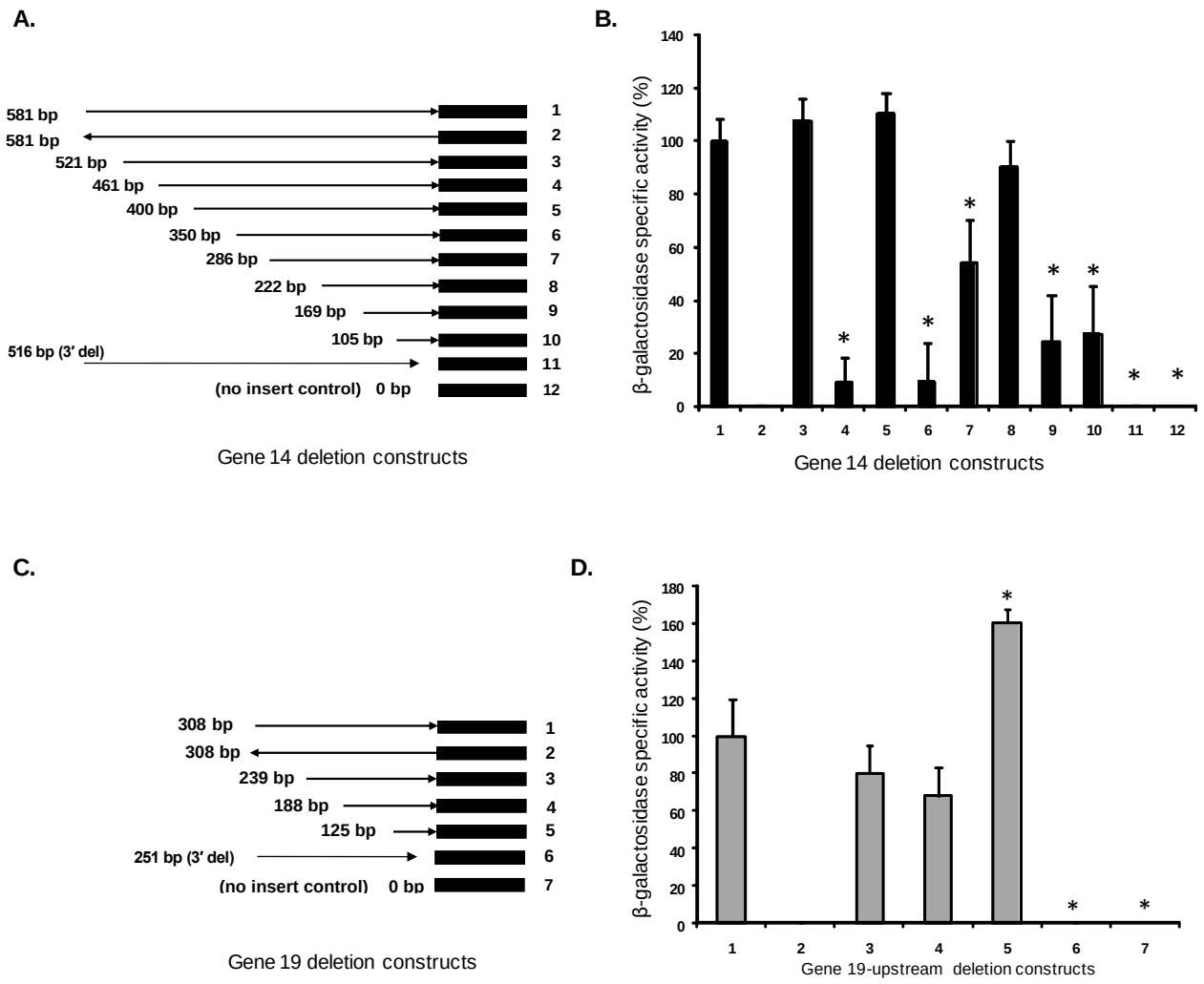


Figure 4.5. Deletion analysis of the genes 14 and 19 promoter regions. β-galactosidase activity of extracts prepared from *E. coli* cultures of bacteria transformed with various deletion constructs was determined. Panels A and C have cartoons depicting deletion constructs and their orientations for the genes 14 and 19, respectively. Panels B and D contained the β -galactosidase activity analysis data. Data are presented with SD values calculated from four independent experiments ($P \leq 0.001$) (Reproduced with permission from *BMC Microbiology*, *In press*).

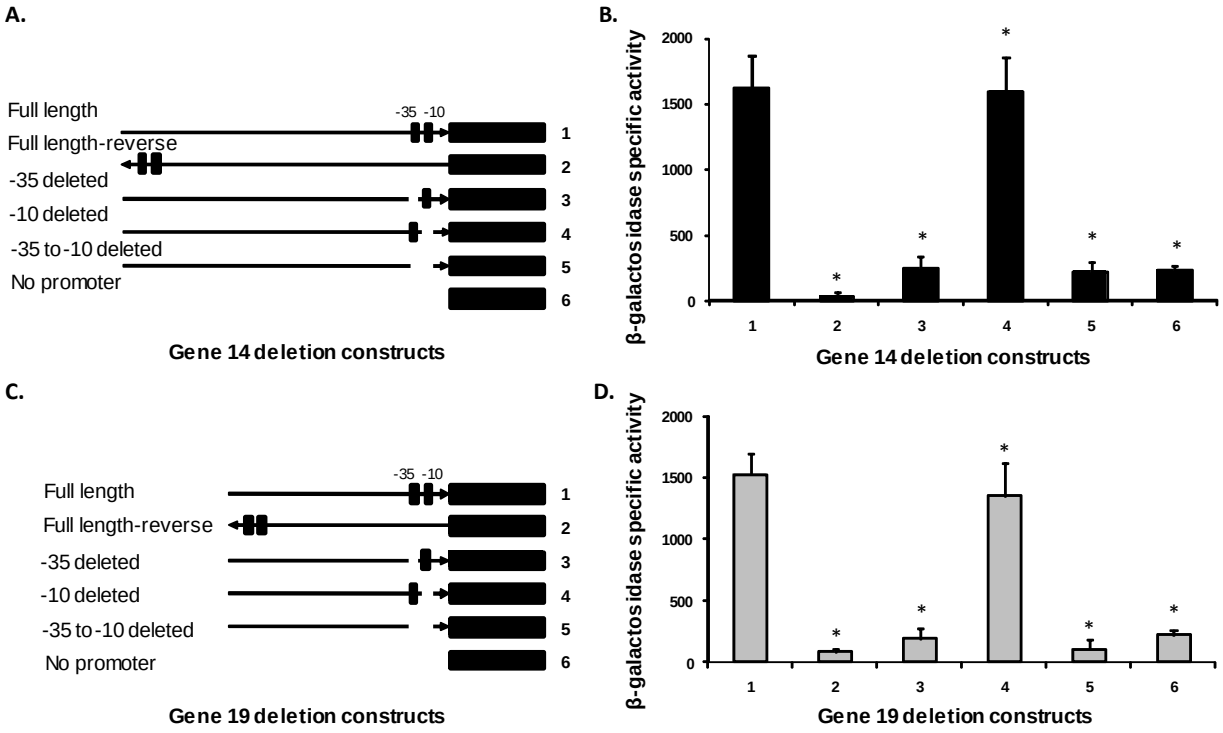


Figure 4.6. Deletion analysis spanning the -35 and -10 regions of the genes 14 and 19. β-galactosidase activity of extracts prepared from *E. coli* cultures of bacteria transformed with -35 or -10 deletions or deletions spanning from -35 to 10 were determined. Panels A and C have cartoons depicting deletion constructs and their orientations for the genes 14 and 19, respectively. Panels B and D contained the β-galactosidase activity analysis data. Data are presented with SD values calculated from four independent experiments ($P \leq 0.001$) (Reproduced with permission from *BMC Microbiology*, *In press*).

Primers	Sequnece	Orientation	Annealing temperature (°C)
PROMOTER ANALYSIS			
Gene 14-upstream sequence primer pairs for PCR			
For cloning into pPROBE-NT			
RRG 183	5' gactctagattgctcaacccataaaataatg	Forward	50
RRG 184	5' agtgagctctttataaaagataataaaaatttaag	Reverse	
For cloning into pBlue-TOPO			
RRG 217	5' attgctcaaccataaaataatggga	Forward	48
RRG 218	5' gttaataaacctttataaaag	Reverse	
RRG 267	5' cagttaactttctgtaaacttc	Forward	48
RRG 218*		Reverse	
RRG 268	5' atcataagtttacaataatgtc	Forward	48
RRG 218*		Reverse	
RRG 269	5' cgttttctgctttattagaatg	Forward	48
RRG 218*		Reverse	
RRG 270	5' gttccgtatttattaatatatg	Forward	48
RRG 218*		Reverse	
RRG 271	5' catgtactgaatttgtgatttg	Forward	48

RRG 218*		Reverse	
RRG 272	5' ggataagtactttagcaagtgg	Forward	48
RRG 218*		Reverse	
RRG 273	5' taagtagtaaagttaactatag	Forward	48
RRG 218*		Reverse	
RRG 274	5' acttttgttgtaaatttgaaag	Forward	48
RRG 218*		Reverse	
RRG 217*		Forward	50
IG14-35 del R	5' (PO_4) gtctagaatataaaatttctttc	Reverse	
IG14-10 del F	5' (PO_4) taaatttttattatcttttataaaaggtttattaac	Forward	56
IG14-10 del R	5' (PO_4) atgaaagaaataaagaaaagcaagtctag	Reverse	
IG14-35 del F	5' (PO_4) ttctttatttctttcattattc	Forward	48
IG14-35 del R*		Reverse	
IG14-10 del F*		Forward	51
IG14-35 del R*		Reverse	

Gene 19-upstream sequence primer pairs for PCR

For cloning into pPROBE-NT

RRG 185	5' gactctagacttttaattttattattgccacatg	Forward	61
RRG 186	5' agtgagctcaatagtgacaaataaattaacaatag	Reverse	

For cloning into pBlue-TOPO

RRG 185*		Forward	60
RRG 445	5' atataacctaatagtgacaaataaattaac	Reverse	
RRG 275	5' gtggcaaaagaatgtagcaataag	Forward	50
RRG 445*		Reverse	
RRG 276	5' gtgctgtttttctcacctttacac	Forward	63
RRG 445*		Reverse	
RRG 277	5' ctgacgtaatatattaaattttcc	Forward	55
RRG 445*		Reverse	
RRG 185*		Forward	50
IG19-35 del R	5' (PO_4) gtcagaatataaatttttgtataaaatatcg	Reverse	
IG19-10 del F	5' (PO_4) taatttatttgtcactattaggttat	Forward	56
IG19-10 del R	5' (PO_4) gtagaagtgtcatataaaagcaag	Reverse	
IG19-35 del F	5' (PO_4) ttatatgacacttctactattgttaatttatttg	Forward	61.5
IG19-35 del R*		Reverse	
IG19-10 del F*		Forward	58
IG19-35 del R*		Reverse	

Table 4.1. Primers used for cloning into pPROBE-NT and pBlue-TOPO vectors

Promoter deletion clones	Primer pair (Forward/Reverse)	Deletion type	Promoter orientation	Amplicon length(bp)
Gene 14 promoter deletion clones				
pPROBE-NT clones	RRG183/RRG184	Full length	Forward	596
pBlue TOPO clones				
1	RRG217/RRG218	Full length	Forward	581
2	RRG217/RRG218	Full length	Reverse	581
3	RRG267/RRG218	5' deletion	Forward	521
4	RRG268/RRG218	5' deletion	Forward	461
5	RRG269/RRG218	5' deletion	Forward	400
6	RRG270/RRG218	5' deletion	Forward	350
7	RRG271/RRG218	5' deletion	Forward	286
8	RRG272/RRG218	5' deletion	Forward	222
9	RRG273/RRG218	5' deletion	Forward	169
10	RRG274/RRG218	5' deletion	Forward	105
	RRG217/IG14-35 del R	3' deletion	Forward	516

11				
-10 deletion	IG14-10 del F/IG14-10 del R	-10 deletion	Forward	8366
-35 deletion	IG14-35 del F/IG14-35 del R	-35 deletion	Forward	8366
-10 to -35 del	IG14-10 del F/IG14-35 del R	-10 to -35 del	Forward	8343

Gene 19 promoter clones

pPROBE-NT clones	RRG185/RRG186	Full length	Forward	334

pBlue TOPO clones

1	RRG185/RRG445	Full length	Forward	308
2	RRG185/RRG445	Full length	Reverse	308
3	RRG275/RRG445	5' deletion	Forward	239
4	RRG276/RRG445	5' deletion	Forward	188
5	RRG277/RRG445	5' deletion	Forward	125
6	RRG185/IG19-35 del R	3' deletion	Forward	267

-10 deletion	IG19-10 del F/IG19-10 del R	-10 deletion	Forward	8112
-35 deletion	IG19-35 del F/IG19-35 del R	-35 deletion	Forward	8112
-10 to -35 deletion	IG19-10 del F/IG19-35 del R	-10 to -35 del	Forward	8088

Table 4.2. List of the p28-Omp genes 14 and 19 promoter clones

Chapter 5

Establishment of *in vitro* transcription and transcription coupled translation systems for *Ehrlichia chaffeensis*

Abstract

Host-specific differential gene expression in response to host cell environments has been reported for a tick-borne rickettsial pathogen, *Ehrlichia chaffeensisEhrlichia chaffeensisEhrlichia chaffeensis.* Molecular characterization of *E. chaffeensis* promoters and identification of proteins that regulate gene expression will provide important insights about the pathogen strategies in support of its survival and persistence. Study of the molecular events that aid in accomplishing the differential gene expression *in vivo* in *E. chaffeensis* is not possible at the present time due to lack of an established DNA delivery and mutational analysis methods. Development of *in vitro* transcription assays to study the mechanisms of gene regulation will be a desirable alternative to carryout genetic analysis for this intracellular pathogen. Experiments discussed in Chapter 4 and studies reported in the literature for other closely related rickettsial pathogens suggest that *E. coli* RNA polymerase (RNAP) can recognize the promoters of these organisms. Compressions of amino acid sequences of RNAP subunits (α, β, β', ω and σ70) of *E. chaffeensis* with those of *E. coli* revealed close similarities. Here, we described the heterologous cell free *in vitro* transcription and transcription coupled translation assays to drive transcription from *E. chaffeensis* promoters. These *in vitro* assays were validated for their use in assessing the activities of the p28-Omp genes 14 and 19 promoters. The molecular assay systems established in this study will be useful in detailed promoter characterization *in vitro* and to identify *E. chaffeensis* regulators that contribute to alterations in gene expression in response to the pathogen's growth in its tick vector and vertebrate hosts.

Introduction

Ehrlichia chaffeensisEhrlichia chaffeensisEhrlichia chaffeensis is a tick-transmitted bacterial pathogen that causes emerging illnesses in people and animals (78,93,94,281,302,427,433). The pathogen requires its adaptation and propagation in a tick vector and vertebrate hosts for the completion of its life cycle (8,79,215,297). Host cell-specific gene expression for several *E. chaffeensis* genes including from the 28 kDa outer membrane protein multigene locus has been reported for this pathogen (221,274,347,352,398). Biological significance of differentially expressed *E. chaffeensis* genes can be better understood by functional disruption of target gene though alteration of its sequences and then studying its impact *in vivo*. Similarly, mutations to regions spanning promoter sequences of a gene of interest may aid in defining role of various regulatory elements that may influence the gene expression. Studies pertaining to introduction of genetic mutations and defining their effect *in vivo* for *E. chaffeensis* is not possible due to the lack of an established genetic transformation system.

Mutational analysis is also challenging for other intracellular bacteria such as *Rickettsia* species and *Chlamydia* species. To overcome this challenge, several studies reported the use of *in vitro* transcription and translation assays (2,22,341,351,375,434). *In vitro* transcription methods were extensively used to map the promoters and regulatory proteins of gene expression for several Chlamydial genes (2,238,239,341,351,375,434). The assays are also useful in mapping differences in Chlamydial gene expression during different developmental stages. *In vitro* transcription assays are similarly used for characterizing several genes of *Rickettsia* species, which are closely related organisms to *Ehrlichia* species (5,89,290,305). Here, we utilized similar strategies to develop *in vitro*

transcription assays for use in mapping the *E. chaffeensis* promoters and regulatory proteins. The establishment of *in vitro* transcription and *in vitro* transcription coupled translation assays are described.

Material and Methods

Bioinformatics analysis of RNA polymerase subunits of *E. coli, E. chaffeensis,* and *C. trachomatis*: To identify sequence homologies protein coding sequences of the RNAP subunits of *E. chaffeensis* (GenBank #CP000236), *E. coli* (GenBank #CP000247) and *C. trachomatis* (GenBank #NC010287) were compared using the GCG program, OldDistances (87).

Assessment of promoter activity *in vitro*: For use in *in vitro* assays, promoter region and reporter gene segments were amplified by PCR using pBlue-TOPO promoter constructs as the templates. The amplicons were then used for *in vitro* transcription reactions. The entire gene 14 promoter region in forward or reverse orientation or a segment lacking a 180 bp portion at the 5' end in forward orientation along with a 301 bp of lacZ gene fragments were amplified from the pBlue-TOPO recombinant plasmid constructs utilizing primers described in Table 5.2. Amplicons containing a shorter gene 14 promoter and 196 bp lacZ segment were also prepared by using a different 3' primer (Table 5.2). Similar strategy was also followed to generate p28-Omp gene 19 promoter region templates for use in *in vitro* transcription analysis. For gene 19 promoter templates, the complete upstream sequence inserted in forward and reverse orientations and a shorter segment in forward orientation which lacks 183 bp from the 5' end were used. The PCR products were purified using the QIAquick PCR Purification Kit (Quiagen, Valencia, CA).

In vitro transcription analysis was performed by following protocol described previously (418) with minor modifications. Briefly, the assays were performed in a 10 µl reaction containing 50 mM Tris-acetate (pH 8.0), 50 mM potassium acetate, 8.1 mM magnesium acetate, 27 mM ammonium acetate, 2 mM dithiothreitol, 400 µM ATP, 400 µM GTP, 400 µM UTP, 1.2 µM CTP, 0.21 µM [α-^{32}P] CTP, 18 U of RNasin, 5% glycerol, 100 ng of purified PCR templates and 0.03 U of *E. coli* RNAP holoenzyme saturated with σ70 (Epicentre, Madison, WI). The reaction was incubated at 37°C for 15 min and then terminated by adding 4 µl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Four micro liters of reaction contents each were resolved in a 6% polyacrylamide gel containing 7 M urea. The gel was transferred to a Whatman paper, dried, and exposed to an X-ray film and the *in vitro* transcripts were detected after developing the film using a Konica film processor (Wayne, NJ).

***In vitro* transcription coupled translation assay:** *In vitro* transcription coupled translation assay was performed using *E. coli* S30 extracts (Promega, Madison, WI). The p28-Omp gene 19 complete upstream sequences in forward orientation plus the complete GFP coding sequence was amplified from a pPROBE-NT recombinant plasmid (described above). PCR products were purified using a QIAquick PCR purification kit (Cat# 28104, Quiagen, Valencia, CA) and the *in vitro* transcription coupled translation reaction was performed in a 50 µl mixture containing 20 µl of S30 linear extracts, 15 µl of S30 premix without amino acids, 5 µl of amino acid mixture and 1.5 µg of DNA template. The assay controls included all of the reagents except amino acid mixture or all of the reagents except S30 extracts or templates. The assays were performed by

incubating at 37°C for 90 min and then the reaction was stopped by transferring the assay mixtures to 4°C. Reaction products were assessed for the presence of GFP by performing Western blot analysis using a GFP polyclonal antibody (Rockland Immunochemicals, Inc., Gilbertsville, PA).

Results

Sequence analysis of RNA polymerase subunits of *E. coli, E. chaffeensis,* and *C. trachomatis*: Promoter analysis of the differentially expressed genes, p28-Omp 14 and 19, in *E. coli* demonstrated that *E. coli* RNAP can recognize and initiate transcription from the *E. chaffeensis* promoters (Discussed in Chapter 4). Amino acid sequences of all four subunits, α, β, β', and σ70, of *E. chaffeensis* RNAP were compared with *E. coli*. The analysis also included the *C. trachomatis* RNAP subunits (Table 5.1) as this is also an intra phagosomal pathogen similar to *E. chaffeensis*. Moreover, *E.coli* similarly recognizes *C. trachomatis* promoters. . The homology between *E. chaffeensis* and *E. coli* RNAP subunits ranged from 56 to 64%, which is grater than that observed for the *C. trachomatis* and *E. coli* RNAP subunits (48 to 62%). The homology between the RNAP subunits of *E. chaffeensis* and *C. trachomatis* (46-59%) is also lesser as compared to the homology observed between *E. chaffeensis* and *E. coli* or *E. coli* and *C. trachomatis* polymerases.

***In vitro* transcription analysis:** Comparative analysis of amino acid sequences reveled that *E.coli* RNAP complex proteins have greater homology with those of *E. chaffeensis*. *E. chaffeensis* promotes are recognized by *E.coli* RNAP *in vivo*. *In vitro* transcription assays were performed using complete promoter regions or a segment of the promoter having similar promoter activity. Predicted *in vitro* transcripts, as estimated from the

transcription start sites mapped by primer extension and RNase protection assays were detected only when the p28-Omp 14 and 19 complete upstream sequences were present in the forward orientation (Figure 5.1A). *In vitro* transcripts were absent for the reactions contained the complete gene 14 and 19 promoter regions ligated in reverse orientation (Figure 5.1A). Similar results were obtained when shorter lac Z segments were used in the analysis with shorter segments of genes 14 and 19 upstream sequences (Figure 5.1B).

***In vitro* transcription coupled translation assay:** To further verify the promoter activity *in vitro*, the full length gene 19 upstream sequences tagged to the complete GFP coding sequence was utilized in the *in vitro* transcription coupled translation assay (Figure 5.2). The *in vitro* translated products were detected, as judged by the presence of GFP using GFP-specific sera, only when the reaction contained all the reagents and the template, but not in the assays that contained all the reagents except amino acids or a DNA template or a reaction containing only the DNA template but no other assay reagents.

Discussion

Studies described in Chapter 3 and our recent proteome analysis demonstrated differential gene expression by *E. chaffeensis*, including from the p28-Omp genes 14 and 19, in a host cell-specific manner (347,352). Data reported in the previous Chapter also demonstrate that deletions from the p28-Omp genes 14 and 19 promoters altered their activities in *E. coli* (Chapter 4). Although it is possible to assess the impact of one protein at a time, the analysis in *E. coli* will not provide the flexibility to assess the contributions of coordinated regulation of multiple *E. chaffeensis* regulatory proteins influencing the gene expression. This can be best addressed using an *in vitro* assay similar to the one described in this study.

Bioinformatics analysis of RNA polymerase subunits of *E. chaffeensis*, *E. coli* and *C. trachomatis* suggested that *E. chaffeensis* has greater homology to *E.coli* RNAP, compared to that of *C. trachomatis*. This is encouraging because *E.coli* RNAP can be used to drive expression from *E. chaffeensis* promoters. *E. coli* RNAP has also been extensively used to understand the regulation of gene expression for several *C. trachomatis* genes (2,90,238,239,341,351,374,375,434). Moreover, recognition of *E. coli* promoters by *C. trachomatis* RNAP has also been reported (239). Considering these data, we anticipated that *E. coli* RNAP can similarly be useful in mapping the regulation of gene expression in *E. chaffeensis*. Indeed our deletion analysis reported in the previous Chapter demonstrates that *E. coli* is a good system to use in mapping *E. chaffeensis* promoters. As reported for *C. trachomatis*, *in vitro* transcription assays may be developed and used to study the activity *in vitro* for *E. chaffeensis* genes of interest. *In vitro* assay will be particularly useful in assessing the impact of host cell environments.

In this study, precisely for this reason, we developed *in vitro* transcription and *in vitro* transcription coupled translation assays using *E. coli* RNAP. The methods described are similar to those reported for *R. prowazekii* and *C. trachomatis* and utilized *E. coli* RNAP. In particular, our studies clearly demonstrated that *in vitro* assays using *E. coli* RNAP identify the same transcription start sites as observed for the transcripts in *E. chaffeensis* (reported in Chapter 3). This is consistent with the reports that *E.coli* RNAP supports transcription, including initiating the transcription from the same transcription start sites as described for *Rickettsia* species and *Anaplasma* species (5,22,89,290,305). These assays can now be used to assess the impact of host cell environments or to study how

various regulatory proteins of *E. chaffeensis* interact with the pathogen's gene promoters. Similar assays have extensively used to study regulatory mechanisms for several *C. trachomatis* genes. *C. trachomatis* studies also compared *in vitro* assays using *E. coli* RNAP and *C. trachomatis* RNAP (2,90,238,239,341,351,374,375,434). *E. coli* RNAP functioned very similar to that of *C. trachomatis* for several *Chlamydia* genes (111,239,375). These observations are encouraging for *E. chaffeensis* studies as at this time we do not have purified or recombinantly expressed *E. chaffeensis* RNAP. However, the *in vitro* methods described here may need to use *E. chaffeensis* RNAP. The efforts to purify *E. chaffeensis* RNA polymerase and recombinantly express various subunits of this enzyme are currently in progress in our laboratory.

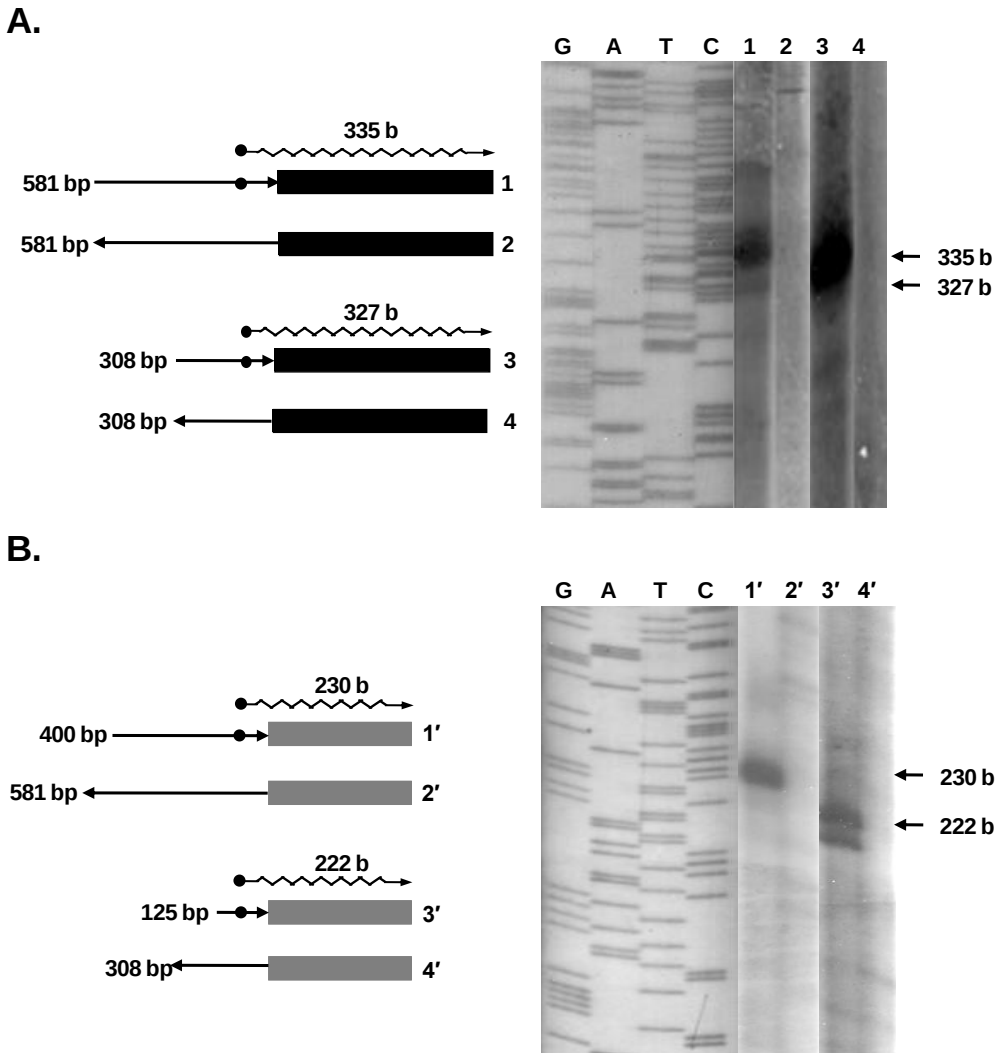


Figure 5.1. *In vitro* transcription analysis. *In vitro* transcription analysis was performed for the A) complete upstream sequences of the genes 14 and 19 in forward and reverse orientations ligated to a lacZ segment (301 bp); B) The analysis was also performed using shorter segments of the gene 14 and 19 upstream sequences ligated to a shorter lacZ segment (196 bp). Genes 14 and 19 sequence segments (straight arrow) and the predicted transcripts (wiggled arrow) were shown as cartoons on the left and the observed transcripts were shown on the right of the panels. Puc18 plasmid DNA was used as the template to generate sequence ladder using the M13 forward primer. (1, 2, 1'

and 2' refer to the constructs for *in vitro* transcription for gene 14 and 3, 4, 3', and 4' refer to *in vitro* transcription templates for gene 19) (Reproduced with permission from *BMC Microbiology, In press*).

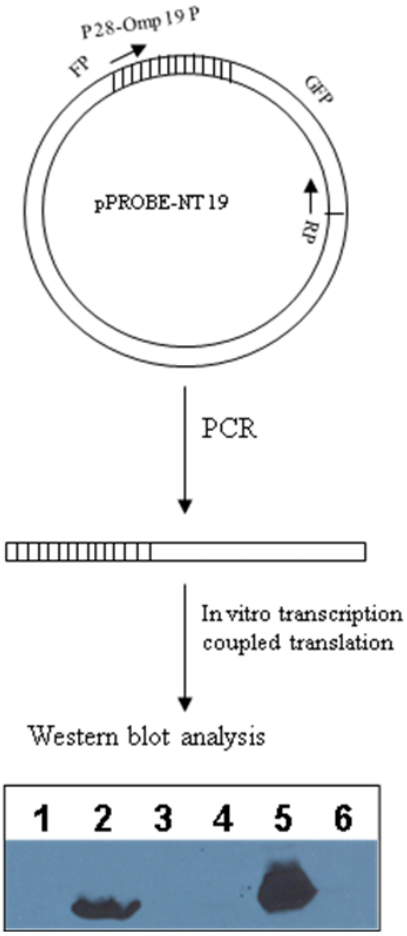


Figure 5.2. *In vitro* transcription coupled translation assay. *In vitro* transcription coupled translation assay was performed using PCR product containing the gene 19 upstream sequence with the complete GFP coding sequence. FP, forward primer; RP, reverse primer. Lanes: 1, template with reagents only for transcription using *E. coli* RNAP; 2, template with transcription and translation assay reagents; 3, contained only the template, but no enzyme and reagents were added; 4, contained all of the assay reagents, but no template was added; 5, lysates prepared from *E. coli* transformed with pPROBE-NT plasmid containing the complete gene 19 upstream sequences in forward orientation (positive control); 6, lysates prepared from *E. coli* containing the pPROBE-NT plasmid alone (negative control).

α subunit

	E. coli	*E. chaffeensis*	*C. trachomatis*
E. coli	1.0000	0.5593	0.4802
E. chaffeensis		1.0000	0.4633
C. trachomatis			1.0000

β subunit

	E. coli	*E. chaffeensis*	*C. trachomatis*
E. coli	1.0000	0.6401	0.6158
E. chaffeensis		1.0000	0.5895
C. trachomatis			1.0000

β' subunit

	E. coli	*E. chaffeensis*	*C. trachomatis*
E. coli	1.0000	0.6411	0.6067
E. chaffeensis		1.0000	0.5931
C. trachomatis			1.0000

ω subunit

	E. coli	*E. chaffeensis*	*C. trachomatis*
E. coli	1.0000	0.4066	-
E. chaffeensis		1.0000	-
C. trachomatis			-

σ70 subunit

	E. coli	*E. chaffeensis*	*C. trachomatis*
E. coli	1.0000	0.5628	0.5201
E. chaffeensis		1.0000	0.5131
C. trachomatis			1.0000

Table 5.1: RNA polymerase subunits' comparisons were made using the GCG programs PileUp and OldDistances. Similarity values shown in the Table are the number of matches between each sequence pair divided by the length of the shorter sequence.

Chapter 6

Promoter DNA interactions of the *Ehrlichia chaffeensis* p28-Omp gene 14 and 19 Promoters

Abstract

Host-specific gene expression including from the p28-Omp genes 14 and 19 by *E. chaffeensis* has been reported during the pathogen's growth in tick and vertebrate host cells, respectively. Analysis of the p28-Omp genes 14 and 19 promoters of *E. chaffeensis* identified several putative regulatory sequences that influenced the promoter activity. Identification of proteins that interact with these putative regulatory elements of the p28-Omp genes 14 and 19 promoters will be important to map gene regulatory mechanisms of *E. chaffeensis*. In the current study, electrophoretic mobility shift assays (EMSA) were performed utilizing protein lysates of cell free *E. chaffeensis*, to evaluate their interaction with the promoters of the p28-Omp 14 and 19 genes. The protein extracts were prepared from the tick cell- and vertebrate macrophage-grown *E. chaffeensis* organisms. The EMSA experiments demonstrated the interaction of *E. chaffeensis* proteins with promoter sequences of both the p28-Omp 14 and 19 genes. Sequence analysis of *E. chaffeensis* *g*enome identified five open reading frames that encode for putative regulatory proteins. These proteins were recombinantly expressed in *E. coli* and their interactions with the p28-Omp genes 14 were evaluated. The analysis revealed a minor trend for decline in the gene 14 promoter activity, although these changes were not statistically significant ($P \geq 0.05$). More detailed investigations are needed to map regulation of the p28-Omp genes 14 and 19 promoters of *E. chaffeensis*.

Introduction

Ehrlichia chaffeensisEhrlichia chaffeensisEhrlichia chaffeensis, similar to other tick-transmitted pathogenic bacteria, requires its survival and persistence in tick vector and vertebrate hosts for its continued maintenance in nature. To date, strategies employed by *E. chaffeensis* for its dual host adaptation and persistence are not known. Differential host cell-specific expression for several *E. chaffeensis* genes including two outer membrane protein genes (the p28-Omp 14 and 19) is well documented (discussed in Chapter 3; Sirigireddy and Ganta unpublished data, 2007) (347,352). The molecular mechanisms employed by *E. chaffeensis* to achieve host cell-specific gene regulation remain unclear. Bioinformatics analysis of the p28-Omp genes 14 and 19 promoters identified several promoter-specific putative regulatory sequences. Deletion analysis involving the elimination of one or more of the putative regulatory sequences has been shown to alter the activity of p28-Omp genes 14 and 19 promoters (reported in the Chapter 4). These findings suggest that the host-specific expression of the p28-Omp 14 and 19 genes may be regulated with the involvement of these sequences.

Pathogenic organisms adapt to changes in the environment they reside by sensing those signals and by modulating their gene expression accordingly. Regulatory proteins are shown to play an important role in the process of altering the gene expression in support of pathogen's survival and persistence in a host (15,37,63,67,166,197,224,295,308,417). For example, studies in *Anaplasma phagocytophilum*, a close relative of *E. chaffeensis*, identified a transcription regulator (ApxR) that has a regulatory role in p44-Omp genes transcription (408). Involvement of a two component regulatory system comprising of two alternate sigma factors and a

response regulatory protein in the regulation of two host-specifically expressed outer membrane proteins in *B. burgdorferi* is well documented (35,165,430). In *Chlamydia trachomatis*, an intraphagosomal organism, developmental stage-specific gene regulation is accomplished by involvement of various transcription regulators (27,197,423). Thus, identification of *E. chaffeensis* proteins that interact with the promoters of host-specifically expressed genes will provide important insight into mechanisms of gene regulation by this bacterium.

We hypothesized that the differential gene expression in *E. chaffeensis* is accomplished by the involvement of regulatory proteins made by the pathogen in response to the hose cell signals. In the current study, we performed electrophoretic mobility sift assay (EMSA) to examine if any of the *E. chaffeensis* proteins interact with the p28-Omp genes 14 and 19 promoters. We also recombinantly expressed five putative regulatory proteins of *E. chaffeensis* and their interaction with the p28-Omp gene 14 promoter was tested.

Materials and Methods

***E. coli* strains:** XL1Blue and BL21 (DE3) strains of *E. coli* were obtained from commercial vendors (Stratagene, La Jolla, CA; Novagen Inc., Madison, WI; respectively). XL1-Blue strain of *E. coli* were cultured in Luria-Bertani (LB) liquid medium or agar plates in the presence of tetracycline antibiotic, whereas, BL21 (DE3) cells were grown in plain LB medium or agar plates without any antibiotic.

Preparation of *E. chaffeensis* soluble protein lysates: *E. chaffeensis* organisms were cultivated *in vitro* in canine macrophage (DH82) cell lines at 37°C by following the protocols described earlier (61). Twenty five ml of about 80-100% *E. chaffeensis* infected macrophage cultures were harvested with the help of glass beads. The cultures were centrifuged at 15,560 x *g* for 15 min to recover infected host cells and cell free *Ehrlichia*. To release *Ehrlichia* organisms from host cells, the cell pellet was resuspended in 10 ml SPK buffer (0.5 M K_2HPO_4, 0.5 M KH_2PO_4, and 0.38 M sucrose) and sonicated twice for 30 sec at a setting of 6.5 in a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA). To collect the host cell debris, the cell lysates were centrifuged at 400 x *g* for 5 min at 4°C. The supernatant containing the cell free *E. chaffeensis* was collected and filtered through a 5 μm and 3 μm sterile isopore membrane filters (Millipore, Billerica, MA). The filtrate was collected and cell free organisms were concentrated through centrifugation at 15,560 x *g* for 15 min at 4°C. The resulting cell pellet containing the *E. chaffeensis* organisms was washed twice with 1.5 ml of lysis buffer (150 mM Tris-HCl pH 8.0, 100 mM KCl, 10 mM Magnesium Acetate, 1 mM EDTA, 2 mM DTT and 10% glycerol) and the pellet was resuspended in 1ml of lysis buffer containing protease inhibitors (Roche Diagnostic Labs, Indianapolis, IN). The cell suspension was sonicated four times at 8.5 setting, 30 sec each time to lyse the *E. chaffeensis* organisms. The cell lysates were centrifuged at 15,560 x *g* for 15 min at 4°C to pellet out the insoluble fractions and the supernatant containing soluble proteins of *E. chaffeensis* was collected into sterile micro centrifuge tubes as 25 μl aliquots and stored at -80°C until use.

Electrophoretic mobility shift assay (EMSA): The full-length promoter fragments of the p28 Omp 14 and 19 genes were released from the pBlueTOPO recombinant plasmids (described in Chapter 4) by performing HindIII restriction enzyme digestion. The resultant promoter fragments containing 5' overhang were end-labeled with Klenow DNA polymerase (Promega; Madison, WI) using α-^{32}p [dATP] by performing fill in reaction. Binding of *E. chaffeensis* soluble proteins (5 μg) with 1 ng of the end-labeled p28-Omp genes 14 and 19 promoter fragments were performed in a 20 μl reaction by following previously established protocols (321). Fifty nano grams of unlabelled (cold) DNA was used to serve as a competitor in binding reactions. Ten microliters of the assay products from each reaction were resolved on a 3.5% nondenaturing polyacrylamide gel by subjecting to 250V current for 90 min. The gel was dried and exposed to X-ray film for at least 24 h at −80°C and the film was developed using Konica film processor (Wayne, NJ).

For use in subsequent EMSA experiments, partial promoter fragments that contained one or more putative regulatory sequences (discussed in Chapter 4) were amplified using sequence specific forward primer and 5' end biotin labeled reverse primer listed in Table 6.1. About 1 ng of biotin label containing partial promoter fragments were evaluated for their binding ability in the presence of 5 μg *E. chaffeensis* soluble proteins prepared from macrophage derived bacteria. About 50 ng of unlabeled gene 14 or 19 full length promoter DNA was used as a competitor in binding reactions. The binding experiments were performed utilizing LightShift Chemiluminescent EMSA Kit containing streptavidin-HRP conjugate and by following the manufacturer's instructions (Cat # 20148, Pierce Biotechnology, Rockford, IL).

Genome analysis: *E. chaffeensis* whole genome sequence was obtained from GenBank (accession number, CP000236) and searched for the presence of genes that encode for putative regulatory proteins (162).

Oligonucleotides: The oligonucleotides used in this study were custom synthesized from a commercial vendor (Integrated DNA Technologies, Coralville, IA). The oligonucleotides used for amplifying the p28-Omp genes 14 and 19 partial promoter fragments were listed in Table 6.1. The primers used to amplify the putative regulatory coding sequences were listed in the Table 6.2.

Cloning and expression of putative regulatory proteins of *E. chaffeensis*: Coding sequences of putative regulatory sequences were amplified utilizing sequence specific forward and reverse primers listed in Table 6.2. The forward and reverse primers were engineered to include NcoI and XhoI restriction enzymes sites at their 5' ends, respectively. This is to aid in the directional cloning into the pET32a (+) expression vector (Novagen Inc., Madison, WI). The PCR amplicons and pET32 vector were digested with NcoI and XhoI restriction enzymes and recombinant clones were generated by following the standard molecular cloning procedures (336) (outlined in Figure 6.1). Initial amplification of the recombinant plasmids was performed in XL1-Blue strain of *E. coli* (Stratagene, La Jolla, CA) and recombinant proteins were expressed after transforming the plasmids into in BL21 (DE3) strain of *E. coli* (Novagen Inc., Madison, WI).

Sodium dodecyl sulfate polyacrylamide gel electrophorosis (SDS PAGE): BL21 (DE3) cells containing the recombinant pET32 plasmids were grown in LB medium containing ampicillin (50 μg/ml) to 0.6 OD (at 562 nm) and the recombinant protein expression was induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). To assess the expression of recombinant proteins, total protein lysates were prepared from *E. coli* cultures obtained at various time points (0h, 1h, and 3h) following induction with IPTG. Approximately, 10% of the protein preparations were resuspended in 1x SDS poly acrylamide gel electrophorosis buffer and resolved on a 12% SDS PAGE gel by subjecting to 90V current for 60 min. To visualize the presence of recombinant protein, the gels were stained with 0.01% Coomassie G-250 stain (J. T. Baker Chemical Co., Phillipsburg, NJ), prepared in 50% methanol and 10% acetic acid. Excess stain was removed by de-staining with a solution containing 10% methanol and 1.4% acetic acid. The presence of recombinant proteins was assessed by comparing proteins made from non recombinant pET32 plasmids in *E. coli*.

Preparation of the p28-Omp 14 promoter-lacZ constructs: Full length lacZ coding sequence along with the p28-Omp gene 14 promoter was obtained after Pme I restriction enzyme digestion of pBlueTOPO recombinant plasmid containing p28-Omp gene 14 promoter clones (described in Chapter 4). The Pme I restriction enzyme sites, located at both the 5' end of the p28-Omp gene 14 promoter and 3' end of the lacZ coding sequence in the pBlue-TOPO recombinant plasmid (Figure 2). The promoter lacZ fragments were then cloned into EcoRV site of pACYC184 plasmid (New England Biolabs, Beverly, MA) as both Pme I and EcoRV digestion produce blunt ended products. Figure 6.2 illustrates the molecular cloning procedure and construction of recombinant plasmid.

The recombinant clones were selected after transforming into XL1-Blue strain of *E. coli* by following the standard molecular procedures (336). The recombinant pACYC184 plasmids containing the p28-Omp gene 14 promoter and lacZ fragments (pACYC184-14F-lacZ) were verified by restriction enzyme digestion analysis.

Co-transformation: BL21 (DE3) strain of *E. coli* containing the pET32 plasmid alone or containing putative regulatory protein coding sequences were transformed with pACYC 184 promoter-lacZ fusion plasmids. To allow the plasmids compatibility for co-expression in the same *E. coli* host, the vectors for cloning the desired inserts were selected to contain different origins of replication and having different antibiotic resistance genes (382). *E. coli* clones expressing both the recombinant plasmids were selected after confirming the resistance to ampicillin and chloramphenicol antibiotics. Figure 6.3 describes the strategy used to clone two plasmids into *E. coli*. The presence of the both plasmids was verified after isolating the plasmid DNA and subsequent evaluation by agar gel electrophorosis and restriction enzyme digestion analysis.

Evaluation of β-galactosidase enzyme activity in *E. coli* clones expressing the recombinant regulatory proteins: *E. coli* transformants containing both the recombinant plasmids (pET32 and pACYC184-14FlacZ) were cultured overnight in the LB medium containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol antibiotics. The following day, *E. coli* transformants were subcultured in the presence of antibiotics to 0.5 OD (at 562 nm) and recombinant putative regulatory proteins expression was induced using 1 mM IPTG at 37°C for 2 h. Following induction, β-galactosidase activity

of soluble protein lysates prepared from the *E. coli* cultures was assessed by following the methods described in the Chapter 4.

Statistical analysis: Statistical analysis for the promoter activity data were performed using repeated measures of analysis of variance method utilizing GraphPad InStat Software (La Jolla, CA). Multiple comparisons were adjusted by Bonferroni method. *P*-value of ≤ 0.05 was considered significant.

Results

Electrophoretic mobility shift analysis of complete p28-Omp 14 and 19 genes promoter regions: Promoter deletion analysis described in the previous Chapter demonstrated the presence of several putative DNA regulatory sequences, which influenced the promoter activity of the p28-Omp genes 14 and 19 (discussed in the Chapter 4). Electrophoretic mobility shift assay was performed with full length promoter sequences of these two genes in the presence of *E. chaffeensis* soluble proteins prepared from macrophage and tick cell-derived organisms. Both the gene 14 and 19 promoter probes migrated at a slower rate in the presence of 5 μg of macrophage- or tick cell-derived *E. chaffeensis* proteins (Figure 6.4; A, lanes 1, 3 and B, 2, and 4) compared to control reactions that contained probe alone (Figure 6.4; A, lane 5 and B, lane 1). The shift in probe migration was abolished when 50 ng of unlabelled promoter probe was added as a competitor (Figure 6.4; A, lanes 2, 4 and B, 3, 5).

EMSA utilizing short promoter segments of the p28-Omp genes 14 and 19 promoters: EMSA experiments utilizing the complete promoter regions of the p28-Omp genes 14 and 19 of *E. chaffeensis* revealed promoter-specific binding of tick cell or

macrophage-derived *Ehrlichia* proteins. The probes used for the above described EMSA experiments included sequences for RNA polymerase binding sites. To assess the impact of various putative regulatory sequences present upstream to the RNA polymerase binding sites, six biotin labeled short promoter fragments (P1-P6) for the p28-Omp genes 14 and 2 for gene 19 (P7-P8) were utilized in the binding assays (Figure 6.5, A and B). All 6 probes for the p28-Omp gene 14 promoter exhibited a slower migration in the presence of *E. chaffeensis* proteins compared to probes migration in the absence of *Ehrlichia* proteins or in the presence of non-specific protein, bovine serum albumin (BSA) (Figure 6.6, A-F). Addition of 50 fold excess of unlabeled full length promoter DNA in the binding reactions significantly reduced the shift in the probes migration (Figure 6.6, A-F). Similarly, both the p28-Omp gene 19 promoter probes migrated at a slower rate in the presence of *E. chaffeensis* proteins compared to migration of probe alone, or probe in the presence of BSA (Figure 6.6, G-H). Addition of 50 ng competitor DNA also significantly abolished the shift in probe 8 migration but not for probe 7 (Figure 6.6, G-H).

Genome analysis: Gel mobility shift experiments demonstrated the binding of *E. chaffeensis* proteins to both the p28-Omp gene 14 and 19 promoter sequences. *E. chaffeensis* genome was searched to identify open reading frames that encode for putative regulatory proteins (162). The analysis aided in identification of five putative regulatory protein coding sequences, which included; a transcription regulator of MerR family (GenBank accession number, YP_506990.1), a DNA binding response regulator (GenBank accession number, YP_507798.1), a putative transcriptional regulator (tr1) located upstream to the p28-Omp multigene locus (GenBank accession number,

YP_507902.1), a DNA-binding histone like protein, HU (GenBank accession number, YP_507602.1), and a homolog to the transcription regulator of *A. phagocytophilum*, ApxR (GenBank accession number, YP_507593.1) (115,162,408) (Table 6.3).

Expression of recombinant putative regulatory proteins of *E. chaffeensis*: The putative regulatory protein coding sequences were cloned into pET32 expression vector and the expression of recombinant proteins in *E. coli* was assessed by SDS PAGE analysis (Figures 6.7, 6.8, and 6.9). The expression of recombinant proteins was visible in total *E. coli* cell lysates for all five clones from 1 and 3 h of IPTG induction. No recombinant protein expression was observed in the absence of IPTG induction. IPTG induction of *E. coli* transformants that contained pET32 plasmid without any insert expressed ~20 kDa histidine-tagged thioredoxin protein, which served as a control for comparison of other recombinant proteins (Figures 6.7, 6.8, and 6.9). The predicted recombinant proteins size for the genes YP_506990.1 (ECH_0163), YP_507798.1 (ECH_1012), YP_507902.1 (ECH_1118), YP_507602.1 (ECH_0804), and YP_507593.1 (ECH_0795) are 29.5 kDa, 45 kDa, 39.2 kDa, 26.2 kDa, and 27.7 kDa, respectively. Two of the five clones were expressed predominantly as inclusions (YP_507798.1, YP_507602.1), while the remaining three were expressed in both soluble and insoluble forms (YP_506990.1, YP_507902.1, YP_507593.1).

Co-transformation: Interaction of a putative regulatory protein with a gene promoter can be tested by inducing its protein expression in *E. coli* containing recombinant promoter plasmid and then assessing its impact by measuring the reporter gene expression driven by that promoter (115,197,408). BL21 (DE3) strain of *E. coli*

transformants containing the pET32 alone or recombinant plasmids expressing putative regulatory protein were assessed after transforming with a second plasmid, pACYC184-14F-lacZ, containing *E. chaffeensis's* p28-Omp gene 14 promoter driving the expression of lacZ. This is to examine if the regulators have a role in influencing the p28-Omp gene 14 promoter activity. *E. coli* containing two plasmids was confirmed by growing in presence of both ampicillin and chloramphenicol antibiotics. The presence of double plasmids was further verified by agarose gel electrophorosis (Figure 6.10) and restriction enzyme analysis.

Induction of recombinant putative regulatory protein expression and assessment of β-galactosidase assay: The effect of putative regulatory proteins on the p28-Omp gene 14 promoter activity to drive the lacZ gene expression was assessed by measuring the β-galactosidase specific activity. The clones expressing regulatory proteins had slightly lower β-galactosidase activity compared to controls containing gene 14 promoter plasmid (pACYC184-14F-lacZ) co-transformed with the non-recombinant pET32 plasmid (Figures 6.11, A-E). A reduction in the β-galactosidase activity in the presence of putative regulators ranged from 9-23%. This reduction is consistent with both IPTG induced and non-induced samples. However, the decline the β-galactosidase activity was not statistically significant (n=3, $P \geq 0.05$).

Discussion

Transcriptional differences in the p28-Omp genes 14 and 19 of *E. chaffeensis* in response to host cell environment was described in detail in the Chapter 3. Bioinformatics analysis of the p28-Omp genes 14 and 19 promoter sequences and their

subsequent deletion analysis in *E. coli* suggested the presence of regulatory domains that influenced the promoter activity (Chapter 4). These findings suggest that *E. chaffeensis* proteins may interact with these putative regulatory domains and influence the gene expression in a host cell-specific manner. Identification of the proteins interacting with the promoters of p28-Omp 14 and 19 genes and mapping their regulatory role will be important in defining pathogen's molecular strategies to achieve its dual host adaption and persistence.

In this study, EMSA experiments were performed to determine binding of *E. chaffeensis* proteins to the promoter sequences of p28-Omp 14 and 19 genes. A shift in the probe migration is anticipated when complete promoter regions are used in the gel mobility shift assay, as *E. chaffeensis* RNA polymerase (RNAP) is expected to bind to both the p28-Omp genes 14 and 19 promoter regions. Consistent with this hypothesis, we did observe shift for both genes 14 and 19 complete promoter sequences. We also anticipate a shift when there is a binding of *E. chaffeensis* regulatory proteins to sequences within the gene 14 and 19 promoter regions upstream to RNAP binding regions. In subsequent experiments, short fragments of *E. chaffeensis* p28-Omp genes 14 and 19 promoters spanning one or more putative regulatory sequences, but lacking RNA polymerase binding regions, were utilized. All the gene 14 and 19 promoter probes interacted with *E. chaffeensis* proteins as evidenced by shift of probes in EMSA experiments. The probes shift is specific to *Ehrlichia* proteins as addition of unlabelled specific competitor DNA in 50 fold excess abolished the shift and non-specific protein, BSA, did not cause any shift. These results indicate that one or more of *E. chaffeensis*-specific proteins interact with its promoters and they may possibly influence promoter

activity. This is consistent with other rickettsial pathogens, including *E. chaffeensis*, where the regulatory proteins have been shown to interact with the promoters of several genes (63,115,380,408). More detailed experiments are needed to identify the specific proteins involved in causing the shift in *E. chaffeensis* promoter probes migration in a gel.

Establishing the identity of *E. chaffeensis* proteins interacting with the gene 14 and 19 promoters is somewhat challenging as it may require purification of large quantities of *E. chaffeensis* proteins that are binding to the promoters. Another approach to test the interaction of *E. chaffeensis* proteins with its promoters is to evaluate the interaction of or more putative regulators, which are likely candidates for gene regulation. In this study, we took the later approach, where we searched the entire genome of *E. chaffeensis* for the presence of genes that encode for putative regulatory proteins. Our analysis identified five genes encoding putative regulatory proteins sequences. One of them is a transcription regulator (tr1) (YP_507902.1) located upstream to the p28-Omp locus of *E. chaffeensis* (162), whose homolog was also found in *E. canis*, *E. ruminantium*, *A. phagocytophilum*, and *A. marginale* pathogens (212,218,272,402). This is a logical choice to include in our analysis as it is located upstream to the p28-Omp multigene locus. Similarly, *E. chaffeensis* homolog (YP_507593.1) of *A. phagocytophilum* transcription regulator, ApxR, which has been recently reported to have a regulatory role in tr1 expression and p44 mRNA expression (115,408) was also included in this analysis. In *C. trachomatis*, a homolog of *A. phagocytophilum* ApxR, CpxR, was demonstrated to have a regulatory role in the pathogen's developmental stage-specific gene expression (197). Other *E. chaffeensis* putative regulatory genes included

in this analysis are; a MerR family transcription regulator (YP_506990.1), histone-like DNA binding protein (YP_507602.1), and a putative response regulator DNA binding protein (YP_507798.1).

Influence of these five putative regulatory proteins on the p28-Omp gene 14 promote activity was tested by following methods previously described for *A. phagocytophilum* and *C. trachomatis* (197,408,408,409). Our analysis in *E. coli* identified a minor decline in gene 14 promoter activity, which ranged from 9-23%. This decline is, however, statistically not significant ($P \geq 0.05$). It is possible none of these proteins may have a regulatory role in the p28-Omp gene 14 expression. Alternatively, coordinated binding or interaction of two or more of the putative regulatory proteins may be required for the gene 14 regulation. It is also possible that there may be yet unidentified *E. chaffeensis* proteins that influence the p28-Omp gene 14 promoter activity. The effect of these proteins on gene 19 promoter activity remains to be studied. Additional experiments are needed to map the regulatory proteins that are binding to the promoter regions of p28-Omp genes 14 and 19 promoters. As discussed previously, use of short promoter fragments containing putative regulatory sequences in binding experiments and protein purification using affinity column chromatography and mass spectrometry methods may aid in identification of *E. chaffeensis* proteins interacting with these promoter sequences. Subsequent analysis of bound *E. chaffeensis* proteins by mass spectrometry methods will help in identification of these proteins.

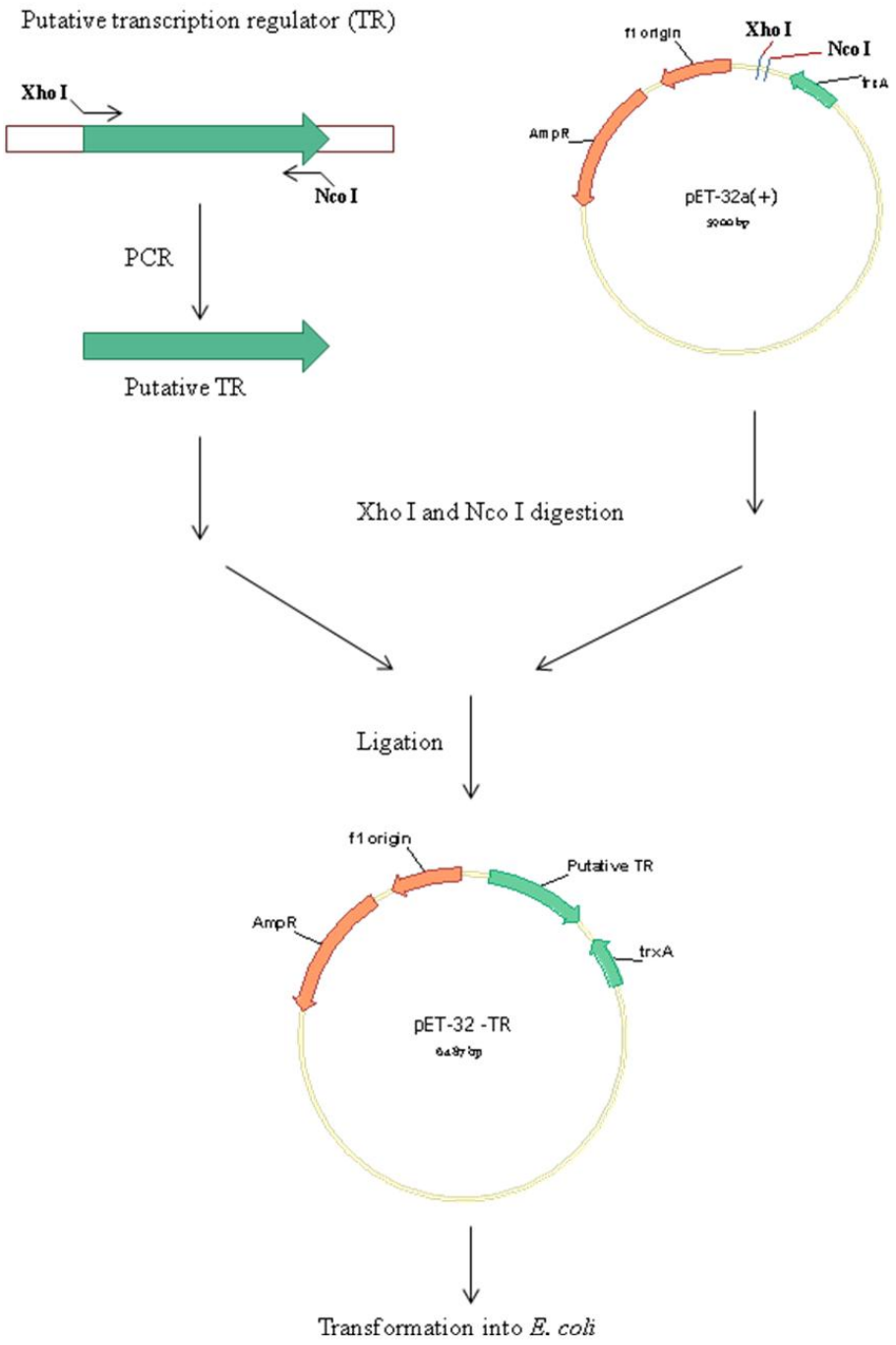


Figure 6.1. Cloning of putative transcription regulator coding sequence into pET 32 a (+) expression vector

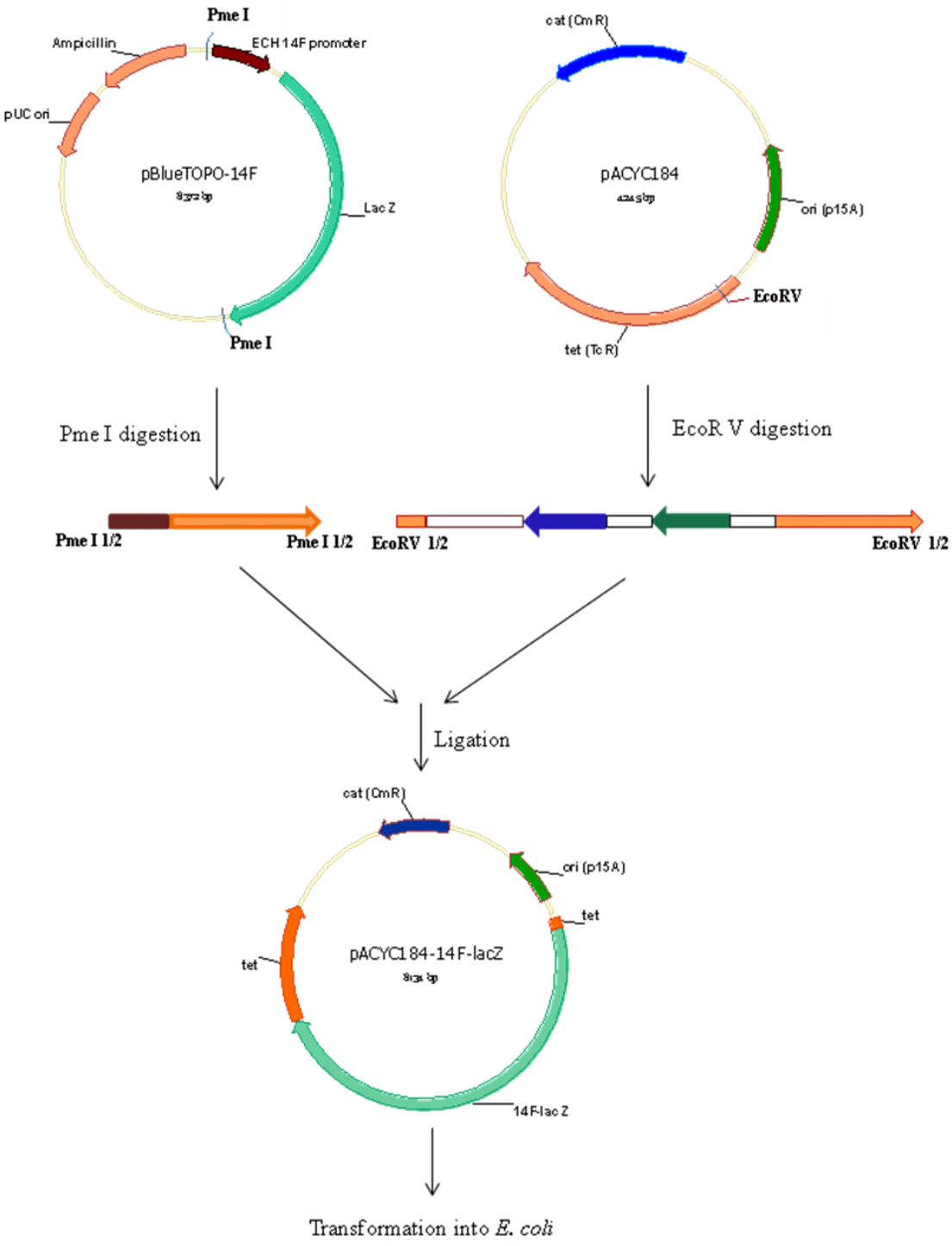


Figure 6.2. Construction of lacZ and p28-Omp 14 promoter fusion plasmids

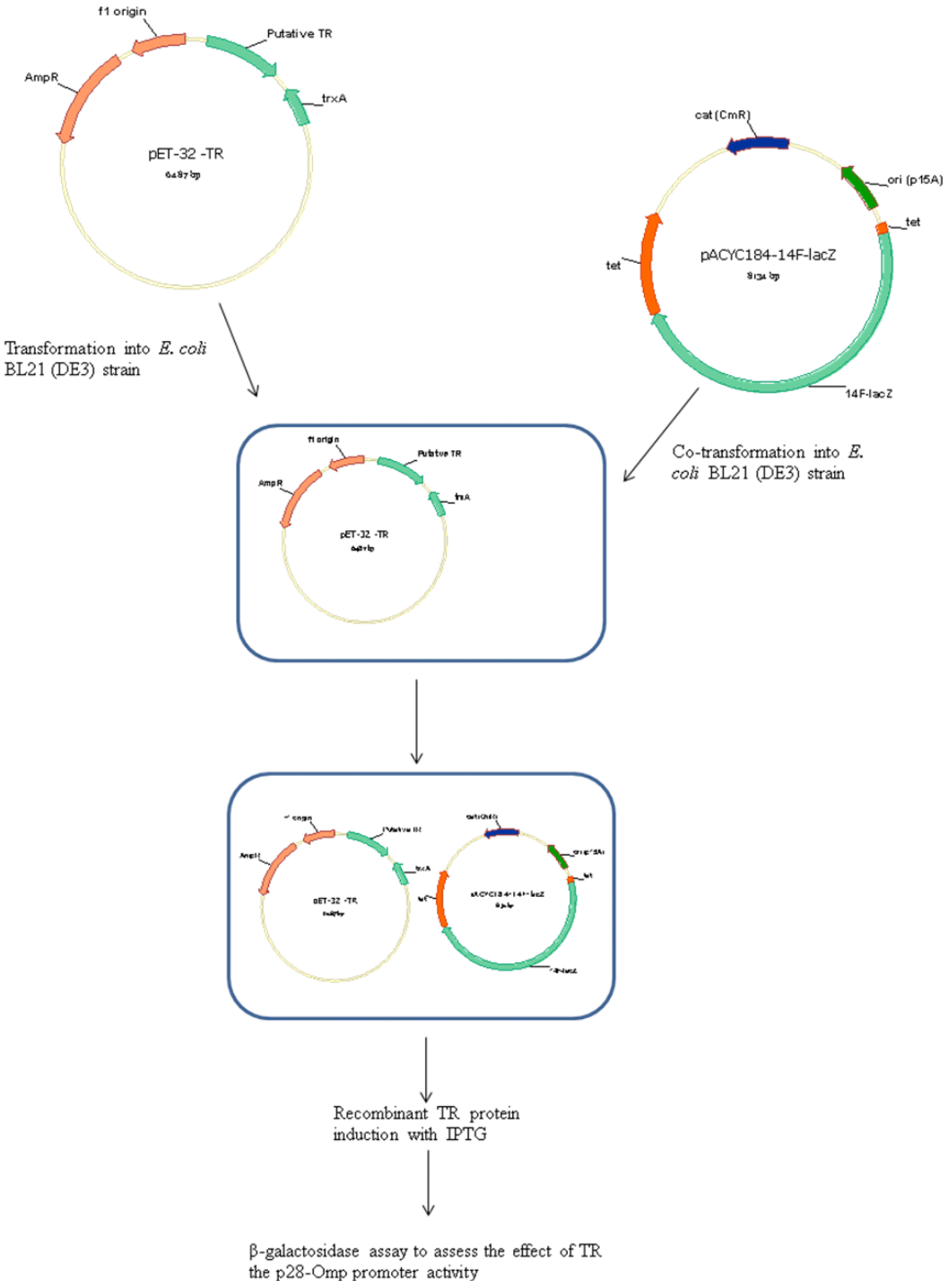


Figure 6.3. Co-transformation of recombinant pET32 and pACYC 184 plasmids

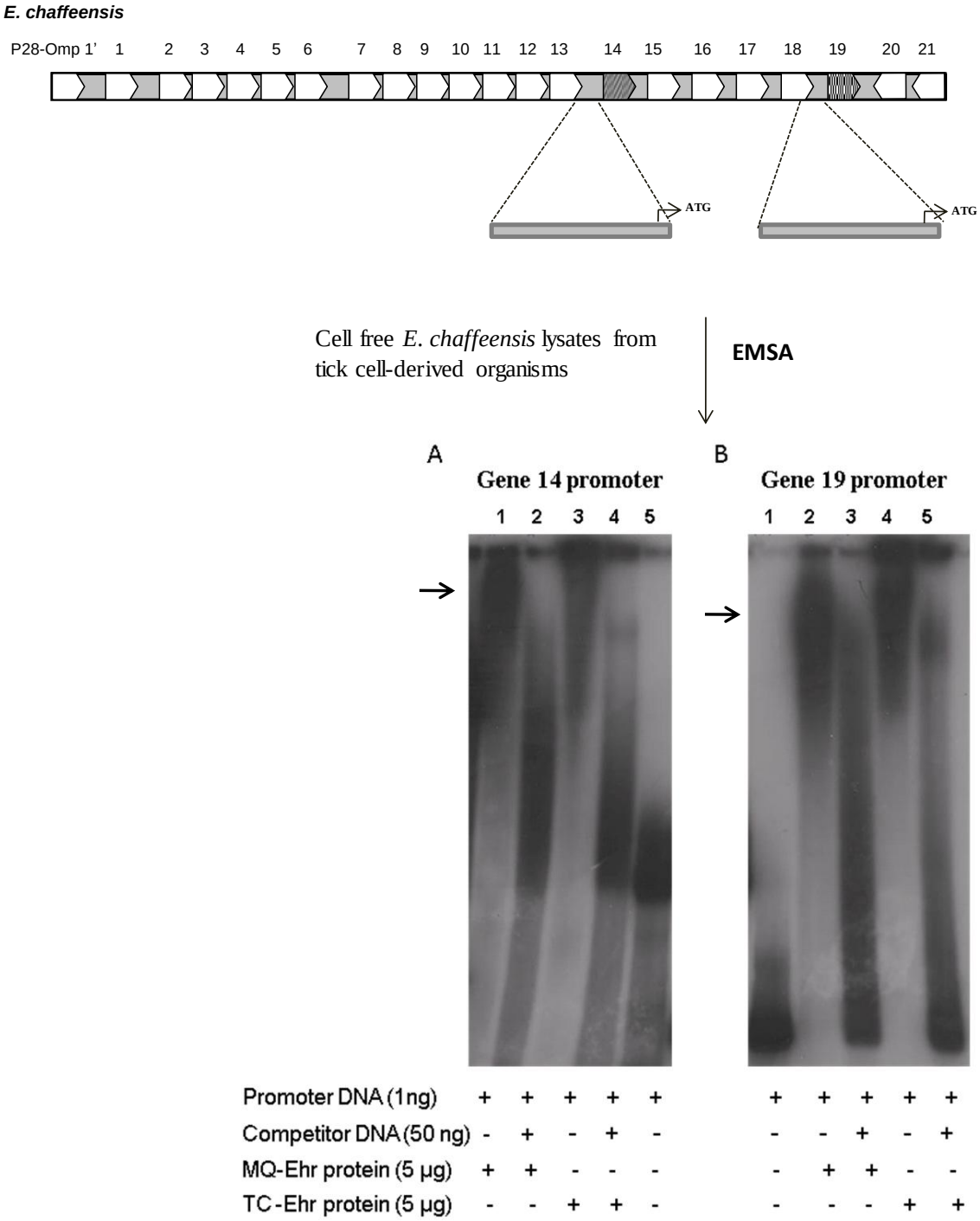
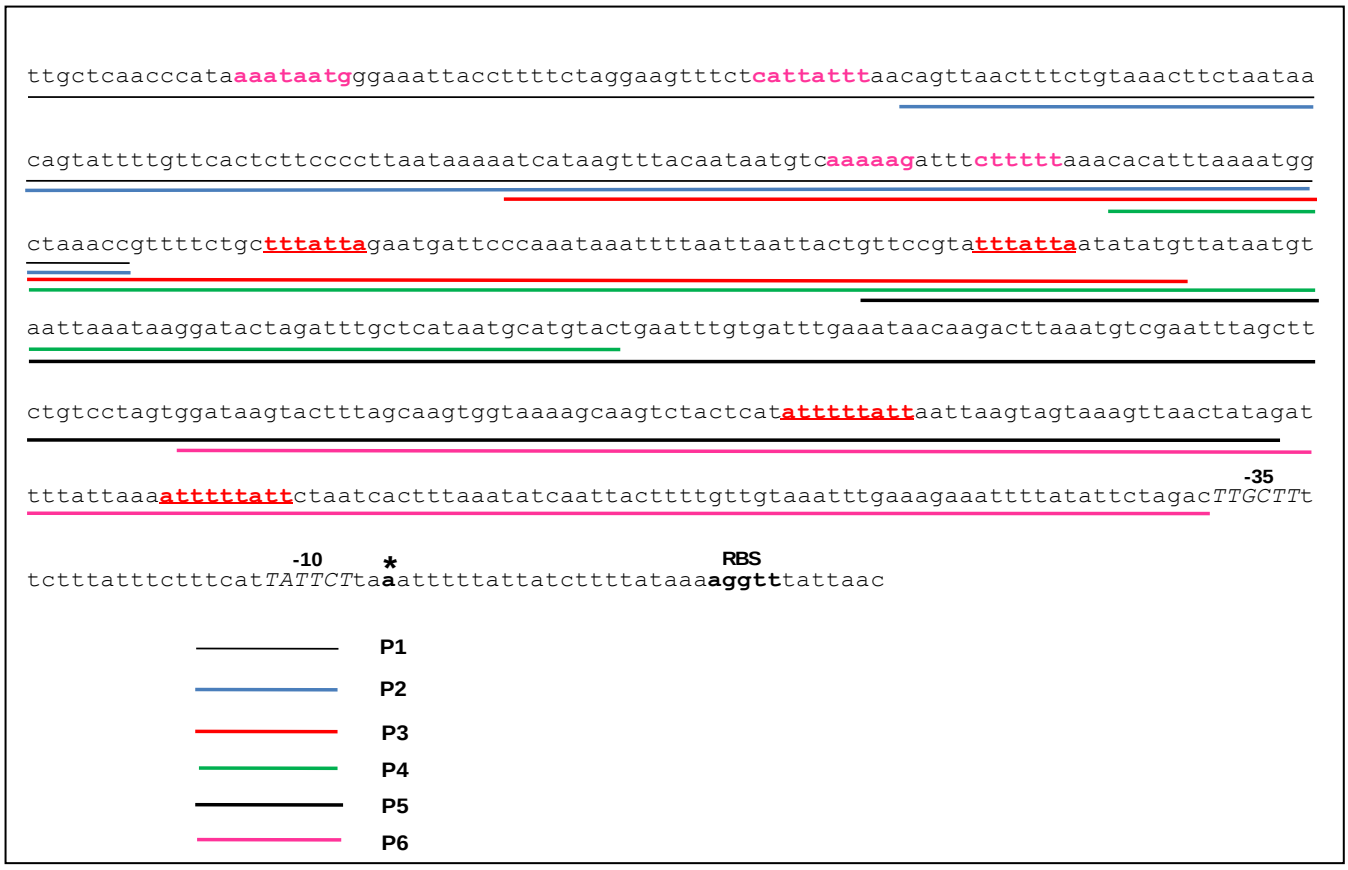


Figure 6.4. Electrophoretic gel mobility shift assay: Binding assay was performed with p28-Omp gene 14 and 19 full length promoter sequences in the presence of *E. chaffeensis* soluble proteins. A, Lanes 1 to 5 contained 1 ng of 32p-labeled, p28-Omp

gene 14 complete promoter fragments; B, lanes 1 to 5 contained 1 ng of 32p-labeled, p28-Omp gene 19 complete promoter fragments. Shifted probe in the presence of *E. chaffeensis* proteins is indicated with arrows. MQ-Ehr protein, macrophage derived *E. chaffeensis* proteins; TC-Ehr protein, tick cell derived *E. chaffeensis* proteins. Addition of *E. chaffeensis* proteins and unlabeled promoter DNA as a competitor are indicated in captions at the bottom of the Figure.

A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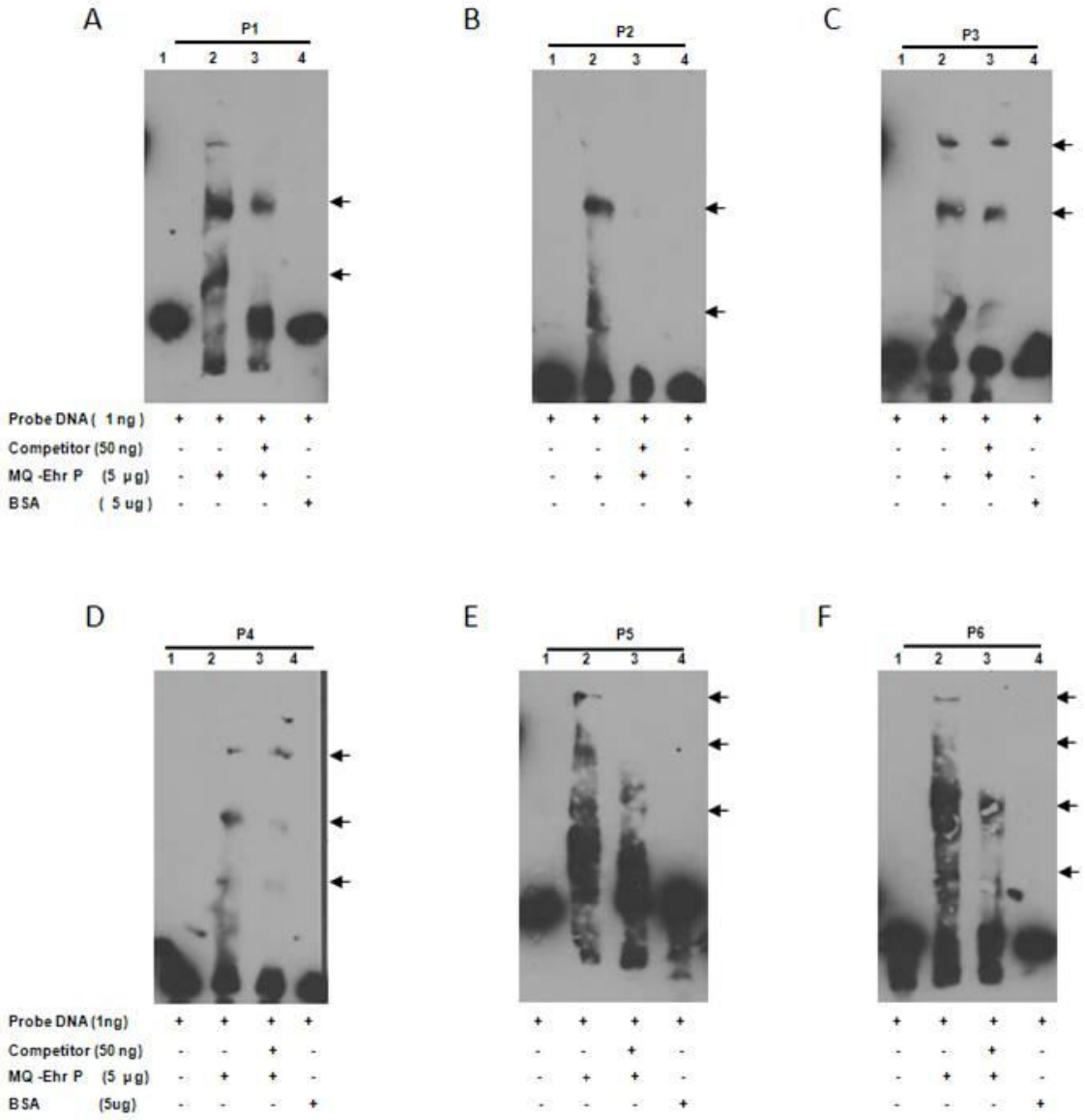
cagtattttgttcactcttccccttaataaaaatcataagtttacaataatgtcaaaaagatttctttttaaacacatttaaaatgg
ctaaaccgttttctgctttattagaatgattcccaaataaatttttaattaattactgttccgtatttattaatatatgttataatgt
aattaaataaggatactagatttgctcataatgcatgtactgaatttgtgatttgaaataacaagacttaaatgtcgaatttagctt
ctgtcctagtggataagtactttagcaagtggtaaaagcaagtctactcatatttttattaattaagtagtaaagttaactatagat
tttattaaaatttttattctaatcactttaaatatcaattacttttgttgtaaatttgaaagaaattttatattctagacTTGCTTt
-35
-10
*
RBS
tctttatttctttcatTATTCTtaaattttttattatctttttataaaaggttattaac
P1
P2
P3
P4
P5
P6

B


ttttattattgccacatgttaaaaataatctaaacttgtttttattattgctgcaggtaaataaaaatagtggcaaaagaatgtagc
aataagaggggggggggggggactagtttataagtgctgtttttctcacctttacacatgatactatacttaaccagttttttgct
attacttacctgacgtaatatattaaattttccttacaaaagttaccgatattttatacaaaaatttatattctgacTTGCTTttat
-35
-10
*
RBS
atgacacttctacTATTGTtaatttatttgtcactattaggttatat
P7
P8

Figure 6.5. Sequences of the p28-Omp genes 14 and 19 partial promoter probes utilized in the EMSA experiments: Biotin labeled **s**hort probes spanning the promoter sequences containing one or more putative regulatory sequences were amplified utilizing sequence specific primers. P1 to P6, partial promoter probes amplified from the p28-Omp gene 14 promoter sequences (A). P7-P8, partial promoter probes amplified from the p28-Omp gene 19 promoter sequences (B). The sequences of each probe were underlined with different colored lines and listed under each panel. Putative regulatory sequences were identified in the Figure as different colored text; direct repeats (red text), palindromic sequences (pink text) and for the presence of unique sequences (G-rich region), consensus -35 and -10 regions (uppercase, italics) and ribosome binding sites (bold text). The transcription start sites for the genes mapped by primer extension analysis are identified with bold letter and with an asterisk.

Figure 6.6.


A
P1
1 2 3 4
Probe DNA (1 ng) + + + +
Competitor (50 ng) - - + -
MQ -Ehr P (5 µg) - + + -
BSA (5 ug) - - - +
B
P2
1 2 3 4
+ + + +
- - + -
- + + -
- - - +
C
P3
1 2 3 4
+ + + +
- - + -
- + + -
- - - +
D
P4
1 2 3 4
Probe DNA (1ng) + + + +
Competitor (50 ng) - - + -
MQ -Ehr P (5 µg) - + + -
BSA (5ug) - - - +
E
P5
1 2 3 4
+ + + +
- - + -
- + + -
- - - +
F
P6
1 2 3 4
+ + + +
- - + -
- + + -
- - - +

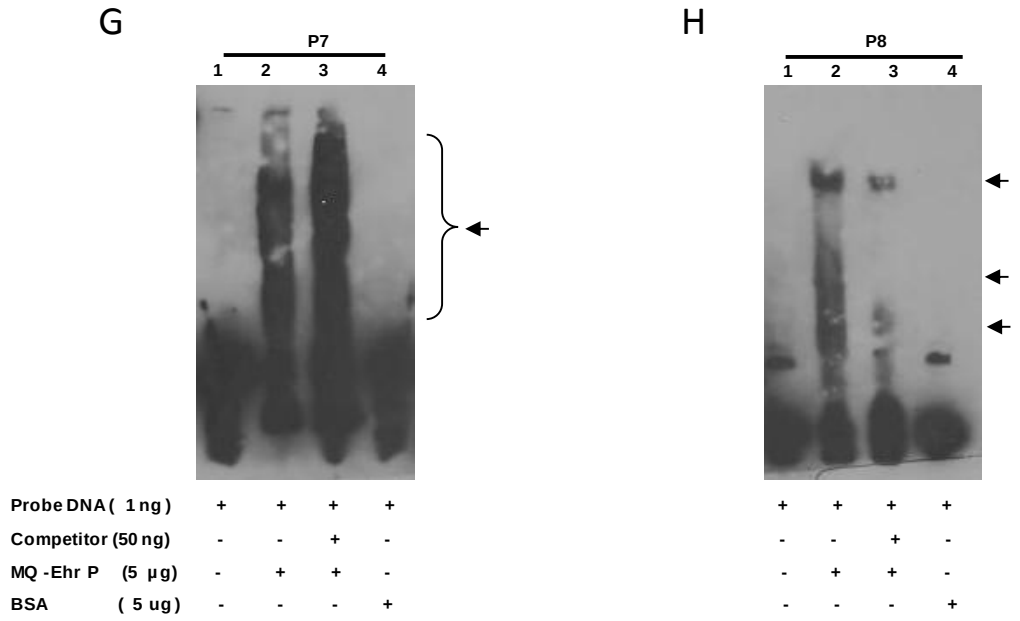
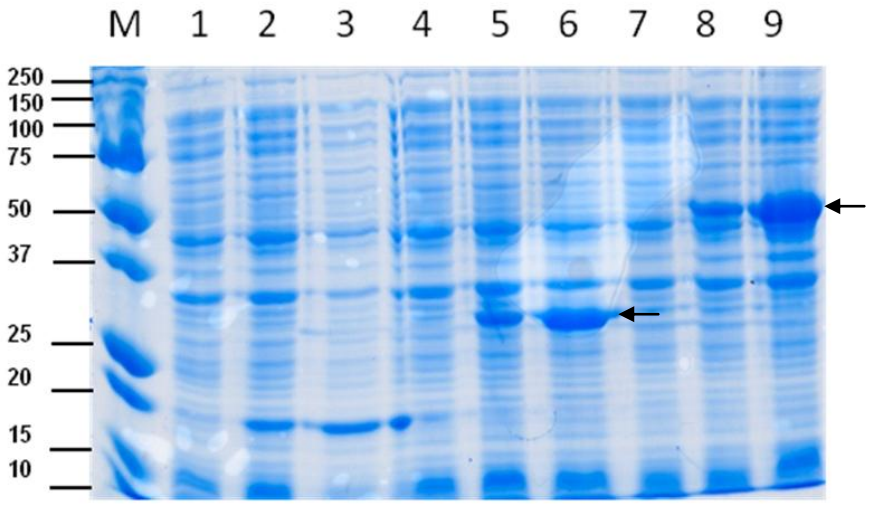
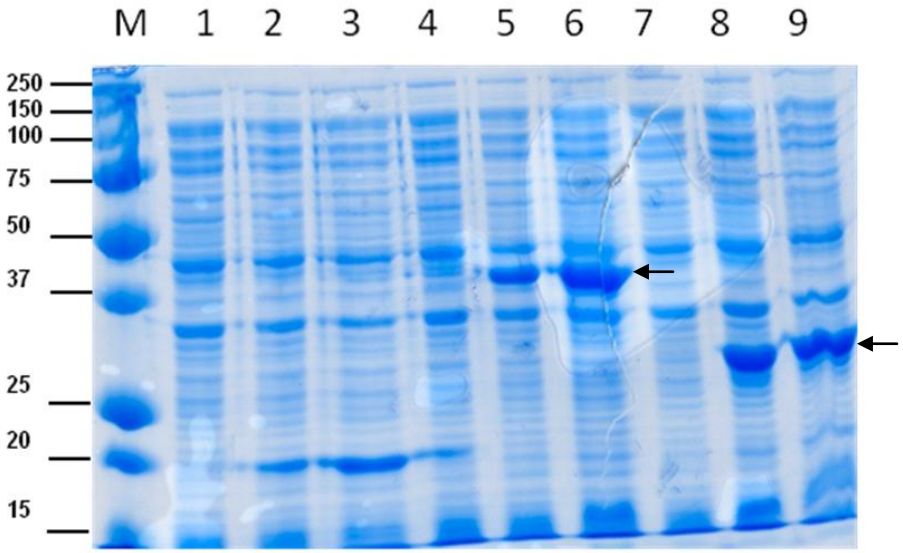


Figure 6.6. EMSA experiments utilizing biotin labeled short segments of the p28-Omp genes 14 and 19 promoters: Short promoter fragments were amplified using sequence specific forward primer and 5' end biotin labeled reverse primer and evaluated for their interaction with the *E. chaffeensis* protein lysates prepared from macrophage-derived *Ehrlichia* organisms. Panels A-F, represent the EMSA experiments using p28-OMP genes 14 promoter probes (P1-P6); Panel G-H, represent EMSA experiments using p28-OMP genes 19 promoter probes (P7-P8). MQ-Ehr P, soluble proteins prepared from macrophage grown *E. chaffeensis* organisms. Shifts in the probe migration are indicated by arrows. Details about the addition of *E. chaffeensis* proteins, unlabelled probe DNA as competitor, and BSA to a reaction are presented under each panel.



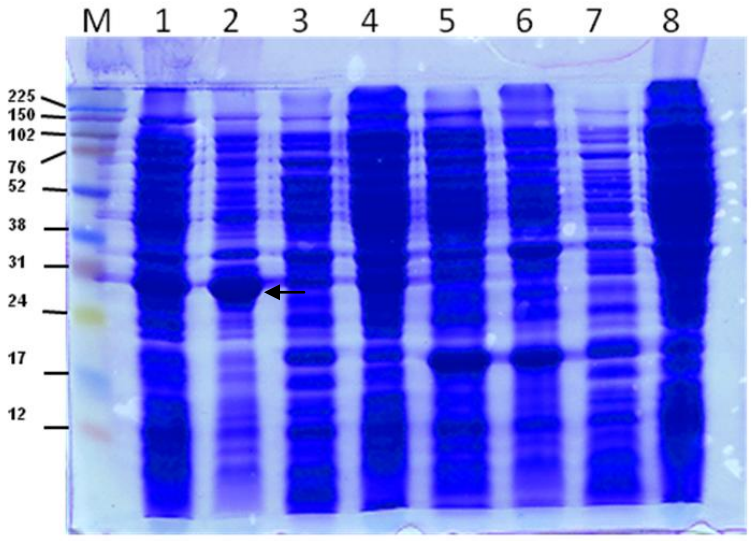
M = Precision plus protein standard (kDa)
1 = pET32 alone plasmid 0h IPTG induction
2 = pET32 alone plasmid 1h IPTG induction
3 = pET32 alone plasmid 3h IPTG induction
4 = ECH_0163 plasmid 0h IPTG induction
5 = ECH_0163 plasmid 1h IPTG induction
6 = ECH_0163 plasmid 3h IPTG induction
7 = ECH_1012 plasmid 0h IPTG induction
8 = ECH_1012 plasmid 1h IPTG induction
9 = ECH_1012 plasmid 3h IPTG induction

Figure 6.7. SDS PAGE gel showing the recombinant protein expression from ECH_0163 and ECH_1012 clones. The expression of recombinant regulatory proteins is indicated with arrows. The details of samples loaded in each well are provided at the botton of gel picture.



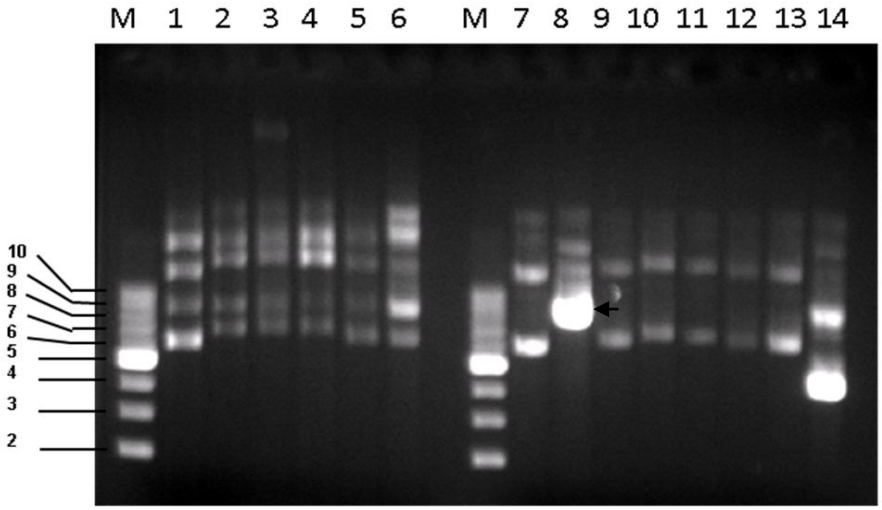
M = Precision plus protein standard
1 = pET32 alone plasmid 0h IPTG induction
2 = pET32 alone plasmid 1h IPTG induction
3 = pET32 alone plasmid 3h IPTG induction
4 = ECH_1118 plasmid 0h IPTG induction
5 = ECH_1118 plasmid 1h IPTG induction
6 = ECH_1118 plasmid 3h IPTG induction
7 = ECH_0804 plasmid 0h IPTG induction
8 = ECH_0804 plasmid 1h IPTG induction
9 = ECH_0804 plasmid 3h IPTG induction

Figure 6.8. SDS PAGE gel showing the recombinant protein expression from ECH_1118 and ECH_0804 clones. The expression of recombinant regulatory proteins is indicated with arrows. The details of samples loaded in each well are provided at the botton of gel picture.



M = Full-Range Rainbow molecular weight marker (kDa)
1 = ECH_0795 plasmid 3h IPTG induction, soluble fraction
2 = ECH_0795 plasmid 3h IPTG induction, insoluble fraction
3 = ECH_0795 plasmid uninduced, soluble fraction
4 = ECH_0795 plasmid uninduced, insoluble fraction
5 = pET32 alone plasmid 3h IPTG induction, soluble fraction
6 = pET32 alone plasmid 3h IPTG induction, insoluble fraction
7 = pET32 alone plasmid uninduced, soluble fraction
8 = pET32 alone plasmid uninduced, insoluble fraction

Figure 6.9. SDS PAGE gels showing the recombinant protein expression from ECH_0795 clone. The expression of recombinant regulatory protein is indicated with arrows. The details of samples loaded in each well are provided at the botton of gel picture.



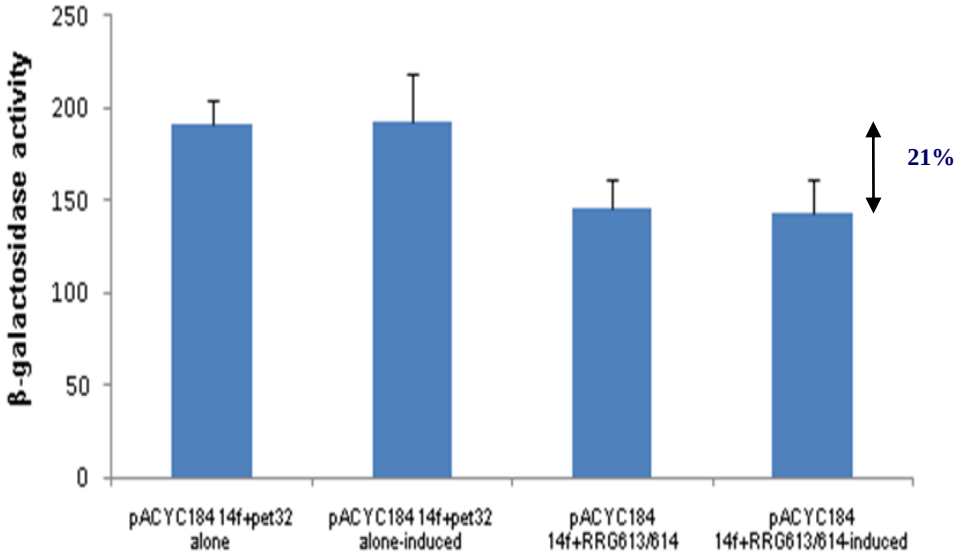
M = Super coiled DNA ladder (kb)
1 = pET32 alone plasmid + pACYC184 -14F-lacZ plasmid
2 = ECH_0163 + pACYC184 -14F-lacZ plasmid
3 = ECH_1012 + pACYC184 -14F-lacZ plasmid
4 = ECH_1118 + pACYC184 -14F-lacZ plasmid
5 = ECH_0804 + pACYC184 -14F-lacZ plasmid
6 = ECH_0795 + pACYC184 -14F-lacZ plasmid
7 = pET32 plasmid with no insert
8 = pACYC184 -14F-lacZ plasmid
9 = ECH_0163
10 = ECH_1012
11 = ECH_1118
12 = ECH_0804
13 = ECH_0795
14 = pACYC184 plasmid with no insert

Figure 6.10. Plasmid DNA extracted from double transformants of *E. coli* and comparison with plasmid DNA extracted from *E. coli* transformed with single plasmid. Lanes 1-6 contain plasmid DNA extracted from *E.coli* containing two plasmids double plasmids. Lanes 7-14 contain PLASMID dna extracted from *E. coli* containing single plasmid. Gene 14 promoter plasmid, which is present in all double transformants of *E. coli* is indicated with an arrow. The details of Plsmid DNA samples loaded in each well are presentd at the botton of gel picture.

Figure 6.11.

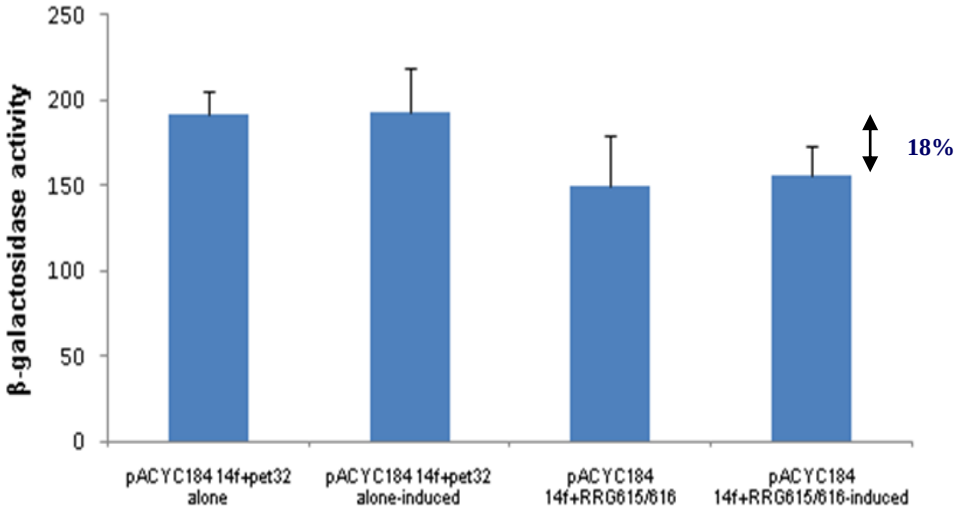
A.

β-galactosidase activity in the presence of ECH_0163 (pET32 RRG613/614) clone



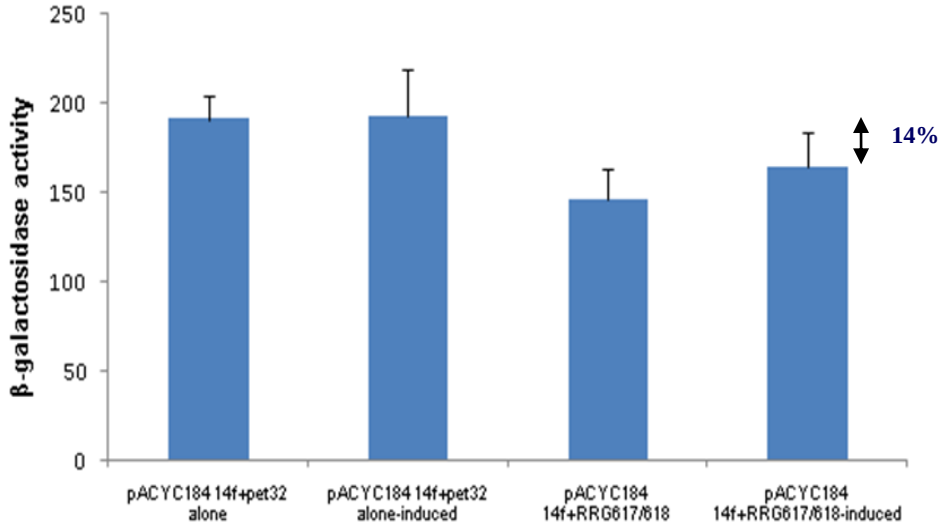
B.

β-galactosidase activity in the presence of ECH_1012 (pET32 RRG615/616) clone



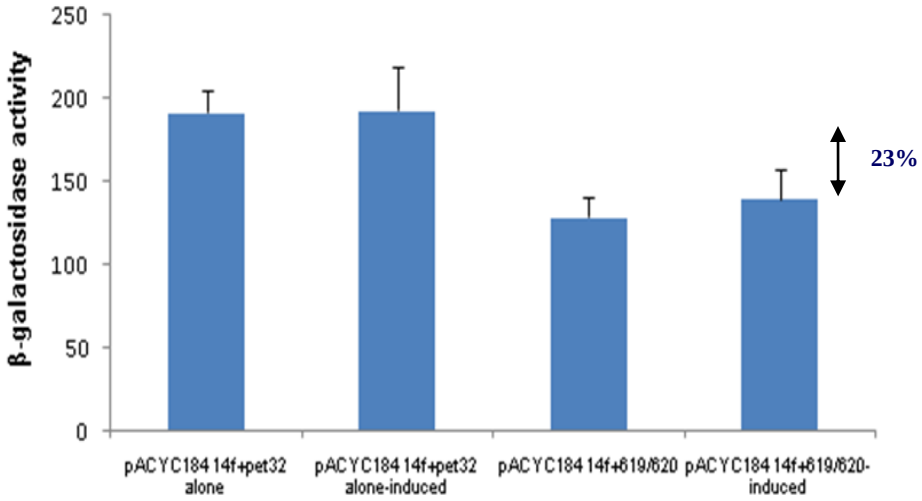
C.

β-galactosidase activity in the presence of ECH_1118 (pET32 RRG617/618) clone



D.

β-galactosidase activity in the presence of ECH_0804 (pET32 RRG619/620) clone



E.

β-galactosidase activity in the presence of ECH_0795 (pET32 RRG 621/622) clone

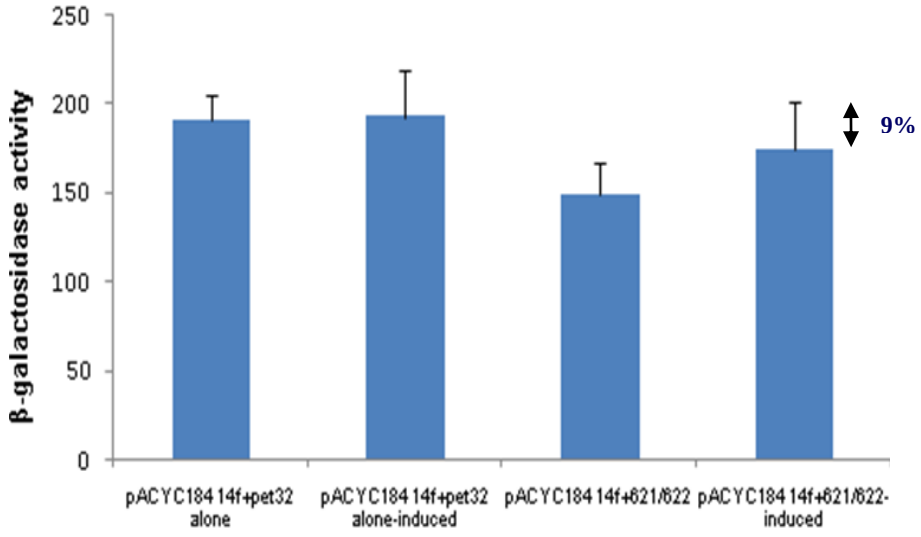


Figure 6.11. Effect of recombinant putative transcriptional regulatory proteins of *E. chaffeensis* on the p28-Omp gene 14 promoter activity. Putative regulatory proteins expression was induced in BL21 strain of *E. coli* transformants containing recombinant pET32 plasmid and pACYC184-14F-lacZ plasmid. β-galactosidase activity of protein extracts prepared from double transformants of *E. coli* cultures was determined. Panels A and E contained the β -galactosidase activity analysis data obtained from double transformants of *E. coli*, induced for protein expression from ECH_0163, ECH_1012, ECH_1118, ECH_0804, and ECH_0795 clones, respectively. Data were presented with SD values calculated from three independent experiments ($P \geq 0.05$).

Primers used	Sequence	Orientation	Amplicon size (bp)	Annealing temperature (°C)
p28-Omp gene 14 promoter EMSA probes				
Probe 1 (P1)				
RRG217	5' attgctcaaccataaaataatggga	Forward	181	50
RRG623-rev-5'-Biotin	5' ggtttagccattttaaatgtg	Reverse		
Probe 2 (P2)				
RRG267	5' cagttaactttctgtaaacttc	Forward	122	50
RRG623-rev-5'-Biotin	5' ggtttagccattttaaatgtg	Reverse		
Probe 3 (P3)				
RRG268	5' atcataagtttacaataatgtc	Forward	133	45
RRG624-rev-5'-Biotin	5' catatattaataaatacggaac	Reverse		
Probe 4 (P4)				
RRG269	5' cgttttctgctttattagaatg	Forward	121	55
RRG625-rev-5'-Biotin	5' gtacatgcattatgagcaaatc	Reverse		
Probe 5 (P5)				
RRG270	5' gttccgtatttattaatatatg	Forward	203	50
RRG626-rev-5'-Biotin	5' ctatacttaactttactactta	Reverse		

Probe 6 (P6)

RRG272	5' ggataagtactttagcaagtgg	Forward	157	50
RRG627-rev-5'-Biotin	5' gtctagaatataaaatttctttc	Reverse		

p28-Omp gene 19 promoter EMSA probes

Probe 7 (P7)

RRG185	5' gactctagacttttaattttattattgccacatg	Forward	150	50
RRG628-rev-5'-Biotin	5' gcacttataaactagtccc	Reverse		

Probe 8 (P8)

RRG276	5' gtgctgtttttctcacctttacac	Forward	96	50
RRG629-rev-5'-Biotin	5' cttttgtaaggaaaatttaatata	Reverse		

Table 6.1. List of primers used to amplify short fragments for the p28-Omp genes 14 and 19 promoters for use in EMSA experiments

Primers used	Sequence	Orientation	Amplicon size (bp)	Annealing temperature (°C)
Clone: BL21-ECH 0163 (pET32 RRG613/614)				
RRG613	5' gtaccatgggtgaaaagaaaatattac	Forward	394	50
RRG614	5' agtctcgagctacattccgttaaacttc	Reverse		
Clone: BL21-ECH 1012 (pET32 RRG615/616)				
RRG615	5' gtaccatggctatgcgtatattattaatag	Forward	818	45
RRG616	5' agtctcgagttatgcttcctcaacatac	Reverse		
Clone: BL21-ECH 1118 (pET32 RRG617/618)				
RRG617	5' gtaccatggctatgtctacacatgcgaaaaac	Forward	662	45
RRG618	5' agtctcgagttactgtttgttatctaaag	Reverse		
Clone: BL21-ECH 0804 (pET32 RRG619/620)				
RRG619	5' gtaccatggctatgagtaaggatatggtagtta	Forward	305	50
RRG620	5' agtctcgagttaattatctaaaaggttaa	Reverse		

Clone: BL21-ECH 0785 (pET32 RRG621/622)				
RRG621	5' gtaccatggctatgacaacaataagtaaccaaa	Forward	347	52
RRG622	5' agtctcgagttaatcttctttttgtatta	Reverse		

Table 6.2. List of primers used for cloning putative transcription regulator coding sequences of *E. chaffeensis* into pET 32a (+) expression vector

Clone	Primers (Forward/reverse)	Putative regulatory protein	Protein ID	Gene ID
ECH_0163	RRG613/RRG614	Transcriptional regulator, MerR family	YP_506990.1	3927338
ECH_1012	RRG615/RRG616	DNA-binding response regulator	YP_507798.1	3927952
ECH_1118	RRG617/RRG618	Putative transcriptional regulator	YP_507902.1	3927407
ECH_0804	RRG619/RRG620	DNA-binding protein HU	YP_507602.1	3927490
ECH_0795	RRG621/RRG622	Hypothetical protein	YP_507593.1	3927528

Table 6.3. Details of *E. chaffeensis* transcription regulators recombinantly expressed in *E. coli*

Chapter 7

Major Conclusions and Future Directions

1. Transcriptional analysis of *E. chaffeensis* RNA revealed host cell-specific gene expression for the p28-Omp gene 14 in tick cells and gene 19 in macrophages.

2. Quantitative RT-PCR analysis of *E. chaffeensis* RNA isolated at various time points post-infection also reveled similar host-specific gene expression for these two genes.

3. The major expression was reversed from gene 14 to gene 19, when tick cell grown bacteria were inoculated into vertebrate macrophages both *in vitro* and *in vivo*. These data are consistent with our previous protein data demonstrating switch in expression of these genes in response to bacterial growth in tick cells and macrophages.

4. These findings confirm that host cell-specific protein expression for the p28-Omp genes 14 and 19 are the result of differences in their transcription.

5. Primer extension analysis and ribonuclease protection assays aided in identifying the transcription start sites and in locating the putative promoter regions for the p28-Omp gene 14 and 19.

6. Transcription is initiated for both the genes at adenine nucleotide, which is located at 34 and 26 nucleotides upstream of ATG for the p28-Omp 14 and 19 gene.

7. Both the putative promoters of the p28-Omp genes 14 and 19 are functional in *E. coli*.

8. Analsyis of the p28-Omp gene 14 and 19 promoter regions revealed several promoter-specific differences, which included variation in their length, presence of several direct repeats, palindromic sequences and a unique 14-bp long ‘G-rich’ sequence specific to gene 19 promoter.

9. Deletion of one or more putative regulatory sequences significantly altered the p28-Omp genes 14 and 19 promoter activity.

10. The fluctuations in gene 14 promoter activity ranged from partial or complete loss to restorations in promoter activity.

11. For gene 19, the 5' end deletions up to 120 bp upstream of ATG resulted in a minor decline in promoter activity, which was further increased to 60% with an additional 60 bp deletion. The increase in the β-galactosidase activity was unique to the gene 19 deletions.

12. Promoter region sequence comparisons with those of *E. coli* consensus sequences aided in the identification of putative -10, -35 and ribosomal binding sequences.

13. Subsequent deletion analysis experiments suggested that those sequences conatin RNA polymerase sites.

14. Promoter characterization studies demonstrated that *E. coli* RNA polymerase recognizes *E. chaffeensis* gene 14 and 19 promoters.

15. *In vitro* transcription assays utilizing *E. coli* RNA polymerase were developed for use in driving the expression from the p28-Omp gene 14 and 19 promoters of *E. chaffeensis.*

16. *E. coli* RNAP recognized the same transcription start site *in vitro* for both the p28-Omp genes 14 and 19 promoters as that recognized by *E. chaffeensis in vivo.*

17. Electrophoretic mobility shift assays of the p28-Omp genes 14 and 19 full length promoters revealed interaction of *E. chaffeensis* proteins with these sequences.

18. Partial promoters spanning one or more putative regulatory sequences but lacking the RNAP binding regions also demonstrated interaction of *E. chaffeensis* proteins with these sequences.

19. *E. chaffeensis* genome was searched to identify genes that encode for putative regulatory proteins, which aided in the identification of five putative regulatory protein coding sequences.

20. These proteins were expressed in *E. coli* and assessed for their possible role in gene 14 promoter activity.
21. All five proteins showed a minor trend for a decrease in gene 14 promoter activity. The decline promoter activity ranged from 9-23% but the data is not statistically significant ($P \geq 0.05$)

Future directions

1. Promoter characterization of p28-Omp genes 14 and 19 *in vivo* in *E. chaffeensis* may help in identifying the sequences that are involved in gene regulation.

2. Promoter characterization using *in vitro* transcription sytems in the presence of native or recombinant *E. chaffeensis* RNA polymerase may also provide important information about sequences that are critical for promoter activity

3. Evaluation of the impact of five putative regulatory proteins identified in this study on the p28-Omp gene 19 promoter activity to identify they have any regulayory role in its expression.

4. Identification of *E. chaffeensis* proteins interacting with the p28-Omp gene 14 and 19 promoter sequences and assessing their precise role in gene regulation.

Chapter 8

General Molecular Biology Methods Used in Study

Polymerase chain reaction (PCR): All PCR‘s were performed in final reaction volume of 25 µl. Each reaction contains a final concentration of 1X PCR reaction buffer, 50 nmoles of MgCl2, 10 nmoles of dNTP's, 10 pico moles each of forward and reverse primers, about 1ng of template DNA, and 1 unit of taq DNA polymerase (Invitrogen technologies, Carlsbad, CA). For PCR reactions that require proof reading, the PCR conditions are; 1x Pfx amplification buffer, 1 U of platinum Pfx DNA polymerase (Invitrogen Technologies, Carlsbad, CA), 5 nmoles of dNTP's, and rest of the reagents concentrations are maintained same as in above described reaction. The PCR temperature cycles include an initial heating to 95°C for 2 min, followed by 40 cycles of 95°C denaturation for 30 sec, primer annealing for 30 sec and carried out at appropriate temperatures calculated for each primer set, 72 °C extension for 30 sec. The extension temperature for platinum Pfx DNA polymerase is 68 °C. The extension temperatures were increased to 1min per each kb of expected amplicons length. Each reaction set included a negative control, which lacked a template but contained all the other reaction components. After reaction is complete the products were resolved on agarose gels containing ethidium bromide and visualized under UV light.

Restriction enzyme digestions: Typically restriction enzyme digestion reaction is performed in a 20 µl volume. The reaction included 1x restriction enzyme reaction buffer, 1 µg of DNA, 1-5 units of enzyme, 0.5 µl of BSA (10 µg/ µl), and water to a final volume of 20 µl. Typically the restriction enzyme digestion is carried out at 37°C for 2 h unless an enzyme requires a specific temperature. For all the reactions that utilized two restriction enzymes, a buffer optimal for both the enzymes is utilized.

Blunt ending of 3'or 5' overhangs: The 3' 'A' overhangs of DNA fragments resulted from a PCR reaction using taq DNA polymerase or a 3' overhang producing restriction enzyme digestion products are blunt ended by performing a T4 DNA polymerase reaction. This reaction typically includes approximately 100 ng of template DNA, 1 unit of T4 DNA polymerase, and 1x reaction buffer containing 100 μM dNTP's (New England Biolabs, Ipswich, MA). The reaction is usually performed for 15 minutes at 12°C. The reaction is stopped by adding 10 mM EDTA and enzyme is inactivated by heating at 75°C for 20 min. To create blunt ends of DNA fragments containing 5' overhangs, a fill-in reaction is performed using large fragment (Klenow) of DNA polymerase I. This reaction is carried at 25°C for 15 min and includes 1 unit of Klenow enzyme, and 1X reaction buffer containing 0.33 μM each of dNTP's (New England Biolabs, Ipswich, MA). The reaction is terminated by adding EDTA to a final concentration of 10 mM followed by heat inactivation of enzyme at 75°C for 10 min. The blunt ended products are purified by following a standard phenolchloroform, ethanol precipitation method described below. The final products after purification are suspended in 10 μl of TE buffer (10 mM Tris-HCl, pH,8.0 and 1 mM EDTA).

Phenol purification of DNA: DNA fragments from PCR, restriction enzyme digestion, and filling-in reactions are purified phenol purification method. Typically, 3 M sodium acetate is added to final concentration of 0.3 M into a microcentrifuge tube containing DNA and final volume was adjusted to 200 μl with TE buffer. Two hundred micro liters of phenol (pH, 8.0) is added, vertexed to mix and centrifuged at 15,000 g for 15 min at 4°C. The top aqueous layer is transferred into a clean microcentrifuge tube and added a 200 μl of phenol:chloroform:isoamylalcohol (25:24:1) mixture. The contents are mixed

by vertexing and centrifuged at 4°C at 15,000 g for 15 min. The top layer is transferred into another clean microcentrifuge tube. Thease steps were repeated with pheol:chloroform:isoamylalacohol and then with chloroform:isoamylalacohol. To the final removed aqueous layer, 0.5 ml absolute cold ethanol is added, incubated at -20°C for 15 min followed by centrifugation at 15,000g for 15 min. The DNA pellet is washed with 0.5 ml of 70% ethanol. Final pellet was air dried, resuspended in 20 µl TE buffer and stored at -20 °C until use.

Ligation reactions: A typical ligation reaction included approximately 25 ng of linearized purified plasmid vector DNA, 5 to10 molar excess of insert DNA, 1x ligation buffer, 5 units of T4 DNA ligase (Promega Corporation, Madison, WI) in a 20 µl reaction volume. The ligation reaction is carried out by incubating the contents at 15°C for 16 h. Following the ligation, 1 µl of ligation mix is used for transformation by chemical method. Alternatively, for use in transformation by electroporation method, the DNA is purified by phenol:choloform:isoamyl alcohol method described above.

Preparation of *E. coli* cells for use in chemical transformation methods: The *E. coli* strains utilized to prepare competent cells included Top 10 cells (Invitrogen Technologies, Carlsbad, CA), DH5α (Stratagene, La Jolla, CA), and BL21 (DE3) (Novagen Inc., Madison, WI). Top 10 stain of *E. coli* is always grown in the presence of streptomycin (35µg/ml). DH5α and BL21 (DE3) stains are grown in a plain LB medium. To prepare chemical competent cells, an *E. coli* colony of a desired strain is cultured in 3 ml of LB medium overnight in a 37°C incubator, shaking at 250 rpm. Subsequently, *E. coli* culture is re-inoculated into 100ml LB medium and grown in a 37°C incubator. After the cells are grown to 0.4 OD (measured at 600nm), the cultures are harvested by

centrifuging at 2,500 g for 5 min at 4°C. The cell pellet is resuspended in 10 ml of freshly prepared 10 mM Tris-HCl (pH, 7.5) and 50 mM $CaCl_2$, and incubated on ice for 30 min. The cultures was centrifuged again at 2,500 g for 5 min at 4°C and the pellet is suspended in 2 ml of 10 mM Tris-HCl (pH, 7.5) and 50 mM $CaCl_2$ and stored at 4°C. The competent cells made by this procedure are utilized within 24 h after their preparation.

Electro competent *E. coli* cells preparation: For use in transformation by electroporation method, a desired strain of *E. coli* is cultured in 5 ml LB medium overnight at 37°C shaker incubator set at 250 rpm. The overnight grown *E. coli* cultures are used to re- inoculate into 500 ml of LB medium and are grown to a 0.4 OD (measured at 600 nm) at 37°C. For the rest of the procedure, the *E. coli* cells and the reagents used for making the electro competent cells are maintained at 4°C. After reaching to 0.4 OD, the cultures are transferred into 125 ml capacity and incubated in the ice for 30 min. The *E. coli* cells are harvested by centrifugation at 1000 g for 20 min at 4°C. Cell pellet in each tube is resuspended in 100 ml of ice cold de-ionized water and centrifuged at 1000 g for 20 min at 4°C. The cell pellet in each centrifuge tubes is resuspended in 50 ml of ice cold water. The *E. coli* suspension from two tubes were combined into one tube and centrifuged at 1000 g for 20 min at 4°C. After discarding the supernatant, the cell pellet is resuspended in 20 ml of ice cold 10% glycerol and centrifuged at 1000 g for 20 min at 4°C. The final pellet was resuspended in 0.5 ml of ice cold 10% glycerol, 55 µl of the *E. coli* suspension is aliquoted into pre-chilled sterile microcentrifuge tubes and frozen immediately by placing the tubes in liquid nitrogen. The cells are stored at -80°C until use.

Transformation: Transformation of ligated products into *E. coli* cells is achieved either by a chemical method or through an electroporation procedure. To transform by chemical method, 200 µl of chemical competent *E. coli* cells are mixed with 50 of 100mM $CaCl_2$ and 49 µl of sterile water. One µl of ligation products are added to this suspension and mixed by gentle tapping of the tube. The contents are then incubated in ice for 15 min, followed by a heat shock at 42°C for 2 min. The cells are incubated at room temperature for 10 min, added 1 ml of LB medium, and incubated at 37 °C in a shaker incubator set at 200 rpm for 1h. To transform by electroporation method, 100 mm gap electroporation cuvetts are used. Fifty microliters of electrocompetant cells and 1 µl of purified ligation mix suspension are transferred into a pre-chilled electroporation cuvette (Eppendorf, Hamburg, Germany) and subjected to an electric shock at 1800 V using an elecroporator (Model # 2510, Eppendorf, Hamburg, Germany). The cells are then transferred into a culture tube containing 200 µl of LB medium and grown at 37 °C in a shaker incubator for 1h.

Preparation of Luria-Bertani (LB) media: The LB liquid medium and LB agar plates were utilized to grow *E. coli* cultures. To prepare 1 lit of LB liquid medium, 15 g tryptone, 10 g of yeast extract and 10 g of sodium chloride were dissolved in 1 lit of double distilled water and pH of the solution was adjusted to 7.0 with the help of 10N NaOH. The LB medium was autoclaved at a liquid cycle. LB agar plates preparation included similar preparation as described above but 15 g of cell culture grade agar powder was added to the medium prior to autoclaving. After autoclaving, the LB agar medium was allowed to cool to nearly 60°C and a desired concentration of appropriate

antibiotic was added to the medium. Approximately 15 ml of medium was poured into sterile agar plates. After solidification of the agar medium, the plates were wrapped and stored at 4°C until use.

Selection of recombinant clones: Two hundred micro liters of transformed bacterial cultures are transferred onto LB plates containing appropriate antibiotic specific to a recombinant plasmid. The culture is uniformly dispersed onto the agar plate using a bacterial culture spreader. To grow the transformed *E. coli* the plates were incubated overnight in a 37°C incubator. The presence of transformants is assessed by comparing plates containing appropriate controls (ligation controls, no transformation controls). Subsequently, several colonies are selected, inoculated in a culture tube containing LB medium with appropriate antibiotic and are grown overnight at 37 °C in a shaker incubator to isolate the plasmid DNA.

Isolation of Plasmid DNA: From overnight grown *E. coli* cultures plasmid DNA is isolated by following boiling preparation method (336). To isolate plasmid DNA, 1.5 ml of overnight grown bacterial cultures are transferred into a micro centrifuge tube and centrifuged 12,000 g for 5min. The supernatant was aspirated carefully with the help of a vacuum device and cell pellet is resuspended in 0.4 ml plasmid lysis buffer (10mM Tris-HCl pH 8.0, 0.1 M NaCl, 1mM EDTA, 5% v/v Triton X-100) with the help of a tooth pick. Twenty five micro liters of freshly prepared lysozyme (10 mg/ml) is added. Lysozyme was prepared by dissolving 10mg of lysozyme powder in 1 ml of 10 mM Tris-HCl buffer (pH, 8.0) to get a final concentration of 10 mg/ml. The contents of the tube are vertexed to mix, placed in a boiling water bath exactly for 40 sec, and centrifuged at

12,000 g for 15 min at 4°C. The pellet containing cell debris is removed with the help of a tooth pick. Four hundred and twenty µl of 100%, cold (-20°C), isopropanol is added to the supernatant and mixed by vertexing, incubated at room temperature for 5 min and centrifuged at 12,000 g for 15 min to recover plasmid DNA. Supernatant is discarded and the DNA pellet is washed with 70% ethanol and dried in a speed-vac system (Labconco Centrivap Concentrator, Kansas City, MO) typically for about 5 min. Final pellet is resuspended in 100 µl of TE buffer and contaminating bacterial RNA was removed by treating with 1 µl of RNase A (1 mg/ml) at 37°C for 5 min. The presence and quality of the plasmid DNA was checked by agarose gel electrophoresis (described seperately).

Isolation of genomic DNA: Genomic DNA of *E. chaffeensis* grown in tick or macrophage cultures is isolated by sodium dodecyl sulfate-proteinase K-phenol, chloroform-isoamyl-alcohol method (336). Briefly, 1.5 ml of *E. chaffeensis* cultures are harvested by centrifugation at 12,000 g for 15 min and the cell pellet is resuspended in 0.5 ml of DNA extraction buffer (10 Mm Tris-Cl pH 8.0, 0.1 M EDTA, and 0.5% SDS) containing 0.5 mg/ml proteinase K (Sigma Chemical Co., St. Louis, MO). The contents are mixed by vertexing and incubated for 2 h at 60°C. Phenol:chloroform:isoamyl alcohol extraction method is used to remove proteinecious material and ethanol is added to concentrate DNA as described previously. DNA pellet is air dried and resuspended in 100 of TE buffer. To remove the contaminating RNA, the DNA is treated with 1 µl RNase A (10 mg/ml) at room temperature for 10 min. The DNA samples were stored at -20°C until use.

Agarose gel electrophoresis: Plasmid DNA, restriction digestion products or PCR products are analyzed by resolving them on a 0.9% agarose gels by subjecting to electrophoresis. The agarose gels are prepared after dissolving agarose powder in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA; final pH 8.0) containing 0.1 μg/ml of ethidium bromide. The contents are poured on a gel holding device and are allowed to solidify at room temperature. The gel is placed in an electrophoresis chamber containing 1X TAE buffer with 0.1 μg/ml of ethidium bromide dissolved in it. About 5 μl of DNA is loaded into the wells. Molecular weights markers are also loaded in a separate well and resolved to help in determining the approximate molecular weight of the DNA. The DNA is subjected to electrophoresis in the agarose gel at 70 V for 60-90 minutes and is visualized under UV illumination. The imahges are captured using Kodak gel imaging system.

Automated sequencing: Recombinant DNA clones were sequenced to verify the accuracy and orientation of the insert DNA using CEQ Genetic Analysis System and by following the manufacturer's recommendation (Beckman & Coulter, Fullerton, CA). Prior to performing a sequencing reaction, the recombinant plasmid DNA is purified by phenol:chloroform:isoamyl alcohol (25:24:1) method as described above. Following the purification, the concentration of the DNA was estimated by Nanodrop method. The purified plasmid DNA is sequenced using a forward or reverse primer (plasmid derived sequence primers) specific to a sequence upstream or downstream to insert DNA, respectively. Sequencing reaction is performed utilizing DTCS sequencing kit by following the manufacturer's recommendations (Beckman and Coulter, Fullerton, CA). Sequence analysis is performed using Genetics Computer Group (GCG) (87) or Vector

NT programs (Invitrogen Technologies, Carlsbad, CA) or by performing BLAST search analysis.

Manual sequencing: The DNA ladders utilized in primer extension, ribonuclease protection assay and *in vitro* transcription assays were synthesized by manual sequencing reaction is perfomed on a PCR product or on a recombinant plasmid DNA. The sequencing reaction is performed utilizing Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit and by following the manufacturer's recommendations (USB Corporation, Cleveland, Ohio).

RNA isolation: Total RNA from *E. chaffeensis* infected canine macrophages or tick cell cultures was isolated using Tri-reagent method (Sigma Chemical Corporation, St. Louis, MO). Briefly, 1.5 ml of *E. chaffeensis* infected tick cell or macrophage cultures are harvested by centrifuging at 12,000 g for 5 min at 4 °C. To the cell pellet, 1ml of tri reagent is added and vertexed to mix. The lysates are incubated at room temperature for 5 min. Two hundred microliters of chloroform is added to the cell suspension, mixed by vertexing and incubated at room temperature for 10 min. Subsequently, the samples are centrifuged at 12,000 g for 15 min at 4°C. The top aqueous layer containing RNA was transferred into a clean microcentrifuge tube, mixed with 0.5 ml of cold isopropanol (-20 °C) and incubated at room temperature for 10 min. The contents are centrifuged at 12,000 g for 15 min at 4°C. The RNA pellet recovered is rinsed with 70% ethanol and then resuspended in 100 µl of nuclease free water. To prevent the degradation of RNA, 40 U RNase inhibitor (Ambion Corporation, Austin, TX) is added and stored at -80°C until use.

DNAse treatment of RNA samples: To remove the contaminating genomic DNA, RNA samples are subjected to DNase treatment using RQ1 RNase-free DNase enzyme (Promega Corporation, Madison, WI). Typically, the reaction is carried out in a 20 µl volume which included 5-10 µg of RNA, 1X RQ1 DNase buffer, 1 unit of RQ1 RNase-free DNase enzyme, and nuclease free water to a final volume of 20 µl. The reaction is incubated at 37°C for 1 hour followed by inactivation of enzyme at a temperature of 70°C for 10 min. The DNase treated RNA samples were stored at -80°C until use.

Real time PCR and RT-PCR: RNA analysis by TaqMan based real-time PCR or RT-PCR was performed by utilizing SmartCycler system (company) and utilizing SuperScript-III One-Step RT-PCR with platinum *Taq* DNA polymerase kit (Invitrogen Technologies, Carlsbad, CA). Real-time RT-PCR reaction is performed in a 25 µl reaction containing 2 µl of RNA, 1x reaction buffer, 5 pmols each of gene specific TaqMan forward and reverse primers, 3.75 pmol of gene specific TaqMan probe, 5 nmol of dNTP's, 125 nmol of $MgCl_2$, and 1 µl of SS-III RT and *Taq* DNA polymerase mix (Invitrogen Technologies, Carlsbad, CA). The temperature cycles for the reaction include an initial reverse transcription step at 48°C for 30 min, heating at 95°C for 3min, followed by 45 cycles of 95°C denaturation for 15 sec, annealing for 30 sec at 50°C , and a 60 sec extension at 60°C. The product amplification was detected in the SmartCycler machine by measuring the fluorescence emitted during the extension phase of a PCR cycle. A reaction considered as positive for the presence of a template when amplification of the product causes 10 fluorescent units. The Cycle at which a positive signal is obtained is called cycle threshold (Ct) value and it is concentration dependent. High concentration of a template results in a low CT value and vice versa.

Chapter 9

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