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The committee for Scott Wesley Long certifies that this is the approved version of the following dissertation:

THE ROLE OF THE *P28* MULTIGENE FAMILY IN THE PATHOGENESIS OF *EHRLICHIA CHAFFEENSIS* INFECTION.

Committee:

_____
Dr. David H. Walker, Supervisor

_____
Dr. Lawrence Stanberry

_____
Dr. Guy Palmer

_____
Dr. Johnny Peterson

_____
Dr. Xue-jie Yu

_____
Dean, Graduate School

THE ROLE OF THE *P28* MULTIGENE FAMILY IN THE PATHOGENESIS OF *EHRLICHIA CHAFFEENSIS* INFECTION.

by
Scott Wesley Long, B.S.

Dissertation
Presented to the Faculty of The University of Texas Graduate School of
Biomedical Sciences at Galveston
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Approved by the Supervisory Committee

Dr. David H. Walker, M.D.
Dr. Lawrence Stanberry, M.D., Ph.D.
Dr. Guy Palmer, D.V.M., Ph.D.
Dr. Johnny Peterson, Ph.D.
Dr. Xue-jie Yu, M.D., Ph.D.

May, 2007
Galveston, Texas

Key words: Host-pathogen interactions, genetic manipulation, family Anaplasmataceae

To Megan and Carter

ACKNOWLEDGEMENTS

In my time at UTMB I have received assistance, guidance and mentorship from a number of individuals, and it would be impossible to acknowledge all of them in the space provided. However, I would like the specifically thank the following people.

First, I would like to thank Dr. David Walker and Dr. Xue-jie Yu. I was fortunate enough to have Dr. Walker as my Pathology Lab instructor in medical school, and his great interest in educating and mentoring students drew me into his research group to complete my PhD research. His great personal interest in my education has been immeasurably beneficial to my training as an academic physician. Dr. Xue-jie Yu served as a mentor to me daily, and it has been an honor to be exposed to his passion for research, his incredible technical skill and his sense of humor. I can only hope to do justice to the vision, integrity, and scientific ability of these men as I go forth in my career.

My other committee members, Dr. Stanberry, Dr. Peterson, and Dr. Palmer have been extremely helpful in the crafting of this dissertation. They had the foresight to reign in my ambitions and helped me to focus my research, providing great insight into my research process. They have been incredibly generous with their time in scheduling my committee meetings and reviewing manuscripts and dissertation drafts, and I thank them for that.

All the members of the greater Walker lab group, including the Yu, Bouyer and McBride labs, and all the visiting scientists, have been great friends to me and often times have helped me further my research. I would like to thank Drs. Bouyer, Ismail and McBride for supporting the students of the Walker lab group and providing critical input and assistance when needed. I would like to thank all of the graduate students and post-docs in the Walker lab for their friendship and support through the years.

In particular, I would like to thank Ted Whitworth for his initial forays into rickettsial transformation and his expert assistance with all my electron microscopy, Mike Woods for his expertise and assistance with confocal microscopy, and Dr. C. Kuyler Doyle for his assistance in discussing research difficulties as well as introducing me to two dimensional proteomics, tick cell culture, and homebrewing. I thank Nagaraja Thirumalapura for his critical discussions of ehrlichial membrane proteins as well as his development of the modified mitochondrial isolation protocol for purifying host cell-free *Ehrlichia*, a significant technological advance for our field.

Finally, I'd like to thank my loving family for a lifetime of support and encouragement, especially my parents, James Scott Long and Priscilla Long, my brother James, and sister Jill. Furthermore, I give eternal thanks to my wife Megan for loving and supporting me through this arduous journey, and to my son Carter for making me smile and laugh when nothing else could.

THE ROLE OF THE *P28* MULTIGENE FAMILY IN THE PATHOGENESIS OF *EHRLICHIA CHAFFEENSIS* INFECTION

Publication No._____

Scott Wesley Long, Ph.D.
The University of Texas Graduate School of Biomedical Sciences at Galveston, 2006

Supervisor: Dr. David H. Walker

Ehrlichia chaffeensis is a gram-negative obligate intracellular bacterium which parasitizes cells of the monocyte/macrophage lineage in mammals. *E. chaffeensis* possesses a unique cell wall that lacks LPS and the classical peptidoglycan found in other gram-negative bacteria. *Ehrlichia* reside inside unique membrane-bound vacuoles in the cytoplasm of host cells, known as morulae. *E. chaffeensis* is transmitted by lone star ticks, *Amblyomma americanum,* and is not transmitted transovarially, but is maintained in the environment by persistent infection of animal hosts.

E. chaffeensis causes the emerging infectious disease, human monocytic ehrlichiosis (HME), a potentially fatal illness. Although easily treated with tetracycline, due to its obligate intracellular parasitism it is difficult to diagnose by routine clinical methods.

E. chaffeensis expresses a 28 kDa outer membrane protein from a multigene family known as P28/OMP-1. Different alleles from this multigene family are expressed in different host cell types, but the role of this membrane protein remains unknown. Also, few host cell receptors, as well as host-pathogen protein interactions, have been identified for *E. chaffeensis*. Genetic manipulation of the family Anaplasmataceae has not been previously achieved, significantly hampering ehrlichial research.

In this work, we describe a method for the transformation of the genus Ehrlichia, and apply this method to study the role of the P28 in ehrlichial pathogenesis by development of a knockout strain. Furthermore, we identify a novel host cell receptor implicated in ehrlichial binding and infection of host cells.

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BCIP/NBT – 5-bromo-4-chloro-3-indolyl phosphate / nitro-blue tetrazolium
BJAB – human B-cell lymphoblastoid cell line
DH82 – canine monocyte cell line
DMEM – Dulbecco/Vogt Modified Eagle's Minimal Essential Medium
DNA – Deoxyribonucleic acid
FBS – fetal bovine serum
HEPES – 4-2-hydroxyethyl-1-piperazineethanesulfonic acid
IPG – immobilized pH gradient
MEM – Eagle's Minimal Essential Medium
NCS – newborn calf serum
NGS – newborn goat serum
OSGE – O-sialoglycoprotein endopeptidase
PBS – phosphate buffered saline
PCR – polymerase chain reaction
TMB – 3,3',5,5'-tetramethylbenzidine
TrEm – Transformed *Ehrlichia muris*

[U1]

CHAPTER 1

EHRLICHIAE, MONOCYTES AND HOST-PATHOGEN INTERACTIONS

EHRLICHIAE AND EHRLICHIAL DISEASES

The history of the genus *Ehrlichia*

Donatien and Lestoquard first described a pathogenic agent of canines in 1935, which they originally named *Rickettsia canis* (42). The genus name *Ehrlichia* was proposed in 1945, to honor Paul Ehrlich (118). Ehrlich was one of the premier medical scientists of the late 19th and early 20th centuries, and is considered to be the founder of many modern disciplines including hematology, histology, chemotherapy and immunology. He received a share of the Nobel prize in 1908 for his work in the field of immunology (147). Through the years, new bacteria were discovered and added to the genus (48). In 1987, Maeda *et al*. published the initial description of a novel infection noticed the previous year by an astute medical intern, incorrectly identifying the infecting agent as the canine pathogen *Ehrlichia canis* (108). The agent of human monocytotropic ehrlichiosis, *Ehrlichia chaffeensis*, was cultured and characterized by Dawson *et al.* in 1991 (5,38), Following the identification of *E. chaffeensis* in 1991, another agent of human ehrlichiosis, *Ehrlichia ewingii*, was distinguished molecularly and formally named in 1993, after originally having been identified by Sidney Ewing in the 1970s (6,58). The murine pathogen, *Ehrlichia muris*, was isolated from infected rodents in Japan, in 1993 (82,161).

In 2001, the genus was reorganized based upon newly discovered phylogenetic relationships. The genus contains the obligate intracellular bacteria *E. chaffeensis, E. canis, E. ewingii, E. muris,* and *Ehrlichia* (formerly *Cowdria*) *ruminantium*. The reorganized genus was then placed into the family Anaplasmataceae, along with the genera *Wolbachia, Neorickettsia,* and *Anaplasma* (48).

The *Ehrlichia* are small, gram-negative obligate intracellular bacteria which parasitize a variety of host cells and possess unique cell walls, that lack LPS and the classical peptidoglycan found in other gram-negative bacterial cell walls. They reside inside membrane-bound vacuoles in the cytoplasm of host cells, known as morulae (Figure 1). Thus far, they are all transmitted by tick vectors and are not transmitted transovarially, but are maintained in the environment by persistent infection of animal hosts (106). They are pleomorphic organisms, appearing coccoid to ellipsoidal. They exist in two morphologic forms, a larger reticulate form and a smaller, more condensed cell type known as the dense-core form (135). Due to their obligate intracellular parasitism, they cannot be cultured in a cell-free medium. From this basis, we can discuss further the four species of the genus *Ehrlichia* of primary importance to our studies.

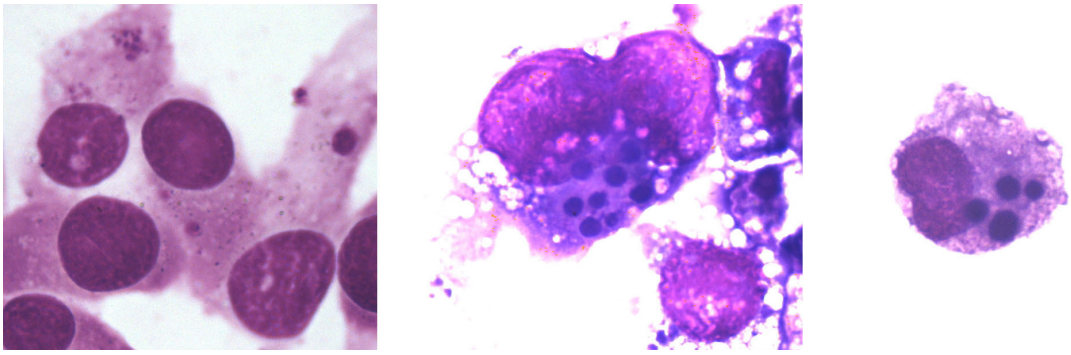


Figure 1 – Light photomicrographs showing morulae from three different species of *Ehrlichia*. *Ehrlichia chaffeensis* (left), *Ehrlichia muris* (center), and *Ehrlichia canis* (right). All three species are shown in DH82 host cells. The *E. muris* sample is heavily infected, with disordering of the host cells undergoing cell death. (Nikon 100X oil immersion).

Ehrlichia canis

E. canis causes canine monocytic ehrlichiosis, and is transmitted by the brown dog tick, *Rhipicephalus sanguineus* (80). *E. canis* has a world wide distribution and has been detected in a variety of different locales (1,155). The major protein antigen is the P28 protein, which varies between 28 kDa and 32 kDa in molecular mass, and is expressed on the outer membrane (114,124,141,166). The major outer membrane proteins of *E. canis* are highly homologous to other *Ehrlichia* and *Anaplasma* species, in

particular *E. chaffeensis* and *A. phagocytophilum* (101). *E. canis* is primarily a canine pathogen although there have been a few case reports of infections of humans and felines (2,20,22,91).

Clinical infection of canines with *E. canis* is typically described as having three distinct stages: an acute phase, a subclincal phase, and a chronic phase. The acute phase typically occurs in the weeks immediately following transmission via an infected tick feeding on the animal (33,74) . The acute phase of infection is generally mild and nonspecific, with clinical signs such as lethargy, fever, anorexia, weight loss, splenomegaly or lymphadenopathy, although the pattern of presentation varies greatly (24,72,74,160). From this initial infection, dogs either clear the bacteria or enter a subclinical stage in which the dog appears to be quite healthy while a low number of organisms remain in the spleen or some other unknown host niche (75). Mild hematological and immunological changes such as thrombocytopenia or hyperglobulinemia may be noted during subclinical infection. It is not known what influences progression from this subclinical phase into the chronic phase, nor is it known what percentage of dogs proceed to chronic infection (32). Often, dogs present to veterinarians offices with previously undiagnosed *E. canis* infection that has entered the chronic phase (65,73).

It is in the chronic phase presentation that some of the more serious manifestations of canine ehrlichiosis occur, including bleeding and coagulation disorders resulting in epistaxis, melena, petechial or ecchymotic hemorrhages, hyphema, retinal hemorrhage or hematuria in anywhere from 25% to 60% of cases studied (65,73,130,154,159). Also, neurological manifestations including ataxia, paraparesis, nystagmus, seizures, head tilt and proprioceptive defects have been observed (65). Glomerulonephritis can also result as complications of chronic infection, as well as secondary infections as a result of pancytopenia (74).

Ehrlichia chaffeensis

It is primarily transmitted by the tick *Amblyomma americanum*, also known as the lone star tick, and is maintained in the environment by persistent infection of animal

hosts, most notably white-tailed deer and wild canids (Figure 2). There is also evidence that *E. chaffeensis* can infect *Dermacentor variabilis.* Like *E. canis,* the major protein antigen is the P28 protein, which varies between 28 and 32 kDa in molecular mass, and is expressed on the outer membrane (124,141,166). The major outer membrane proteins of *E. chaffeensis* are highly homologous to other *Ehrlichia* and *Anaplasma* species, in particular *E. canis, E. muris*, and *A. phagocytophilum.*

The genome size of the Arkansas strain is 1,176,248 bp, consisting of 1,115 identified open reading frames (ORFs) with a GC content of 30.1% (51). The only mouse model of *Ehrlichia chaffeensis* infection currently available utilizes mice with severe immune defects, e.g., SCID mice, as immunocompetent mice clear the infection rapidly and efficiently without manifestations of disease.

The clinical and epidemiological aspects of human monocytic ehrlichiosis will be discussed in greater detail later in this chapter.

Ehrlichia ewingii

Ehrlichia ewingii was originally shown to be an agent of canine granulocytic disease in 1971 (58). More recently, it has been found to be an agent of granulocytic ehrlichiosis in immunocompromised humans (25). It is transmitted by the tick *Amblyomma americanum*. Unlike *E. canis, E. muris,* and *E. chaffeensis,* the neutrophil is the host cell of *E. ewingii*. Much remains unknown about the nature of *E. ewingii,* due to the fact that it has not been successfully cultured *in vitro* in the laboratory.

The clinical presentation of canine granulocytic ehrlichiosis attributable to *E. ewingii* is virtually indistinguishable from that of canine monocytic ehrlichiosis due to *E. canis* infection, with the possible exception of a high incidence of lameness, polyarthropathy and other joint problems (17,34,153).

E. ewingii is responsible for causing a novel human ehrlichiosis, ehrlichiosis "ewingii", which is primarily a disease of the immunocompromised.

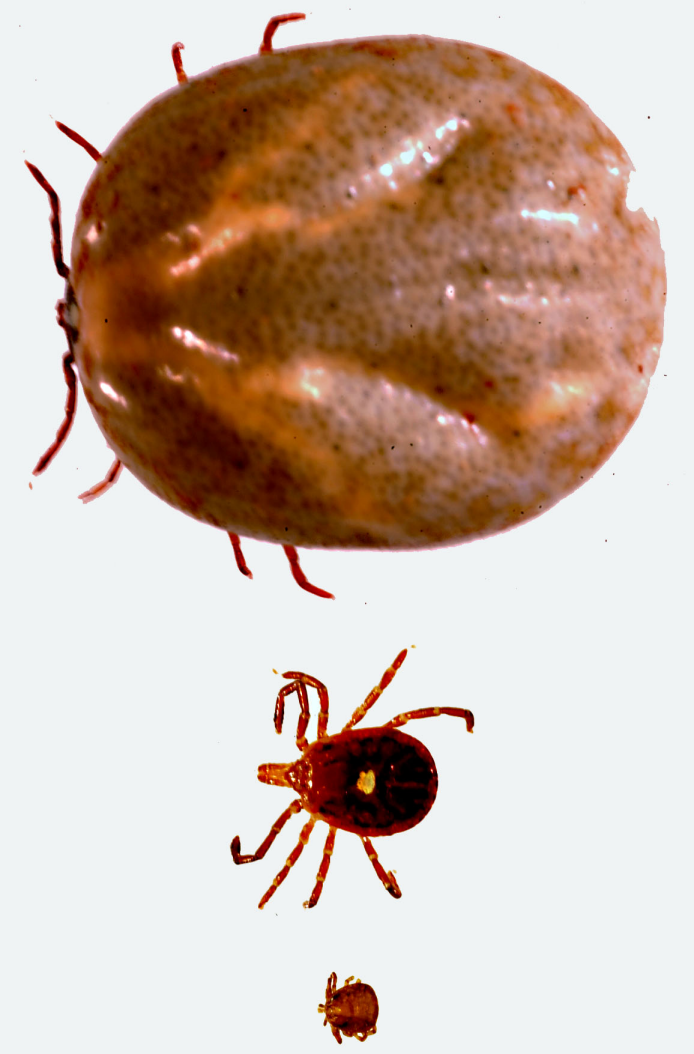


Figure 2 – A comparison of *Amblyomma americanum* in two different stages of its life cycle. From bottom to top, there is an *A. americanum* nymph, an unfed adult female demonstrating the characteristic white spot on the dorsal surface of the thorax, and at the top a fully fed adult female. The larval stage is not pictured, although in general the larvae are approximately the size of the period at the end of this sentence.

Ehrlichia muris

Ehrlichia muris was identified in 1993 as the cause of rodent infection, and is highly homologous to *E. chaffeensis* (82,161). It was first isolated from a vole, *Eothenomys kageus*, in Japan, although samples have also been identified in ticks from Russia as well as other rodent species in Japan (81). Due to the high degree of homology between *E. muris* and *E. chaffeensis*, it has been developed as a mouse model of ehrlichial infection, which was characterized by our group in 2004 (126). It also possesses a P28 locus which is very similar to that of *E. chaffeensis*, with the exception of the deletion of a single allele, *p28-20*[U2] (35). *Ixodes persulcatus* ticks have been incriminated as the primary host in Russia, but *Haemaphysalis flava* ticks have also been found infected with *E. muris* in Japan (4,81,146).

Thus far, no cases of human infection with *E. muris* have been described in the literature. However, there have been human infections in Russia with antibodies reactive to *E. chaffeensis* in areas where ticks have been found infected with *E. muris*. It is possible that these cases are actually human infections with *E. muris,* but further investigations are needed (4,139,146).

Anaplasma phagocytophilum, the agent of human granulocytotropic anaplasmosis

Anaplasma phagocytophilum, the agent of human granulocytic anaplasmosis, was originally named *Ehrlichia phagocytophila*, and was also separately identified as *Ehrlichia equi* and the HGE agent. All of these described strains were unified under the banner of *A. phagocytophilum* in 2001 (48). *A. phagocytophilum* is transmitted primarily by ticks of the genus *Ixodes*, including *Ixodes scapularis, Ixodes ricinus* and *Ixodes pacificus* (140,153).

The genome of *A. phagocytophilum* is 1,471,282 bp consisting of 1,369 identified open reading frames (ORFs) with a GC content of 41.6% (51).

Their morphologic characteristics are similar to those of the *Ehrlichia*, including the existence as two distinct morphologic forms. Two differences include a lack of a fibrillar morular matrix in association with *A. phagocytophilum,* and the lack of close

apposition of the mitochondria in the cell to the morular membrane (135). *A*[U3]. *phagocytophilum* has been reported to cause diverse disease in a variety of hosts, including tick-borne fever of ruminants, equine granulocytic ehrlichiosis, a type of canine granulocytic ehrlichiosis, and human granulocytic anaplasmosis (formerly ehrlichiosis) (9,28,63,69-71,79,107,137,152).

Clinical aspects of canine granulocytic anaplasmosis

The clinical presentation of canine granulocytic ehrlichiosis caused by *A. phagocytophilum* is a mild disease with a lower incidence of lameness or other joint symptoms compared to canine granulocytic ehrlichiosis due to *E. ewingii* (33,71,92). Also, differences in seasonality and gender skew have been reported, with *A. phagocytophilum* canine granulocytic ehrlichiosis appearing in the autumn and affecting predominantly female dogs, while *E. ewingii* occurs more frequently in the spring and summer months in male dogs (33). While the seasonal differences may be accounted for by the habits of the differing vector tick species, the gender bias cannot be as easily explained and at this point, this report is anecdotal.

Clinical aspects of human granulocytic anaplasmosis

In human patients, infection with *A. phagocytophilum* results in a nonspecific spectrum of symptoms including fever, headache, myalgia, anorexia, and chills (50). Serological studies of people living in endemic areas have shown seropositive rates in the range of 15% - 36%, yet diagnosis of symptomatic cases of HGA is far less common. Symptomatic cases can range in severity, with over half of them requiring hospitalization and 5%-7% requiring intensive care (46).

The case fatality rate in humans is reported to be less than 1%; however, severe opportunistic infections may be associated with *A. phagocytophilum* infection (49).

The major constitutively expressed proteins are encoded by the *msp2* multigene family, vary in size between 42 and 49 kDa, and are expressed on the outer membrane (8,47,120,173,174). The amino acid sequences of the major outer-membrane proteins are similar to those of *E. chaffeensis*, *E. canis,* and *E. ruminantium.*

Despite the homology between the P28 proteins of *Ehrlichia* and the Msp2/P44 proteins of *Anaplasma*, the locus structure and mechanism of antigenic variation in expression are quite different. The *Ehrlichia* P28 locus consists of 17 to 22 full length genes which are independently expressed. In *Anaplasma* there is a single polycistronic expression site, in which different *p44* genes can recombine to generate antigenic diversity and facilitate persistence in the host (10). The individual *p44* genes consist of a central hypervariable region with conserved 5' and 3' flanking sequences. Recent genomic sequencing of *A. phagocytophilum* has revealed 22 full-length *p44* genes with start and stop codons and 64 "reserve/silent" *p44* genes that lack start codons. These genes are arranged symmetrically around the *p44* expression site, and are located near the origin of replication. There are also 21 *p44* gene fragments which lack any hypervariable region sequence, and six truncated *p44* genes containing partial sequence from the hypervariable region. These fragments and truncations lack part of the pair of flanking sequence necessary for recombination into the *p44* expression site (51).

This system of homologous recombination into an expression site to generate antigenic diversity aiding in persistent infection is completely lacking in *Ehrlichia*, yet they are still capable of persistent infection. The exact mechanism by which this persistence is achieved in *Ehrlichia* remains one of the great questions of ehrlichiology.

Ehrlichia ruminantium, a pathogen of cattle

This species of *Ehrlichia* was formerly designated as *Cowdria* (157). It is similar to the other *Ehrlichia*, except that it can be found free in the cytoplasm as well as inside membrane-bound vacuoles typical of other *Ehrlichia* species (16). It is transmitted by at least 10 different species of *Amblyomma* ticks (133,134).

The genome of *E. ruminantium* is 1,516,355 bp consisting of 920 identified open reading frames (ORFs) with a GC content of 27.5% (51).

The major constitutively produced protein antigen is encoded by the MAP1 multigene family, varies between 31 and 32 kDa in size, and is expressed on the outer membrane. The amino acid sequence of the MAP1 proteins is highly homologous to the major outer membrane proteins in *E. chaffeensis* (P28), *E. canis,* and *A. phagocytophilum*

(MSP2). When compared to other ehrlichiae, however, the actual number of alleles in the MAP1 locus is reduced, with only 16 paralogs (156).

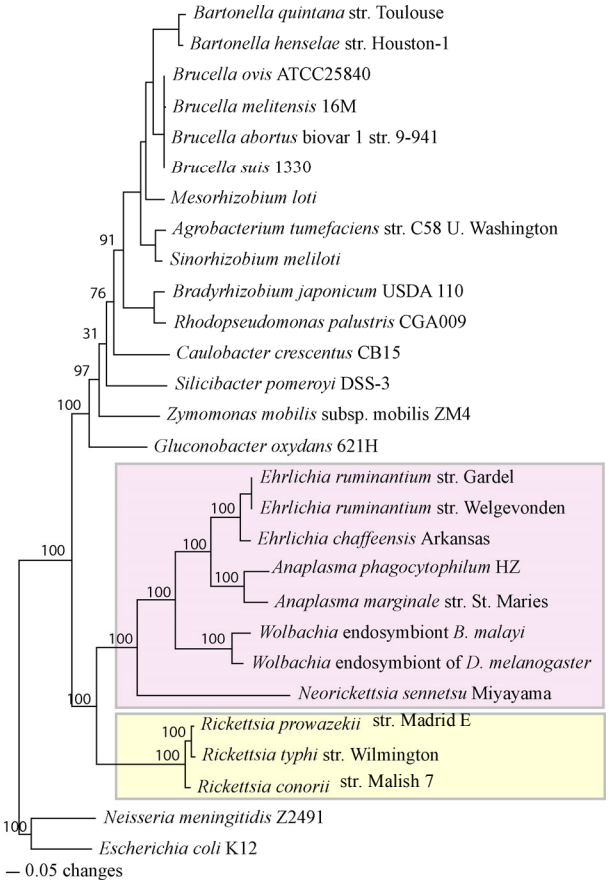


Figure 3 – Phylogenetic tree of the sequenced α-Proteobacteria, with a focus on the family Anaplasmataceae. The protein sequences of 31 housekeeping genes (*frr, infC, nusA, pgk, pyrG, rplA, rplB, rplC, rplD, rplE, rplF, rplK, rplL, rplM, rplN, rplP, rplS, rplT, rpmA, rpoB, rpsB, rpsC, rpsE, rpsI, rpsJ, rpsK, rpsM, rpsS, smpB,* and *tsf*) from complete α-Proteobacteria genomes genes were concatenated and aligned, and a phylogenetic tree was inferred of all sequenced α-Proteobacteria (51). The family Anaplasmataceae (purple) and the family Rickettsiaceae (yellow) are highlighted.[1]

[1] Dunning Hotopp JC, Lin M, Madupu R, Crabtree J, Angiuoli SV, et al. (2006) Comparative Genomics of Emerging Human Ehrlichiosis Agents. PLoS Genet 2(2): e21. Reproduced with permission under the Creative Commons License.

Transmission of *Ehrlichia chaffeensis*

Ehrlichia chaffeensis is transmitted primarily by *Amblyomma americanum*, also known as the lone star tick. The lone star tick is found throughout the south-eastern and south-central United States. Prior to the discovery of their transmission of ehrlichiae, these ticks were thought to be primarily a nuisance species. Subsequently, they have been shown to transmit a variety of pathogenic organisms. *Ehrlichia chaffeensis* is transmitted transstadially but not transovarially by these ticks (106).

The lone star tick *A. americanum* was first suspected as a potential vector of *E. chaffeensis* when the distribution of HME cases was shown to be similar to the host range of *A. americanum* (54). Subsequently, experimental transmission of *E. chaffeensis* was demonstrated between white-tailed deer using adult and nymphal *A. americanum* (57).

The potential for other routes of transmission exist, as *E. chaffeensis* has been shown to be stable in refrigerated anticoagulated blood for at least 11 days, and the blood supply is not currently screened for ehrlichial agents (115). However, there is little evidence to support a significant role for alternative modes of transmission at this time.

There is seasonality to *E. chaffeensis* infection which is directly correlated to the life cycle of *Amblyomma americanum*. Most cases of *E. chaffeensis* infection are reported between the months of March and November, with 70% of them occurring in the period between May and July (60,61,151). Nymphal and adult *A. americanum* are in their peak period of feeding during this time. Late fall and winter cases of *E. chaffeensis* infection are sometimes reported in the more temperate southern extremes of the *A. americanum* range (140).

Ehrlichia chaffeensis survives in the environment by persistently infecting animal hosts, primarily the white-tailed deer *Odocoileus virginianus* (98-100). Other animals, including domestic dogs (39,121)and wild canids such as coyotes (84), have been shown to be infected in the field as well. There is a single report of a persistently infected domestic goat (45). Since *Amblyomma* eggs do not contain ehrlichiae, this persistent infection of animal hosts gives the ehrlichiae a means of persisting in the environment year after year, along with the overwintering of infected nymphal and adult ticks (103).

Humans are accidental hosts, who acquire ehrlichial infection after being bitten by an infected tick. The lone star tick is well known for feeding on a wide variety of host animals, and feeding aggressively (19,31,85). The wide variety of potential hosts for *E. chaffeensis* may provide an explanation for some of the diversity found in its P28 outer membrane protein family.

Expansion of the white tailed deer population in North America, combined with greater incursions into wildlife habitat by human development and expansion of urban centers, has been hypothesized to explain the emergence of human monocytotropic ehrlichiosis and other tick borne infectious diseases (128).

Climate change may lead to expansion of the range of the vector tick species for these *Ehrlichia* as well, possibly leading to a greater incidence of disease (29).

Clinical and epidemiological aspects of human monocytotropic ehrlichiosis

In the year 2000, 200 cases of human monocytotropic ehrlichiosis were reported to the CDC, an increase of more than 30% from the previous year (26). The number of cases reported had increased to 321 in 2003 (27). Despite this relatively low number of reported cases, the actual incidence of *E. chaffeensis* infection is likely much higher due to the difficulties of correctly diagnosing infections as well as the wide variety of illness severity. Also, active surveillance has demonstrated that passive reporting results in drastic underreporting of disease. One study in southeastern Missouri showed provisional estimates of 8 to 14 cases per 100,000 population during 1997 and 1998 (125). Another study of hospitalized patients in Georgia showed a higher incidence of HME than Rocky Mountain spotted fever during the period studied (61).

Immunocompromised patients are at particular risk of dying from fatal ehrlichial infections. In one case series of human immunodeficiency virus-infected persons with human monocytotropic ehrlichiosis, more than half the patients died as a result of their ehrlichial infection (129). There have also been case reports of severe or fatal disease in patients with monoclonal gammopathy (40), asplenia (54,59), sickle beta-thalassemia (145), and Down's syndrome (53). Age >60yr has been shown to be an independent risk

factor for severe or fatal disease (60). It has been suggested that the apparent dependence of *Ehrlichia* on host cholesterol for membrane stability may account for enhanced disease severity in older adults, who tend to have higher serum cholesterol levels; however, no direct evidence exists for this effect (95).

Despite the apparent role that age and immunocompromise play in severe disease, cases of severe or fatal ehrlichiosis have been reported in otherwise healthy children and young adults (18,59,64,109,111,145).

The clinical syndrome is markedly nonspecific, with an onset of symptoms usually one to two weeks after the tick bite. Patients present with symptoms classical for a nonspecific viral illness, which can include fever, headaches, myalgia, nausea, arthralgia, and malaise (56,60). More rarely, patients may experience cough, pharyngitis, lymphadenopathy, diarrhea, vomiting, abdominal pain, and mental status changes (56,60,151).

Neurological complications are typically limited to headaches, but mental status changes are seen in 20% of patients. Symptoms consistent with meningeal irritation, including nuchal rigidity, photophobia, and severe headache are reported in as many as 10% of patients. Complications associated with CNS involvement, including hyperreflexia, seizures, ataxia, cranial nerve palsies, optic neuritis and demyelinating polyneuropathy, have been observed (78).

THE MOLECULAR PATHOGENESIS OF *EHRLICHIA CHAFFEENSIS*

Little is known of the detailed molecular interactions that allow *E. chaffeensis* to bind to host monocytes, invade and survive and replicate inside unique intracellular vacuoles, called morulae, which possess markers for caveolae as well as early endosomes (96). The recent completion of genomic sequencing of *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis, E. canis*, and *Neorickettsia sennetsu* allows for interesting comparisons between the different members of the family Anaplasmataceae and family Rickettsiaceae within the order Rickettsiales (51,112).

Immunoreactive proteins of *Ehrlichia chaffeensis*

The most recent findings in the area of molecular pathogenesis have provided few clues to the larger picture of the pathogenesis of *E. chaffeensis* infection. The immunoreactive glycoprotein GP120 is differentially expressed on dense core cells, and confers an invasive phenotype on *E. coli* transformants which express it (136). Comparative genomic studies have revealed that the *gp120* is unique to *E. chaffeensis* and *E. canis*, as no ortholog could be found in the known sequences of other members of the order Rickettsiales (51). The fact that *E. chaffeensis* and *E. canis* share a common tropism for cells of the monocyte / macrophage lineage combined with the observed behavior of *gp120*-expressing *E. coli* is highly suggestive of a role for *gp120* in host cell recognition, binding, and invasion.

Iron acquisition is critical to the ehrlichial life cycle (12,13). Our group previously identified a 37-kD protein of *E. chaffeensis*, which possesses homology to iron binding proteins in other gram-negative bacteria (165). Our recent work has determined that there are two distinct proteins, a 38-kD ferric binding protein Fbp which has been identified in *E. canis* and *E. chaffeensis*, as well as a 36-kD major immunoreactive protein in *E. canis* which bears homology to a 55-kD immunoreactive protein in *E. chaffeensis* (113).

We sequenced the genes of the locus encoding the p28 outer membrane protein family (168). This family of 22 complete, paralogous genes forms a 27 kilobase locus in the *E. chaffeensis* genome (123).

For many of the surface proteins of *E. chaffeensis,* no functional role has been demonstrated, and only their antigenic nature has been determined. One major factor contributing to this void is the lack of an appropriate antibiotic for transformation studies. The only antibiotic available for selection of *E. chaffeensis* transformants is also the only widely available safe and effective treatment for *E. chaffeensis* infection, and therefore ethically it cannot be used. To circumvent the presently insurmountable obstacle to transformation of *E. chaffeensis*, the homologous murine pathogen *E. muris* was used as a model. The *E. muris* mouse model has been well characterized (126). *E. chaffeensis* and *E. muris* are very closely related, based upon comparisons of groEL and 16S RNA

phylogeny. The *E. muris* groEL amino acid sequence is only 0.9% divergent from that of *E. chaffeensis,* while the 16S rRNA gene sequence of *E. muris* is 97.7% similar to that of *E. chaffeensis* (161,167).

Known adhesins of *Ehrlichia chaffeensis*

Previously, *in vitro* studies using HeLa cells and *E. coli* expressing the 120-kDa outer membrane protein of *E. chaffeensis* (*gp120*) have shown that it is an adhesin which may be involved in the internalization of ehrlichiae (136). However, studies involving knockouts of the *gp120* or another system more closely approximating the natural infection of monocytes by ehrlichiae have not been conducted.

Role of iron in ehrlichial survival

Recent studies have shown that ehrlichiae have developed systems for iron acquisition, which may be essential for survival within host cells. Deferoxamine, which is a known iron chelator, inhibits the growth of *E. chaffeensis,* and interferon gamma has been shown to kill *E. chaffeensis* via iron depletion (12). Early endosomes which contain *E. chaffeensis* accumulate transferrin receptors (TfR) (13,119). Also, cells infected with ehrlichiae increase expression of iron-responsive protein 1, which increases cellular expression of transferrin receptor in the host cells (11). *E. chaffeensis* itself expresses a 37 kDa protein Fbp which is similar to iron binding proteins found in other gram-negative bacteria (165).

Fbp has been demonstrated within the cytoplasm, periplasm and on the surface of ehrlichiae (44,164). It has also been shown to associate with the fibrillar morular matrix associated with dense core forms, as well as the morular membrane (44). Fbp is likely the ~40kD immunoreactive protein found to associate with caveolar membrane preparations from *E. chaffeensis* infected THP-1 cells (96).

E. chaffeensis-infected morulae have been shown to accumulate high levels of TfR in their membranes by 6 hours post-infection, although the exact source of TfR is unclear since the morular membrane lacks the markers typical of endosomal TfR (13). Fbp could provide a crucial link between iron associated with the TfR on the morular

membrane and the ehrlichiae within the morula, shuttling iron from the membrane, through the fibrillar morular matrix to the ehrlichial cell walls for uptake and utilization in metabolic activities.

Subsequent genomic sequencing efforts have revealed orthologs of the ferric binding protein in *E. muris*, *E. ruminantium*, *A. phagocytophilum*, *A. marginale*, *N. sennetsu* and several *Wolbachia* species. Since *fbp* appears widely in the family Anaplasmataceae but is absent from the members of the family Rickettsiaceae that have been sequenced thus far, this further supports the theory that Fbp is involved in important metabolic functions necessary for living within the morulae that would not be critical to *Rickettsia* species living free in the cytoplasm, such as iron acquisition.

The P28 multigene family

The P28 outer membrane protein family of *E. chaffeensis* is a member of the OMP-1/MSP2/P44 protein superfamily of the family Anaplasmataceae, which has also been designated as Pfam_PF01617 in the Pfam protein families database (14,51).

We have demonstrated antigenic diversity in the *p28* multigene family among differing strains of *E. chaffeensis* (105). Also, it has been shown that passive transfer of anti-p28 antibodies protects SCID mice from infection (94). It might be argued that the P28 protein does not play an important role in pathogenesis; however, this alternative seems unlikely given the protective effect of antibodies and the variation of the P28 protein in *E. chaffeensis*.

A great deal of research has been done to investigate the expression of the P28 multigene family in *E. chaffeensis*, at both the transcriptional and translational levels. Different research groups have, on occasion, arrived at different results.

Host cell specific expression of P28 proteins

Originally, Rikihisa *et al*. reported that mRNA transcripts could be found for all the P28 genes of *E. chaffeensis* isolate Arkansas, including the intergenic spacer regions, leading to the conclusion that a polycistronic messenger was transcribed (123). This was followed by the initial report of Long *et al.*, who found mRNA transcripts for 16 of the 22 p28 genes of *E. chaffeensis* isolate Arkansas (Figure 4) (105).

Singu *et al.* followed this work with two dimensional SDS PAGE proteomics analysis, in which he found two proteins, P28-19 and P28-20, expressed in DH82 canine monocytes, while another P28, P28-14, was expressed in ISE6 tick cells. In DH82 cells, the P28-20 was detected in only two of three experiments (148).

He followed this work in 2006 by comparing several different isolates of *E. chaffeensis*, all of which produced P28-19, P28-20 or both, in DH82 cells. He then examined *E. canis* and found that the P30-1, P30 and P30-20 were expressed in DH82 cells, while P30-10 was expressed in ISE6 tick cells. These *E. canis* P30s found in DH82 cells are homologous to the *E. chaffeensis* P28-19, P28-20, and P28-21 while the *E. canis* P30-10 in ISE6 cells is homologous to the *E. chaffeensis* P28-14 (149).

The role of P28 proteins in antigenic variation

The role of the P28s in the pathogenesis of *Ehrlichia* infection has been long debated, with some contention as to their potential role in antigenic variation. While Singu *et al.* proposed in 2005 that the host cell specificity of P28 expression is indicative of an important role in antigenic variation, Zhang *et al*. showed previously that canine hosts rapidly generate an antibody response capable of reacting with almost all P28 proteins, implying either that all had been produced and stimulated the canine immune system, or a sufficient subset had been produced to generate antibodies which cross-reacted with all other members of the P28 family (169).

It seems more likely that the diversity of P28 proteins conserved in the ehrlichial genome relates to a functional role other than antigenic diversity, yet a role that has a great deal of evolutionary significance for an organism that has otherwise tended towards genomic reduction.

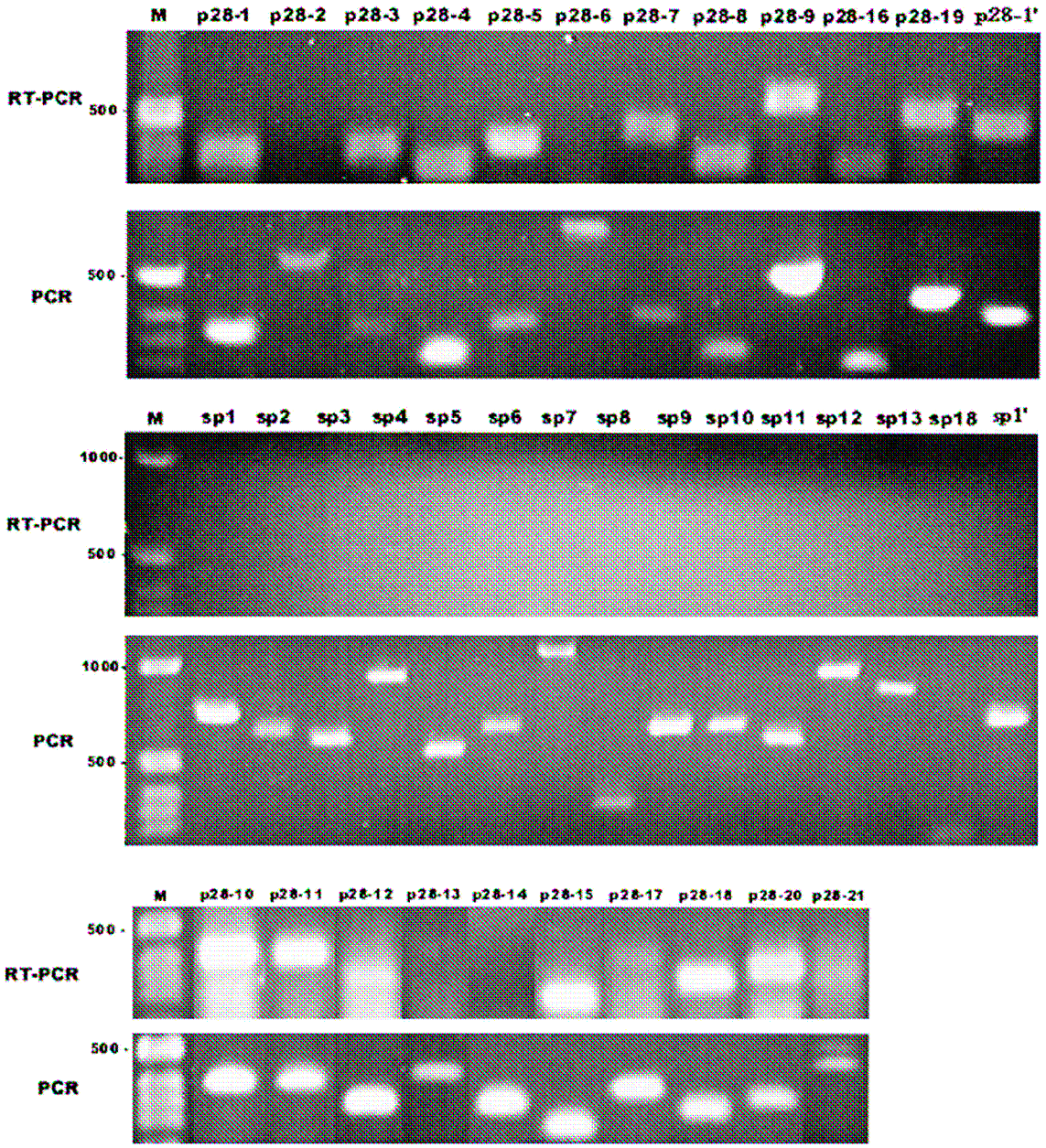


Figure 4 – Transcriptional analysis of the *p28* genes and their intergenic sequences (sp) by RT-PCR. PCR amplification of the DNA template with *Taq* polymerase is shown as a positive control.[2]

[2] Long SW, Zhang X-F, Qi H, Standaert S, Walker DH, Yu X-J. Antigenic variation of *Ehrlichia chaffeensis* resulting from differential expression of the 28-kilodalton protein gene family. *Infect Immun* 2002;70(4):1824-31. *Copyright © 2002, the American Society for Microbiology. All rights reserved.*

Variations in the P28 locus

It is possible that the variety of P28 proteins in the ehrlichial genomes represents a set of host cell receptors for the large number of different host cells which ehrlichiae might encounter during their life cycle, including the tick and animal hosts. Given the large natural host range of the vector tick *Amblyomma americanum*, ehrlichiae would encounter a large number of potential host animals. A wide variety of P28 proteins would provide an optimal array of host cell-specific ligands for cell recognition and binding. This situation also may allow for the stability of the ehrlichial cell membrane in a wide variety of environments during its life cycle.

Small deletions in the P28 locus have been noted in some clinical isolates, indicating that the various P28s may be capable of some degree of complementation under certain circumstances. Variation is also noted in the *p28-19* sequence in separate clinical isolates of *E. chaffeensis* (Figure 5). Different species of *Ehrlichia* have varied P28 loci, in some cases having even fewer P28 members. *E. muris* lacks the P28-21 found in *E. chaffeensis* (35). The Map1 locus of *E. ruminantium* is particularly reduced compared to the P28 locus of *E. chaffeensis*, consisting of only 16 alleles. This may in part explain why *E. ruminantium* is not a pathogen of humans or canines, but mostly of large ruminants.

In contrast to these examples of contraction of the locus, there are also stunning examples of expansion at the *msp2*/*p44* locus of *Anaplasma*. As discussed previously, *Anaplasma* employ an expression site where *msp2/p44* genes can recombine resulting in antigenic diversity that aids in persistent infection of animals. As a result of these recombination events, there are 86 *p44* homologs with and without start codons that are involved in *p44* expression and antigenic variation in *Anaplasma phagocytophilum.*

Recently, the Map1 locus of several *E. ruminantium* isolates has been sequenced and compared, revealing an active process of genome size plasticity (66). Three strains were sequenced, the Gardel strain (designated Erga) and two different Welgevonden derived strains (designated Erwo and Erwe, respectively).

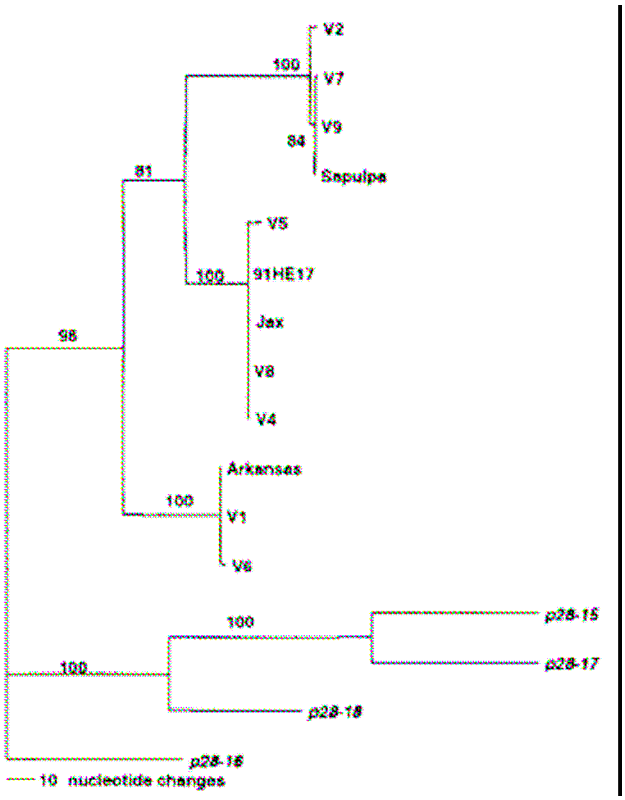


Figure 5 –Phylogenic analysis of the *p28-19* allele of 12 different *E. chaffeensis* isolates. *p28-15* to *p28-18*, which are the closest genes to *p28-19*, were used as the out-group. Bootstrap values are shown at the nodes of the branches.[3]

[3] Long SW, Zhang X-F, Qi H, Standaert S, Walker DH, Yu X-J. Antigenic variation of *Ehrlichia chaffeensis* resulting from differential expression of the 28-kilodalton protein gene family. Infect and Immun 2002;70(4):1824-31. *Copyright © 2002, the American Society for Microbiology. All rights reserved.*

The evolution of an intracellular bacterium is marked by a period of genomic size reduction followed eventually by a stabilization between the competing demands of generating diversity while shedding useless genetic material (117).

Analysis of the *E. ruminantium* strains revealed several interesting features. *E. ruminantium* has one of the lowest coding ratios observed so far among all annotated bacterial and archaebacterial genomes (63-64%). Although a general increase in intergenic spaces is a feature of many intracellular bacteria, this feature is very pronounced in *E. ruminantium*.

The *E. ruminantium* genome is notable for observed expansion and contraction even over short periods of time and in response to changing cell environments, including recombination events in the *map-1* locus in the CTVM-Gardel strain (15).

P28 orthologs in other bacteria

It is interesting to note that proteins belonging to the OMP-1/MSP2/P44 superfamily defined by Pfam_PF01617 can be found in other related alpha-proteobacteria, including facultative intracellular parasites and free living bacteria.

In the order Rhizobiales, three pathogenic species of *Brucella* are found to possess orthologs, only one of which, the heat resistant agglutinin 1 (HRA1) of *Brucella melitensis*, has been characterized. *Agrobacterium tumefaciens* also possesses one ortholog, as does the root nodule bacterium *Rhizobium loti*.

In the order Rhodospirillales, *Gluconobacter oxydans* possesses a homologous outer membrane protein as well. *G. oxydans* is noted for its ability to convert ethanol into acetic acid. As a consequence, it is used in the production of vinegars, as well as implicated in the spoilage of beer and wine. It is interesting to note that *E. chaffeensis* morulae are thought to be weakly acidic, a similar pH environment to that which *G. oxydans* regularly encounters (119).

Moving out of the alpha-proteobacteria, two members of the delta-proteobacteria family Geobacteraceae, *Geobacter sulfurreducens* and *Geobacter metallireducens* strain GS-15 possess orthologs which are homologous to the 26 kD Tia invasion determinant-related protein of *Chlorobium tepidum*, a photosynthetic green sulfur bacterium (3). The

Tia invasion determinant protein was discovered in an invasive enterotoxic *E. coli* strain, and the *C. tepidum* homolog is also highly homologous to HRA1, as well as the HEK adhesion/virulence protein (62). All of these proteins are expressed on the bacterial cell surface and share motifs which are implicated in host-pathogen interactions involving host cell surface heparin sulfate proteoglycans (3).

Considering these data along with the expansion of the OMP-1/MSP2/P44 family in so many *Ehrlichia* and *Anaplasma* species, it seems most likely that the P28s are involved in recognition of host cell surface molecules, allowing for subsequent binding and entry into the host cells. The generation and preservation of such a diverse genomic locus in genomes which have largely been focused on reduction as they adapted to obligate intracellular life must be critical to survival. As these organisms possess natural life cycles involving transitioning between an unpredictable diversity of host types, possessing a wide variety of suitable cell recognition molecules would be paramount to survival.

It also seems probable that the P28s provide significant structural support to the ehrlichial cell membrane, especially considering the lack of peptidoglycan and LPS (95).

The necessity of further research on the P28 membrane protein family

In conclusion, the paucity of research exploring the molecular pathogenesis of *E. chaffeensis* combined with our preliminary observations of the P28 protein family make it clear that there is a need to identify the role of the P28 protein in ehrlichial pathogenesis. Lack of this knowledge is preventing further research into the overall molecular pathogenesis of *E. chaffeensis* and related ehrlichial pathogens. Without taking steps to advance the state of knowledge on the molecular level, progress into development of new therapeutics and vaccine efforts will be hindered.

HOST-PATHOGEN INTERACTIONS

Monocytes

Monocytes are derived from the myeloid lineage of hematopoietic stem cells, and function in antigen presentation and host defense. They have a variety of host cell surface receptors.

The receptors found on monocytes can be divided into several broad categories. These include receptors for cell recognition, cell signaling and cell adherence. We will concern ourselves mainly with one receptor in particular, the P-selectin glycoprotein ligand 1 (PSGL-1) also known as CD162.

Also, the ehrlichiae are capable of growing inside membrane-bound vacuoles within the monocyte. These *Ehrlichia*-containing vacuoles are known as morulae. The exact method by which ehrlichiae exist inside morulae and prevent phagosome-lysosome fusion is unknown. However, it is worth noting that ehrlichial morulae exist as unique vacuolar compartments with some markers consistent with early endosomes, other markers typical of caveolae, and that lysosomal fusion is avoided (119,171).

Membrane cholesterol

E. chaffeensis and *A. phagocytophilum* both lack genes for synthesis of the peptidoglycan and lipid A of lipopolysaccharide (LPS). Consequently, they have been discovered to incorporate significant amounts of membrane cholesterol from their eukaryotic host cells. Extraction of membrane cholesterol results in ultrastructural disturbances of the ehrlichial cell membrane and in decreased infectivity, which is reversible with the addition of exogenous water soluble cholesterol (95).

Cytokine response to *E. chaffeensis* infection

Infection of THP-1 human monocytes with *E. chaffeensis* results in the release of cytokines, although the increase is less profound than that induced by *E. coli* LPS. At 2 hours post-infection, levels of interleukin-1β (IL-1β), IL-8 and IL-10 increase, while IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNF-α) are not induced. The elevated cytokine levels are detected between

21 to 24 hours post-infection, returning to baseline faster than an LPS-induced response. Heat-killed *E. chaffeensis* induce the same response, while periodate-treated ehrlichiae do not, indicating that an ehrlichial carbohydrate is responsible for inducing this pattern of cytokine expression (88).

Subsequently, it was shown that anti-*E. chaffeensis* antibodies, when used to treat ehrlichiae prior to infection, result in a more robust IL-1β expression in THP-1 cells. Antibody binding also results in the induction of TNF-α and IL-6, and the expression levels of IL-1β, TNF-α, and IL-6 are similar to those seen in *E. coli* LPS controls. Reaction of *E. chaffeensis* with anti-*E. chaffeensis* Fab fragments completely abolishes the induction of IL-1β, TNF-α, and IL-6, indicating that ehrlichial binding to the host cell is required for IL-1β induction, while Fcγ receptor binding is required for the TNF-α and IL-6 response (89).

Interferon gamma-mediated killing of *E. chaffeensis*

Interferon gamma (IFN-γ) has been shown to be capable of triggering the killing of intracellular *E. chaffeensis*, via a mechanism that results in iron depletion (12). However, this anti-ehrlichial effect has been shown to vanish 24 hours post-infection. *E. chaffeensis* blocks tyrosine phosphorylation of Stat1, Jak1 and Jak2 within 30 minutes of infection. Phosphorylation of these signaling molecules is part of the normal cellular response to IFN-γ. Heat and proteinase K treatment of *E. chaffeensis* abolish the ehrlichial inhibition of tyrosine phosphorylation by IFN-γ, but periodate treatment does not, indicating that ehrlichial protein and not carbohydrate is responsible for the effect.

Furthermore, reaction of *E. chaffeensis* with anti-*E. chaffeensis* Fab fragments abolishs the inhibitory effect. Treatment with monodansylcadaverine (MDC), a transglutaminase (TGase) inhibitor which blocks ehrlichial internalization but not binding, does not abolish the ehrlichial inhibition of IFN-γ induced tyrosine phosphorylation, indicating that ehrlichial binding alone is sufficient to inhibit Stat1 phosphorylation. Treatment of THP-1 cells with a protein kinase A (PKA) inhibitor partially abolishes the ehrlichial inhibitory effect, suggesting that *E. chaffeensis* avoids the anti-ehrlichial effect of IFN-γ via stimulation of PKA activity (90).

Intracellular signaling events associated with *E. chaffeensis* internalization and growth

Infection of macrophages by *Neorickettsia risticii* is inhibited by cytochalasins, the transglutaminase inhibitor MDC, taxol, calcium channel blockers, calmodulin agonists, and protein tyrosine kinase (PTK) inhibitors by interfering with internalization, resulting in an inhibition of proliferation as well (12,142,172). Calcium also plays a role in the internalization of *Salmonella enterica* serovar Typhimurium (127). Protein tyrosine kinases have been shown to be involved in the internalization of enteropathogenic *E. coli* (EPEC), *Listeria, Yersinia,* and *Campylobacter jejuni* (143,144,158,162).

Given this wealth of prior experience, the effect of inhibition of these pathways on *E. chaffeensis* infection of THP-1 cells was investigated. The TGase inhibitor MDC, the PTK inhibitor genistein, the phospholipase C (PLC) inhibitor neomycin, and the calcium channel blocker verapamil all prevent internalization of *E. chaffeensis* in THP-1 cells. Furthermore, MDC, genistein, verapamil, neomycin, the PLC inhibitor U-73122, and the intracellular calcium mobilization inhibitor 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) inhibit proliferation of *E. chaffeensis* within THP-1 cells (97) .

Infection with *E. chaffeensis* results in a rapid increase in IP_3, a signaling molecule produced by PLC-γ after tyrosine phosphorylation. IP_3 binds to receptors on the endoplasmic reticulum which release stored calcium. Inhibitors of PLC, PTK and TGase all block this increase in IP_3 (97). It was further demonstrated that the activity of the PLC-γ2 isoform, and the subsequent increase in intracellular calcium, is essential in THP-1 cells for *E. chaffeensis* infection. TGase is a calcium-dependent enzyme, and fusion of transferrin receptor (TfR)-containing endosomes with morulae is a calcium-dependent process. A positive feedback loop may exist, consisting of TGase activation of tyrosine kinases which in turn activate PLC-γ2, resulting in further release of intracellular calcium stores. PLC-γ2 and tyrosine phosphorylated proteins have been found localized to the morular membrane (97).

The nature of the morular membrane

The morular membrane defines a unique cytoplasmic compartment. The morular membrane possesses some markers of early endosomes, such as early endosomal antigen 1 (EEA1) and Rab5 (13). However, it lacks clathrin and contains vesicle-associated membrane protein 2 (VAMP-2 or synaptobrevin-2), small quantities of MHC class I and II, and beta2-microglobulin not commonly found in early endosomes (11,13,119).

As discussed previously, membrane cholesterol is very important in ehrlichial infection (95). Since cholesterol is an essential component of lipid rafts and caveolae, their potential role in *E. chaffeensis* was investigated. Cholera toxin B (CTB) binds the GM1 ganglioside associated with caveolae and results in their internalization via caveolae, depleting them from the cell surface. Pretreatment of host cells with CTB results in a 90% inhibition of *E. chaffeensis* or *A. phagocytophilum* infection. Furthermore, the morular membrane contains caveolin-1, a well established caveola marker (96).

GPI-anchored proteins are another common feature of lipid rafts and caveolae, and have been implicated in a few facultative intracellular bacterial infections, including *Brucella*. Pretreatment of host cells with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove GPI-anchored proteins from the cell surface inhibits ehrlichial internalization (96).

The morular membrane is also unique in that it contains several immunoreactive proteins of *E. chaffeensis*, with molecular weights of 30, 40, 55, 70 and 85 kDa. The 30 kDa proteins are likely the P28, and the 40 kDa protein is likely the iron binding protein Fbp. Also, PLC-γ2 colocalizes with the lipid raft fractions of infected THP-1 cells, but is not found in the lipid rafts of uninfected cells (96).

P-selectin glycoprotein ligand-1

P-selectin glycoprotein ligand-1 (PSGL-1) is found on the surface of a variety of leukocytes, including granulocytes, monocytes, and T cells. It interacts to varying degrees with the cell surface proteins, P-selectin, E-selectin, and L-selectin, promoting adhesion and rolling in the inflammatory response. PSGL-1 is a dimeric protein which is

formed from two covalently linked 120 kD monomers, and some forms are extensively glycosylated (36).

The glycosylation found on PSGL-1 consists of primarily core-2 structure O-glycans containing the sialyl Lewis x antigen (116). Treatment of cells with sialidase to remove the glycosylation abolishes the interaction between PSGL-1 and P-selectin (122). Studies in Chinese hamster ovary (CHO) cells, which do not normally express PSGL-1, have shown the necessity of glycosylation for interaction with P-selectin. Co-transfection of the human alpha-1,3-fucosyltransferase IV (FucT-IV) or alpha-1,3-fucosyltransferase VII (FucT-VII) is required to generate a high affinity PSGL-1 ligand for P-selectin (110).

PSGL-1 has also been implicated in cell signaling in neutrophils, and dimerization of PSGL-1 appears critical to these signaling events (36).

A parallel role of PSGL-1 in *Anaplasma phagocytophilum* infection

Some direction may be drawn from the related species, *Anaplasma phagocytophilum*. The major surface protein 2 (Msp2) of *A. phagocytophilum* mediates adherence to human granulocytes, via the P-selectin glycoprotein ligand-1 found on neutrophils (76). Use of monoclonal antibodies against Msp2 results in reduced infection by *A. phagocytophilum in vitro* in both HL-60 cells and human neutrophils. Competition of soluble recombinant Msp2 (rMsp2) results in reduced binding of PSGL-1 specific monoclonal antibodies, indicating that rMsp2 interacts and binds with PSGL-1 in solution (132).

PSGL-1 can be found on monocytes as well as granulocytes (87). It may be that PSGL-1 plays a role in *E. chaffeensis* infection in binding of the P28 outer membrane protein, but the role of PSGL-1 in ehrlichial infections has not previously been studied.

CHAPTER 2

EFFECT OF A P28 GENE KNOCKOUT IN *EHRLICHIA MURIS*

As mentioned in Chapter One, the role of the P28 multigene family in *Ehrlichia* pathogenesis has been the subject of much debate. The transcriptional status of the multigene family has been contested, and the role of the family in antigenic variation is also debated (105,123,148). The role of the P28 multigene family of outer membrane proteins in the pathogenesis of *Ehrlichia* infection has not been described.

MATERIALS AND METHODS

The organism and cell culture

An isolate of *E. muris* (originally obtained from Y. Rikihisa) was cultured in the DH82 canine monocyte cell line. The wild-type cells were cultured in high glucose Dulbecco's Modified Eagle Medium (Gibco, Rockford, IL) with 5% fetal bovine serum (FBS) (Gibco, Rockford, IL) and 1 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (Mediatech, Herndon, VA), at 37°C with 5% CO_2. Transformed *E. muris* were maintained in the same medium, with a reduced amount of serum (2.5% FBS).

Polymerase chain reaction (PCR)

PCR was used to verify the presence of *E. muris* DNA in wild-type and transformant cell cultures. For increased sensitivity, a nested PCR of the 16S rRNA gene was used. The primary PCR was 30 cycles of 94 ^{o}C for 30 s, 58 ^{o}C for 30 s, and 72^{o}C for 45 s using primers ECC and ECB. The nested PCR was 30 cycles of 94 ^{o}C for 30 s, 55^{o}C for 30 s, and 72 ^{o}C for 45 s using primers ECC and HE3.(7,41), The PCRs were carried out using 2.5x HotMasterMix (Eppendorf, New York, NY) and PCR-grade water. All primers and probes were obtained from Sigma Genosys (Table 1).

Oligonucleotide name	DNA Sequence (5' to 3')
ECC	AGAACGAACGCTGGCGGCAAGCC
ECB	CGTATTACCGCGGCTGCTGGCA
HE3	TATAGGTACCGTCATTATCTTCCCTAT
catprobe	ACGGGGGCGAAGAAGTTGTCCATA

Table 1 – Primer and probe DNA sequences.

Transformation of *Ehrlichia*

Infected 150 cm^2 cell culture flasks (Corning, Acton, MA) with near 100 percent of cells containing wild-type *E. muris* were scraped, and the cells collected in 50 ml Falcon tubes (BD Biosciences, Bedford, MA). The cells were centrifuged in a Hettich Universal 32 R centrifuge (Hettich, Tuttlingen, Germany) at 240 x g, at 4 ºC, for 5 min. The supernatant was removed with a vacuum aspirator, and the cell pellets resuspended in 5 ml of sterile 10% glycerol in double-distilled water.

Approximately 5 grams of sterile glass beads were added to the 50 ml tube, and the cells were lysed by vortexing for 3 min. The resulting suspension was removed from the beads by pipetting to a new 50 ml Falcon tube. The lysed cell suspension was centrifuged at 240 x g at 4 ºC for 3 min. The supernatant was removed by aspiration and placed into a sterile Oakridge centrifuge tube, and the cell debris pellet discarded.

The supernatant was then pelleted in a Beckman centrifuge, J20 rotor, at 17,640 x g for 20 min. The supernatant was removed by aspiration, and the ehrlichial cell pellet then resuspended in 1 ml of sterile 10% glycerol prepared as described above; 100 µl of this suspension was placed into a 1 mm-gap GenePulser electroporation cuvette (Biorad, Hercules, CA). One µg of plasmid DNA was then added into the cuvette, along with 1 µl of TypeOne restriction inhibitor (Epicentre, Madison, WI).

The samples were then electroporated at 2.5 Kv, 200 ohms, and 25 microfaradays. For recovery, 1 ml of fresh MEM was then added into the cuvette, and the cells were allowed to recover at 37 ºC for 30 min to 1 hr. The entire contents of the cuvette were then added to a 25 cm^2 cell culture flask (Corning, Acton, MA) containing a monolayer of DH82 cells in fresh DMEM with 2.5% FBS and 1 mM HEPES.

Approximately 24 hr later, chloramphenicol was added at a concentration of 16 µg/ml (Sigma, St. Louis, MO). Fresh antibiotic was added during medium changes, which occurred approximately every three days, typically for two weeks to a month. Appearance of wild-type ehrlichiae after withdrawal of antibiotics after at least 2 weeks of treatment has not been observed.

Restriction endonuclease digestion

Restriction enzyme digestion was performed using enzymes from New England Biolabs (NEB, Ipswich, MA). Manufacturer-provided buffers were used in 50 µl reactions carried out at the optimal enzyme digestion temperature for one hour, followed by enzyme appropriate heat inactivation.

Southern blotting

Southern blots were performed by first separating the DNA samples of interest by gel electrophoresis in 0.5% Tris-Borate-EDTA buffer (Sigma, St. Louis, MO). The gel was prepared for capillary transfer according to the Roche DIG Hybridization protocol. In brief, the gel was depurinated for 10 min in 250 mM hydrochloric acid at room temperature with gentle shaking. The gel was rinsed with deionized water, and then placed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 15 min at room temperature, with gentle shaking. This denaturing wash was repeated, and the gel was then rinsed again with deionized water.

The gel was then placed in neutralization solution (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl) for 15 min at room temperature. This neutralization step was repeated; then the gel was placed in 20X SSC to equilibrate for 10 min. The separated DNA in the gel was then transferred to Nytran SuPerCharge membranes (0.45 µm pore size) using a TurboBlotter (Whatman Schleicher & Schuell, Florham Park, NJ). After transfer was complete, the membranes were placed on a piece of filter paper dampened with 2X SSC and crosslinked by ultraviolet irradiation for 2 min in a CL-1000 UV Crosslinker (Ultraviolet Products, Upland, CA).

An appropriate amount of DIG Easy Hyb solution with poly-A oligonucleotide was warmed to 37 ºC and placed into a hybridization bag with the blot and sealed (Roche, Indianapolis, IN). The blot was rocked in a hybridization oven at 37 ºC for 30 min. The *cat* gene-specific DIG-labeled oligonucleotide probe, catprobe (59.1 pmol), was added to fresh DIG Easy Hyb and boiled for 5 min before being cooled rapidly in an ice bath (Table 1). The prehybridization buffer was poured off the blot, and the probe solution added to the hybridization bag, which was then closed with a heat sealer and placed back into the oven to hybridize at 37 ºC overnight.

After hybridization, the blot was placed into a low stringency wash buffer (2x SSC, 0.1% SDS) for two consecutive 5 minute washes with rocking at room temperature. After this low stringency wash, the blot was placed into high stringency wash buffer (1x SSC, 0.1% SDS), which had been prewarmed to 37 ºC, and incubated for two consecutive 15 minute washes at 37 ºC with rocking.

The blot was then placed into 1X Detector block solution prepared according to the manufacturer's instructions and incubated with gentle shaking for 45 min at room temperature (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The same amount of Detector block solution was prepared, and a 1:10,000 dilution of anti-DIG alkaline phosphatase-conjugated antibody (Roche, Indianapolis, IN) was prepared. The blot was incubated with the conjugate solution for 1 hr at room temperature with gentle shaking. After this, the blot was washed three times for 5 min at room temperature in 1X Phosphatase Wash Solution, with gentle rocking (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The blot was rinsed twice for 2 min each at room temperature with gentle rocking in 1X Phosphatase Assay Buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

For colorimetric detection using BCIP/NBT, the assay buffer was drained from the blot and replaced with BCIP/NBT substrate, then incubated for 1 to 2 hours at room-temperature, followed by a distilled water wash to halt the development of the blot. For higher sensitivity colorimetric detection using TMB[U5], the assay buffer was drained

from the blot and replaced with TMB substrate, incubated for 5 to 15 minutes at room-temperature, followed by a distilled water wash to halt the development of the blot.

In the case of chemiluminescent detection, the assay buffer was drained completely from the blot prior to addition of CDP-Star chemiluminescent substrate, which was incubated with the blot for 5 min at room temperature and then carefully removed (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The blot was then placed between two pieces of plastic film and exposed to Hyperfilm ECL (GE Amersham Pharmacia, Piscataway, NJ) for various exposure times (10 s – 1 min, typically) to detect the hybridized bands.

The oligonucleotide probes were tailed with DIG-labeled uridines using the Oligonucleotide DIG-tailing kit obtained from Roche (Roche, Indianapolis, IN).

Confocal microscopy

DH82 cells infected with wild-type or transformed *E. muris* were centrifuged using a cytospin apparatus onto glass slides, and fixed in cold acetone/methanol (1:1) at minus 20 ºC for 10 min. The slides were then washed in PBS three times, 5 min per wash. After the wash, the slides were placed in a blocking solution containing 1% normal goat serum, 5% BSA (fraction V), and 0.3% Triton X-100 at room temperature for 30 min. Mouse anti-*E. chaffeensis* 1A9 monoclonal antibody (1:500 dilution) was used as the primary antibody to stain ehrlichiae, and sheep anti-CAT digoxigenin-labeled antibody (1:1000) was used as the primary antibody to stain the CAT protein. The primary antibodies were diluted in 1% BSA in PBS, centrifuged at 13,400 x g for 5 min and then added to each slide. The slides were washed in PBS three times for 20 min each.

Rhodamine-labeled sheep anti-DIG Fab fragments (1:100 dilution) and FITC-labeled anti-mouse IgG (1:100 dilution) were used as secondary antibodies and diluted in PBS containing 1% BSA, as described above. Slides were incubated with the secondary antibodies for 2 hr in the dark, and then washed with PBS at room temperature three times for 10 min each wash. The slides were incubated with DAPI at room-temperature for 2 min, fluorescent mounting medium was added, the coverslip was affixed, and the

slides then placed into a dark slide box and stored at 4 ºC until examined by confocal microscopy.

Electron microscopy

Monolayers infected with either wild type or transformed *E. muris* were fixed in a mixture of 0.5% glutaraldehyde, 2.5% formaldehyde, 0.03% trinitrophenol and 0.03% $CaCl_2$ in 0.05 M cacodylate buffer at pH 7.3 (136). The cells were scraped from the flasks and pelleted. For immunogold electron microscopy, the cell pellets were stained *en bloc* with 1% uranyl acetate in 0.1 M cacodylate at pH 5.2. The pellets were then embedded in LR White resin. For ultrastructural examination in plastic, the cell pellets were stained in 1% OsO_4 in 0.1 M cacodylate buffer after primary fixation. The cell pellets were then stained with 1% uranyl acetate in 0.1 M cacodylate, dehydrated in a series of ethanol dilutions, and embedded in Poly/Bed 812. Ultrathin sections were cut using a Sorvall MT-6000 ultramicrotome and examined in a Philips CM 100 electron microscope at 60 kV.

Immunogold staining

LR White sections on nickel Formvar-coated grids were stained with a mixture of a mouse anti-p28 monoclonal antibody (1A9) (136) at a dilution of 1:10 and a rabbit anti-chloramphenicol acetyl-transferase monoclonal antibody (Roche) at a dilution of 1:10. The diluent was 1% BSA in TBS. The sections were incubated with the primary antibodies for 1 hr at room temperature and then overnight at 4^{o}C. The secondary antibodies used to stain the LR White sections were a mixture of goat anti-mouse IgG + IgM (H + L) labeled with 5 nm gold particles (AuroProbe RPN430) and goat anti-rabbit IgG (H + L) labeled with 15 nm gold particles (AuroProbe RPN422), both at a dilution of 1:20 in 1% BSA in TBS. The sections were incubated with the secondary antibodies for 1 hr. at room temperature. The sections were then washed, fixed with 2% aqueous glutaraldehyde, and stained with 2% aqueous uranyl acetate (5 min) and lead citrate (30 s). The grids were then examined in a Philips CM 100 electron microscope at 60 kV.

Preparation of host cell-free *Ehrlichia*

Ehrlichia were separated from their host cells for proteomics analysis using a modification of the Pierce Mitochondrial Isolation Kit designed by Nagaraja Thirumalapura (Pierce, Rockford, IL).

Briefly, infected DH82 cells are processed according to the manufacturer's protocol with the following modifications. The 700 x g centrifugation step for the pelleting of cell debris is carried out at 1,500 x g to facilitate complete removal of host cell debris. Also, instead of the 12,000 x g centrifugation step to pellet mitochondria, the optional 3,000 x g spin was substituted, to facilitate a reduction in lysosomal and peroxisomal contaminants as recommended by the manufacturer. The Halt Protease Inhibitor Cocktail is used as directed in the protocol to inhibit protease activity. This protocol resulted in a very pure preparation of intact, host cell free ehrlichiae.

Two dimensional SDS-PAGE

Two dimensional SDS-PAGE was performed to separate proteins in the first dimension based on pI using immobilized pH gradient (IPG) strips and in the second dimension by size using conventional SDS-PAGE.

Ehrlichia were purified from their host cells using the modified mitochondrial isolation method as described. The cell pellet was then processed for two dimensional SDS-PAGE according to the manufacturer's protocol for the ZOOM IPG Runner system, using ZOOM 2D Protein Solubilizer 1 (Invitrogen, Carlsbad, CA). Lysates were prepared per the protocol with pH 4-7 ampholytes and used to hydrate pH 4-7 ZOOM IPG Strips. The IPG strips were focused in the ZOOM IPG Runner core using the ZOOM Dual Power programmable power supply. The electrofocusing protocol consists of 15 minutes at 175 V, a ramp from 175 V to 2000 V over 45 minutes, followed by a 105 minute focusing at 2000 V. The IPG strips were processed immediately for SDS-PAGE or stored at -80 degrees C.

To separate the IPG strips in the second dimension, they were alkylated and placed into NuPage 4-12% Bis-Tris ZOOM SDS-PAGE gels with 400 microliters of 0.5% agarose in 1X MES buffer (50 mM Tris base, 50 mM 3-(N-morpholino)

propanesulfonic acid, 1 mM EDTA, 0.01% SDS at pH 7.3). Electrophoretic separation was carried out at 200V for 45 minutes in MES buffer according to the manufacturer's directions. Gels were subsequently stained using SafeBlue stain according to the maximum sensitivity protocol (Invitrogen, Carlsbad, CA).

MALDI-TOF/TOF mass spectral analysis

All MALDI-TOF mass spectroscopy was done at the Mass Spectrometry Core in the Biomolecular Resource Facility at UTMB. Protein samples were excised from two dimensional NuPage 4-12% Bis-Tris ZOOM SDS-PAGE gels using a new razor blade and placed in 50 microliters of PCR grade water (Invitrogen, Carlsbad, CA).

Gel pieces were incubated with trypsin (20 µg/ml in 25 mM ammonium bicarbonate, pH 8.0; Promega Corp.) at 37˚C for 6 h. One µL of the digested sample was deposited onto the MALDI plate and allowed to dry. One µL of matrix (alpha-cyano-4-hydroxycinnamic acid; Aldrich Chemical Co.) was then applied on the sample spot and allowed to dry. MALDI-TOF/TOF MS was performed using an Applied Biosystems model 4700 Proteomics Analyzer for peptide mass fingerprinting and MS/MS analysis. Following MALDI MS analysis, MALDI MS/MS was performed on several ions from each sample spot. Applied Biosystems GPS software was used in conjunction with MASCOT to search databases for protein identification (37,131). Protein match probabilities were determined using expectation values and/or MASCOT protein scores. Detailed information related to the MALDI-TOF/TOF instrument and software settings can be found in Appendix A.

Since the entire genome of *E. muris* has not been sequenced, in some instances it was necessary to manually compare the peptide sequence data from the MALDI-TOF instrument with *in silico* protein digests generated using the MS-DIGEST program, part of the Protein Prospector package available from the University of California at San Francisco (http://prospector.ucsf.edu/) (30). The PePeR software program was designed to facilitate the comparison of a peptide peak dataset with a large number of *in silico* protein digests.

Statistical analyses

The transformed and non-transformed *E. muris* from both the plastic embedded cells and the LR White embedded cells were measured (at least 15 bacteria were measured from each group), and the average sizes determined. The average sizes from each group were statistically compared by the chi-square (χ^2) method and were considered statistically significant if $P < 0.05$.

DEVELOPMENT OF A P28 KNOCKOUT STRAIN OF *EHRLICHIA MURIS*

Determination of the antibiotic susceptibility of *Ehrlichia muris*

In order to choose an antibiotic for the selection of *E. muris* transformants, the antibiotic susceptibility of *E. muris* had to be determined. The antibiotics chloramphenicol and tetracycline were chosen because they both have readily available antibiotic resistance genes and have shown utility previously in the treatment of *E. chaffeensis* infection or *in vivo* studies. Similar to the methods of Brouqui and Raoult, cultured P388D1 murine monocytes approximately 25% infected with *E. muris* were exposed to three concentrations of chloramphenicol (8,16, and 32 μg/ml) and 4 concentrations of tetracycline (1,5,10, and 20 μg/ml) in a 24-well plate format (23). Cells were treated for 3 days, then washed with sterile PBS and given antibiotic-free medium to allow for growth of any surviving ehrlichiae. Cells were checked at 24, 48 and 72 hours post-treatment to evaluate for % infection by Diff-Quik staining, as well as at 48 and 96 hours post-antibiotic removal. Slides were blinded and read independently by two investigators, and compared to control cells which received no treatment.

While the control population proceeded towards 100% infection and cell death, all treated cell populations showed complete clearance of morulae, with only the lowest concentration of chloramphenicol showing growth at day 7 (Figure 6).

The treatment with chloramphenicol exhibited slightly less bacterial killing at 24 hours than tetracycline compared to the untreated controls, which given the need of transformed cells to recover from the trauma of electroporation may be advantageous. This combined with the important clinical utility of tetracycline as the gold standard of

ehrlichial treatment led us to choose chloramphenicol as the antibiotic for our transformation model system.

The fact that *E. muris* is susceptible to chloramphenicol while *E. chaffeensis* has been reported previously to not be susceptible *in vitro* leads to some serious questions related to the antibiotic susceptibility of *E. chaffeensis*. Recently, the susceptibility of *Ehrlichia* species was reexamined using real-time PCR; however, the authors did not retest the susceptibility of *E. chaffeensis* to chloramphenicol, deciding instead to simply rely on the previously published results (21).

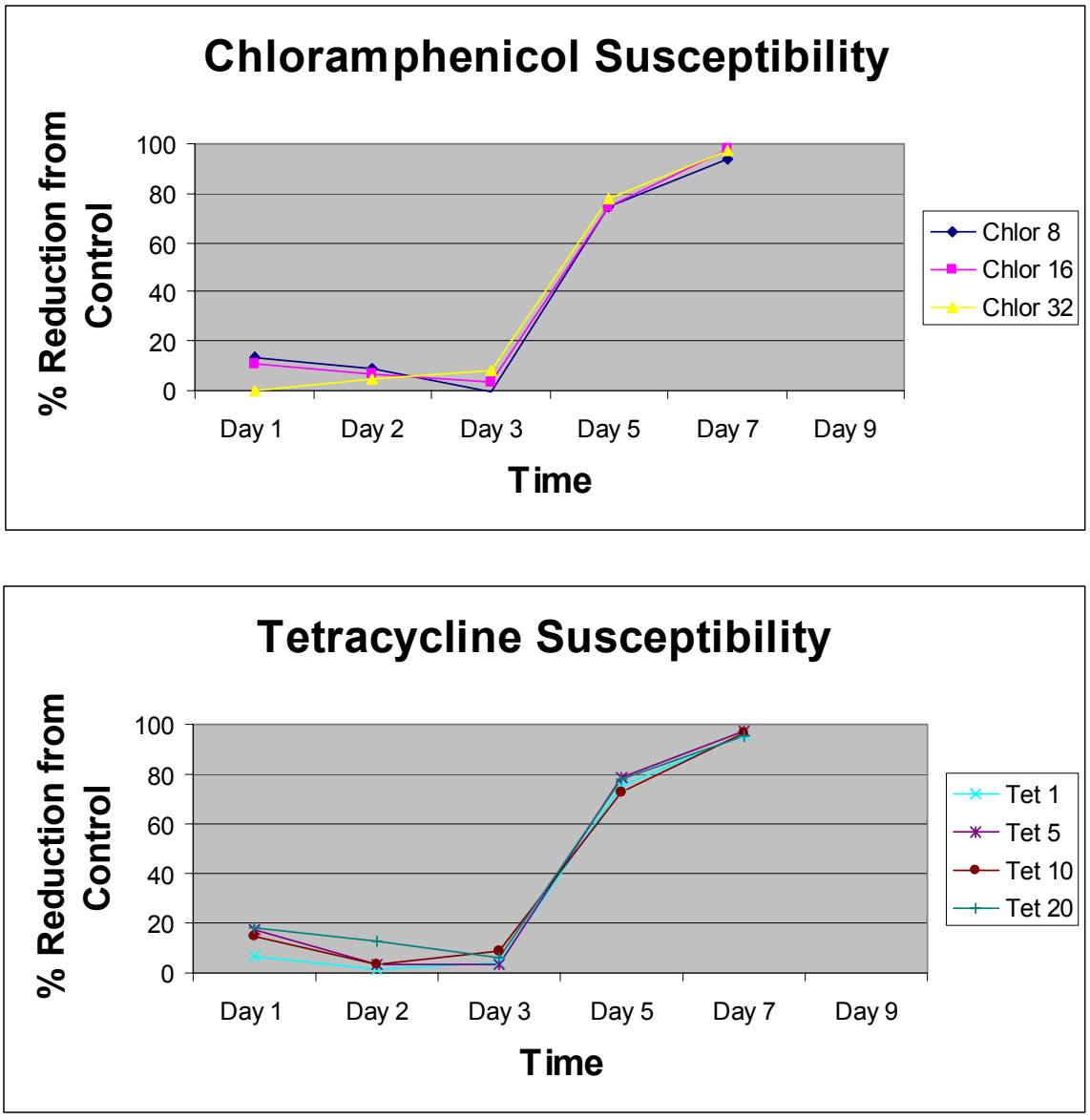


Figure 6 – Antibiotic susceptibility of *Ehrlichia muris*. Cultured P388D1 murine monocytes approximately 25% infected with *E. muris* were exposed to 3 concentrations of chloramphenicol (8, 16, and 32 µg/ml) and 4 concentrations of tetracycline (1, 5, 10, and 20 µg/ml) in a 24-well plate format. Cells were treated for 3 days, then washed with sterile PBS and given antibiotic-free medium to allow for growth of any surviving ehrlichiae. Cells were checked at 24, 48 and 72 hours post-treatment to evaluate for % infection by Diff-Quik staining, as well as at 48 and 96 hours post-antibiotic removal.

Slides were blinded and read independently by two investigators, and compared to control cells which received no treatment[U7].[SWL8]

Transformation

Since *E. muris* is considered to be non-pathogenic for humans, was determined to be susceptible to chloramphenicol, and has a well characterized animal model, it was considered the ideal initial candidate for ehrlichial transformation. We decided to use the plasmid pKK1819, because it contained the chloramphenicol acetyltransferase gene (*cat*) under control of the *E. chaffeensis p28-19* promoter, and had been shown to express *cat* in *E. coli* (105). Transformation was performed using the modified electroporation protocol as described in the methods (104).

PCR confirmation

PCR amplification of the *cat* gene was used to detect the presence and prolonged maintenance of the resistance gene in the transformants (Figure 7). PCR products were cloned, and sequencing verified that the amplicons contained the *cat* gene. Southern blots also verified the identity of the *cat* gene PCR amplicons (Figure 8).

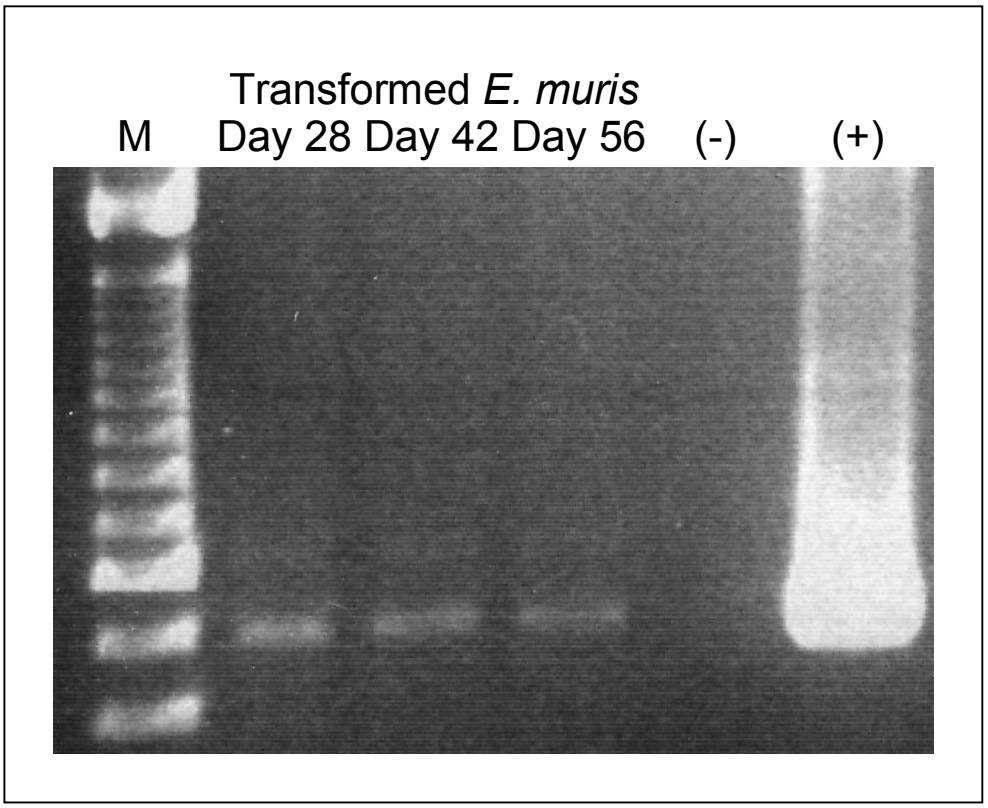


Figure 7 – PCR detection of the *cat* gene in DNA samples taken from transformed *Ehrlichia muris*. These PCR results demonstrate the maintenance of the *cat* gene in a culture of transformed *E. muris* for 56 days. The *cat* gene is maintained in the transformants months after removal of chloramphenicol. (-) indicates the template-free negative control, while (+) indicates the positive control template pKK1819, the transforming plasmid.

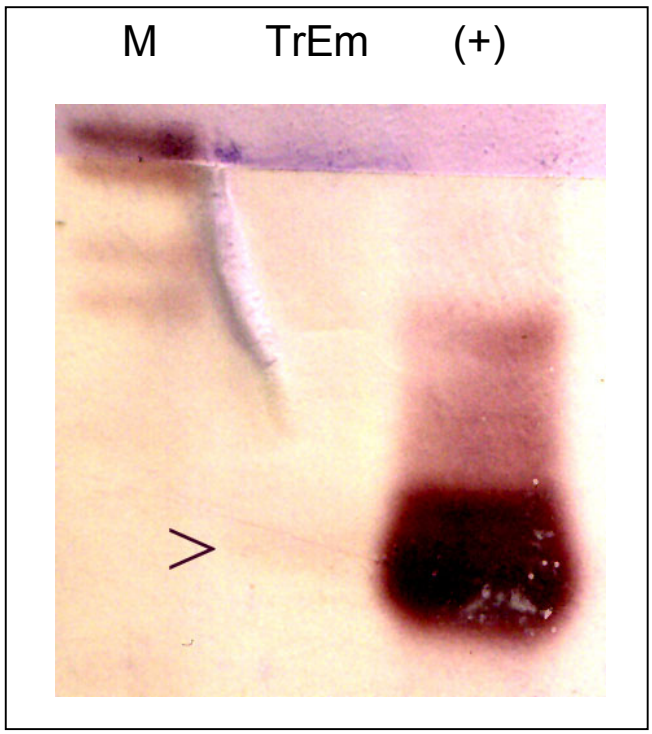


Figure 8 – Southern blot of PCR amplicons from a sample of transformed *E. muris* and the pKK1819 plasmid (used for transformation) as the positive control (+). The DNA probe used was the DIG-tailed catprobe oligonucleotide. The arrow marks the presence of the faint band from the transformed *E. muris* PCR amplification. The negative control showed no hybridization (not shown).

CAT ELISA

The expression of the *cat* resistance gene was verified by detection of the CAT protein in infected host cell lysates using the Roche CAT ELISA kit (data not shown).

Fluorescent microscopy of dsRed-expressing E. muris

A modified version of the pKK1819 plasmid which also contained the fluorescent dsRed protein was produced by Ted Whitworth, and electroporated into wild-type *E. muris*. This procedure produced fluorescent red ehrlichiae which were visible under fluorescent microscopy compared to the non-fluorescent wild-type. These ehrlichiae did not survive in culture beyond four to six weeks, possibly due to the well documented toxicity of the dsRed protein (102). Consequently, this construct was not utilized further. Still, the fluorescent ehrlichiae helped demonstrate that the *E. muris* are successfully transformed and express the transgenes (Figure 9).

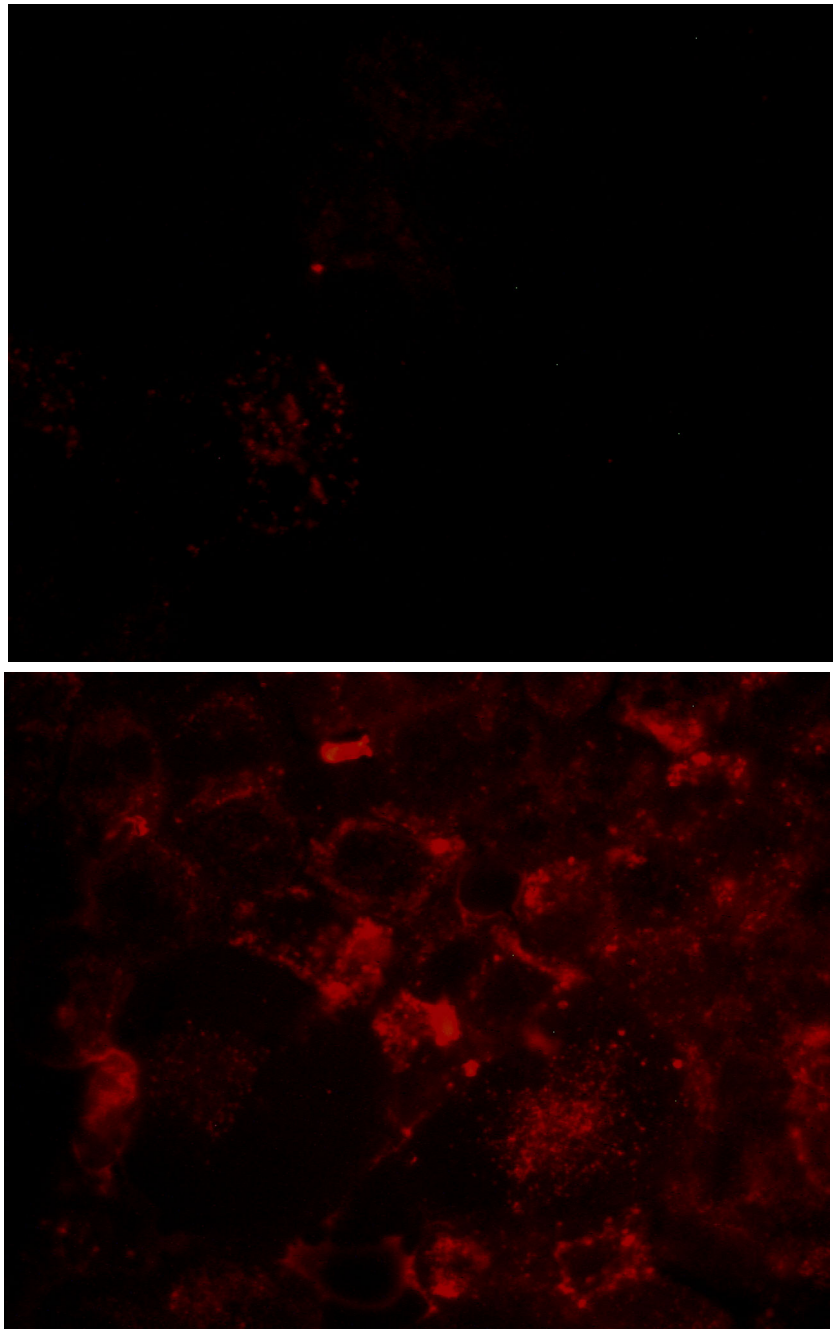
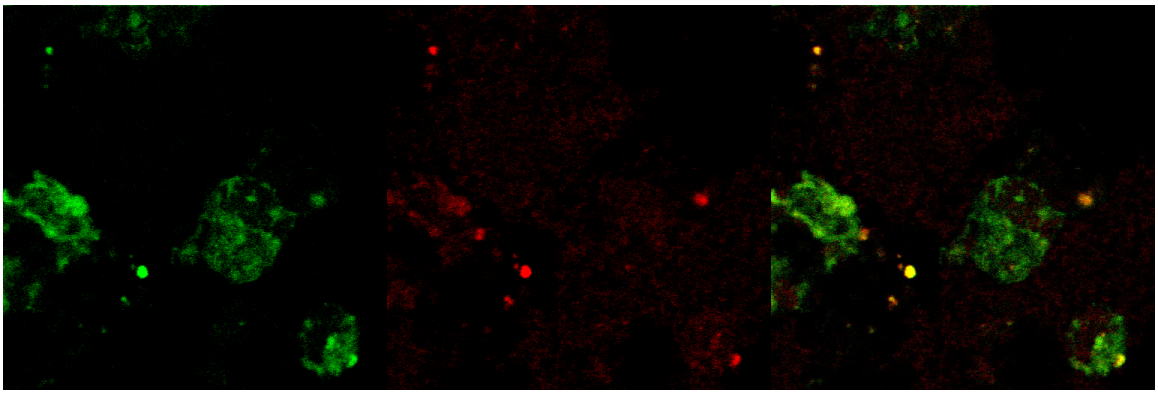


Figure 9 – Fluorescent photomicrographs of wild-type *E. muris* (top) and transformed *E. muris* (bottom) in DH82 cells. In this instance, the cells were transformed with a modified version of the pKK1819 plasmid containing the dsRed gene for a red fluorescent protein. The contrast between the wild-type infected cells and the transformant infected cells is evident.

Confocal microscopy

Confocal microscopy demonstrated the expression of the *cat* gene in transformed ehrlichiae by dual-labeling and co-localization of ehrlichial cells and the CAT protein (Figure 10). Confocal microscopy demonstrated a higher degree of infection than visualized by light microscopy and Diff-Quik staining. The transformant-containing morulae appeared to be smaller than the wild-type morulae with an altered cell wall structure, which may partially account for the difficulty in visualization by Diff-Quik staining.



Anti-*E. muris* Anti-CAT Merged

Figure 10 – Confocal microscopy of transformed *E. muris*, demonstrating the colocalization of *E. muris* and chloramphenicol acetyltransferase (CAT). Murine anti-*E. muris*[U9] polyclonal sera was used to stain *E. muris* (green), resulting in some background staining. Sheep monoclonal anti-CAT antibody, labeled with digoxigenin (DIG), from Roche was used to stain CAT (red). A FITC-conjugated anti-mouse IgG secondary was used to detect the anti-*E. muris* antibodies, and a rhodamine-conjugated anti-DIG IgG secondary was used to stain the anti-CAT antibodies[U10][SWL11].

INFECTION OF C57/BL6 MICE WITH TRANSFORMED *EHRLICHIA MURIS*

The mouse model of ehrlichial infection has been previously established using *E. muris* by Olano *et al* (126). We decided to look for any differences in pathology between the transformed and wild-type *E. muris* using this model. Mice were inoculated with either wild-type *E. muris* or transformed *E. muris*, two mice per group. Both transformed *E. muris* inoculated mice and one wild-type inoculated mouse were treated with chloramphenicol for 3 days, to maintain the transformants under positive selection and to control for the effect of antibiotic killing of any residual wild-type *E. muris.*

While the wild-type infected mice demonstrated an antibody response similar to that previously reported by Olano *et al*., the transformant-inoculated mice did not develop an IgG antibody response by day 60 post-inoculation as detected by IFA (positive = titer > 1:64) (Table 2). Despite the lack of antibody response in the transformed *E. muris*-inoculated mice, *E. muris* DNA was still detectable in the transformant-infected mouse organs at day 60 by real-time PCR. This indicates that the transformed *E. muris* were able to infect and persist within the mice, under chloramphenicol selection. The wild-type *E. muris*-inoculated mouse treated with chloramphenicol actually developed a higher antibody titer earlier than the untreated wild-type infected mouse, so simple antibiotic killing of the inoculum cannot explain the lack of antibody titer in the transformant-inoculated mice.

Minor inflammatory changes were noted in the organs of both wild-type and transformant-inoculated animals consistent with Olano's previous findings.

IFA Titers	Day 17	Day 31	Day 60
WT Untreated	<1:64	1:256	1:512
WT Treated	<1:64	1:512	1:512
DT5-1	<1:64	<1:64	<1:64
DT5-2	<1:64	<1:64	<1:64

Table 2 – Antibody titers of C57/BL6 mice infected with wild-type or transformed *E. muris*. Host cell free *E. muris* was electroporated with pKK1819 (n=2) or electroporated without plasmid (n=2). Both transformant-inoculated mice and one wild-type inoculated mouse were treated with chloramphenicol for 3 days (0.1mg/g total body weight i.p.). Sera were evaluated by immunofluorescence assay on days 17, 31, and 60 post-inoculation. Both wild-type inoculated mice developed an IgG antibody response by day 31. The DT5 (transformant)-inoculated mice remained seronegative at day 60. Cell cultures inoculated in parallel confirmed the viability of the inoculum.

PHENOTYPIC VARIATION OF P28 KNOCKOUT *EHRLICHIA MURIS* IN DIFFERING HOST CELL TYPES

Ultrastructure and electron microscopy in DH82 cells

First, the transformed *E. muris* were grown in the DH82 canine monocyte cell line. Ultrastructurally, wild-type *E. muris* possessed typical gram-negative morphology with an outer membrane separated from the inner membrane by an electron lucent periplasmic space. Ehrlichiae were identified as possessing either dense cored cell morphology or reticulate cell morphology. The non-transformed *E. muris* were typically coccoid although a few coccobacilli were also identified. They ranged from 320 nm to 917 nm in diameter with an average size of 565 nm (Figure 11).

In contrast, the transformed *E. muris* existed as protoplasts completely lacking an outer membrane. They were small, with a range of sizes from 93 nm to 470 nm and an average size of 230 nm. The transformants were identified as either possessing a small electron dense morphology or a slightly larger electron lucent morphology, and both forms were often pleomorphic. Several transformants were observed to possess deep invaginations of the cytoplasmic membrane up to 325 nm (Figure 12).

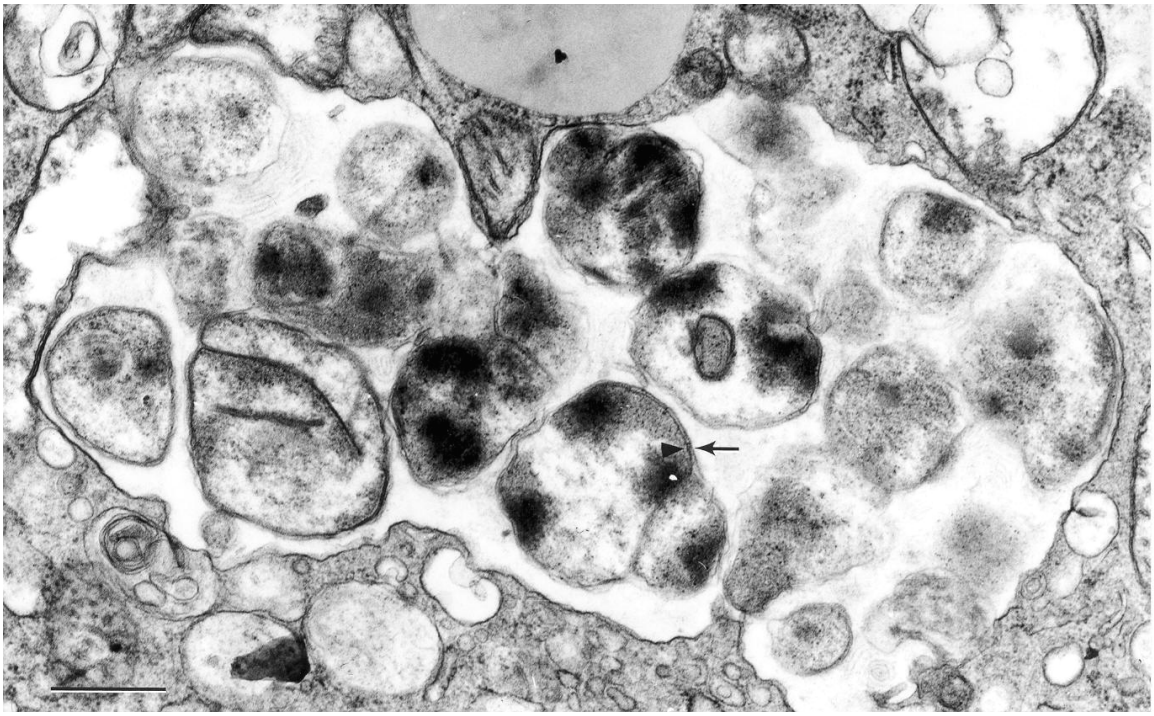


Figure 11 – Electron micrographs showing a variety of *E. muris* cell morphologies. The normal ultrastructure of the ehrlichial cell wall can be appreciated (arrows). Both dense core and reticulate cell types can be seen, as well as one ehrlichia which possesses a large projection, as described by Popov *et al*. Bar = 250 nm.

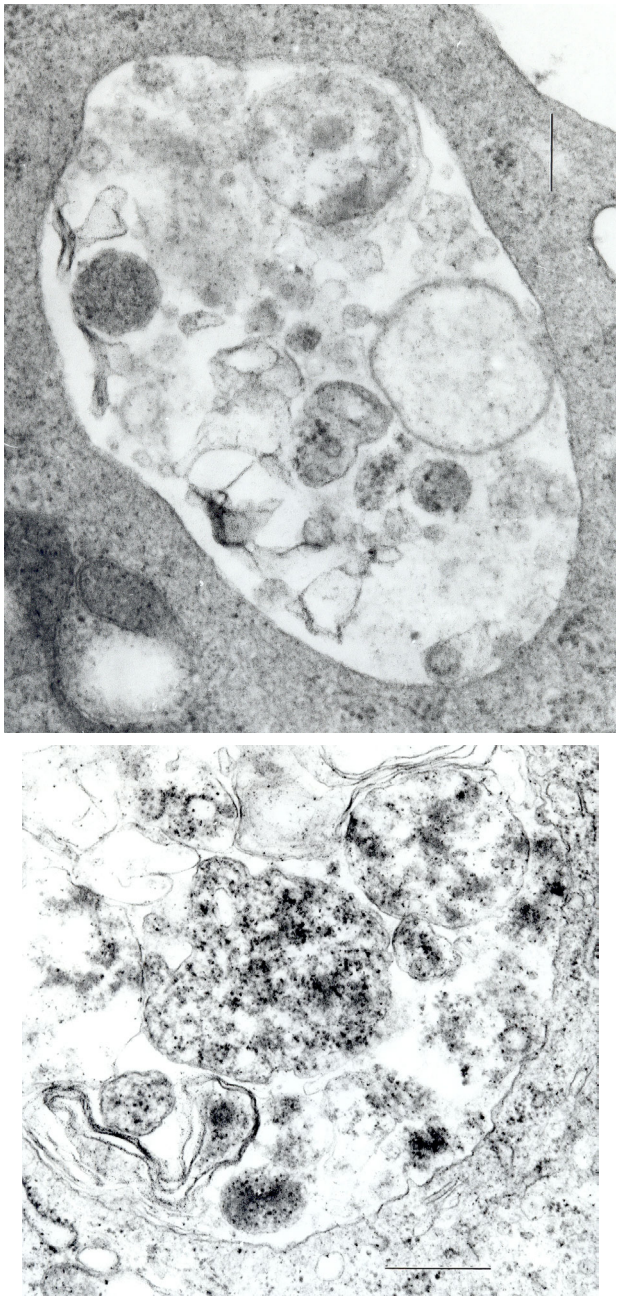
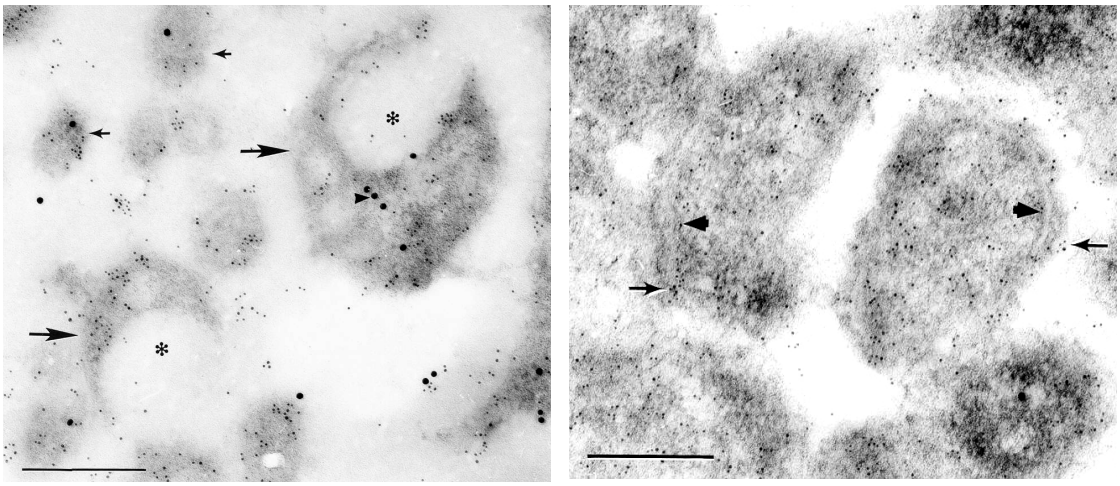


Figure 12 – Electron micrographs of transformed *E. muris* growing in DH82 canine monocytes. The cell membranes appear deranged, lacking the clear definition seen in the wild-type organisms, and are often irregular in shape. The smaller average size of the ehrlichial population can be appreciated. Bar = 250 nm.



Transformed *E. muris* Wild-type *E. muris*

Figure 13 – A comparison of wild-type and transformed *E. muris* processed for immunogold staining. The 5 nm gold particles label anti-P28 monoclonal antibody, while the larger 15 nm gold particles label anti-CAT antibodies. In the wild-type *E. muris,* the arrows highlight clustering of P28 staining on the ehrlichial cell surface, while the arrowheads mark regions where the normal membrane is visible. In the transformed *E. muris,* the arrows highlight areas where the lack of membrane structure is most evident, and the arrowhead marks an area of intraehrlichial CAT staining. The asterisks mark large vacuoles in the transformants. Bar = 250 nm.

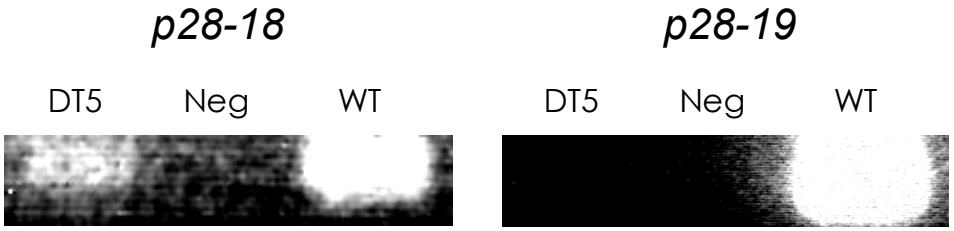
The transformed *E. muris* were significantly smaller than the wild type *E. muris* ($p < 0.001$). Immunogold staining with anti-p28 antibody confirmed that the *E. muris* transformants did indeed exist as smaller particles without an outer cell membrane (Figure 13). Dual immunogold staining with anti-p28 antibody (5 nm particles) and anti-chloramphenicol acetyl-transferase (CAT) antibody (15 nm particles) confirmed the presence of CAT in the transformants and its absence in the wild type *E. muris* (Figure 13). These transformed *E. muris* have a morphological appearance similar to cell wall-deficient bacteria or L-forms described for other gram-negative bacteria.

Ultrastructure and electron microscopy in ISE6 cells

In 2005, Singu *et al.* found that expression of P28 proteins in *E. chaffeensis* was host cell-dependent, with P28-19 the predominant protein expressed in DH82 cells, while P28-14 was the predominant P28 expressed in ISE6 tick cells. He also found that P28-20 was present in two of three DH82 samples which were tested (148). He found that this expression pattern holds true for different clinical isolates of *E. chaffeensis*, as well as in the related species *E. canis* (149).

Initial studies of the P28 locus of our transformed *E. muris* focused on the area surrounding *p28-19*, because the *p28-19* promoter of *E. chaffeensis* had been cloned into pKK1819 to direct expression of the *cat* gene. We discovered that *p28-19* was no longer detectable by PCR in the transformed *E. muris*, while *p28-18*, immediately upstream of the *p28-19*, was still detectable (Figure 14).

Efforts to sequence the P28 locus of the transformed *E. muris* are ongoing.



Friday, January 30, 2004 11:03 PM
Wilbur-Lipman DNA Alignment
Ktuple: 3; Gap Penalty: 3; Window: 20

Seq1(1>237) p1819amplicon	Seq2(1>5659) Emuris p28 contig4	Similarity Index	Gap Number	Gap Length	Consensus Length
(200>237)	(380>417)	100.0	0	0	38

200 210 220 230
TTCTACTATTGTTAATTTATTTGTCACTATTAGGTTAT
TTCTACTATTGTTAATTTATTTGTCACTATTAGGTTAT
TTCTACTATTGTTAATTTATTTGTCACTATTAGGTTAT
380 390 400 410

Figure 14 – PCR amplification of *p28-18*, but not *p28-19*, in transformed *E. muris* DNA samples. The transformed *E. muris* (DT5) amplicon is clearly visible for *p28-18* but not *p28-19*. Wild-type *E. muris* (WT) is used as a positive control, the negative control (Neg) lacks template DNA. The region of sequence homology from the *p28-19* promoter of *E. chaffeensis*, which was cloned into pKK1819, and the *p28-19* promoter of *E. muris*, is displayed below the gel images.

Since the transformed *E. muris* lack *p28-19,* I decided to passage them into ISE6 cells to further study their growth and morphological characteristics. After passage into ISE6 cells, we found that the transformed *E. muris* resumed their normal morphologic appearance when compared to wild-type *E. muris* (Figure 15).

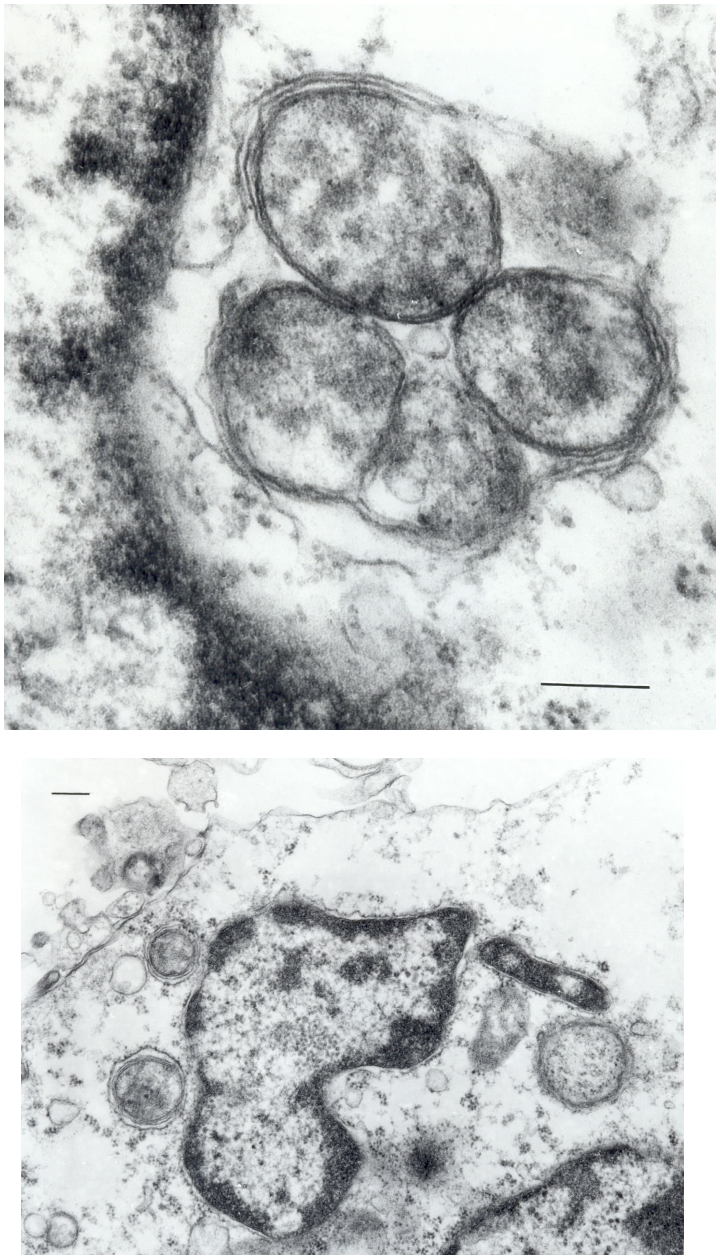


Figure 15 – Electron micrographs of transformed *E. muris* growing inside ISE6 tick cells. These ehrlichiae were passaged into the ISE6 cells from DH82 cells, where they possessed an abnormal morphologic appearance. After passage into ISE6 tick cells, they resumed the normal morphology of wild-type *E. muris*. Bar = 0.25 μM.

TRANSFORMED *EHRLICHIA MURIS* ARE NOT THE RESULT OF L-TRANSFORMATION

The field of rickettsial disease research had been held back for many years due to the lack of suitable transformation systems, and only recently have experimental options begun to be elucidated. These systems have not yet been applied to the *Ehrlichia*, and certain technical modifications were required to develop a system that was suitable for use with these related rickettsial pathogens.

Transformed *E. muris* exhibited ultrastructural changes typical of cell wall deficient forms, which are observed as a minority population of wild-type ehrlichial cultures (135). It was originally postulated that *Ehrlichia* formed these cell wall-deficient forms in response to harsh environmental conditions as a means of survival, similar to the L-transformation observed in other gram negative bacteria in response to antibiotics or nutrient restriction (135).

The appearance of the transformants as L-forms may be indicative of a decreased fitness for replication within the environment of the monocyte. In some cases, the appearance of L-forms in other gram-negative bacteria was attributed to decreased production of structurally important membrane proteins due to interference of antibiotics with protein expression.

It is important to note that exposure to chloramphenicol alone has been shown to induce L-transformation of *Staphylococcus aureus* in the presence of serum at the concentrations of chloramphenicol used in our transformation experiments (68). In order to eliminate this as a possible explanation for the morphologic variation observed in our transformants, wild-type *E. muris*-infected DH82 cells were briefly exposed to chloramphenicol (32 μg/ml) for 2 and 4 days and then allowed to recover in normal medium. These infected cultures were examined by electron microscopy for morphologic variation. The *E. muris* cells post-exposure to chloramphenicol were morphologically similar in both size and overall appearance to wild-type *E. muris*.

HOST CELL SPECIFIC EXPRESSION OF P28 PROTEINS IN TRANSFORMED *EHRLICHIA MURIS*

Expression of P28 proteins in wild-type *Ehrlichia muris*

Although Singu *et al.* reported the expression patterns of *E. chaffeensis* and *E. canis* in DH82 and ISE6 cells, no data were published for *E. muris* (148,149). In order to establish if the homologous P28 proteins were expressed in *E. muris*, isolation of host-cell free protein lysates followed by two dimensional SDS-PAGE and MALDI-TOF/TOF analysis was performed.

When wild-type *E. muris* was grown in DH82 cells, an interesting pattern emerged. P28-19 was identified in four separate preparations of *E. muris* lysates taken from DH82 cells (Table 3). However, in one gel, P28-17 was identified as well, with one spot existing as an apparent mixture of P28-17 and P28-19. This indicates a greater diversity of expression of the P28s than has previously been demonstrated for *E. chaffeensis* or *E. canis*. While *E. chaffeensis* isolates have been found to express P28-19, P28-20 or both proteins, P28-17 has not been reported previously as expressed by *E. chaffeensis* in DH82 cells. The *E. canis* homolog of P28-17, P30-3, is also not reported as expressed in DH82 cells (148,149).

Analysis of the P28 expression of wild-type *E. muris* in ISE6 tick cells is ongoing; however, given the fact that P28-14 is expressed by *E. chaffeensis* and its homolog P30-10 is expressed by *E. canis*, we anticipate that the P28 expressed by wild-type *E. muris* in tick cells will be predominantly P28-14.

Spot	Gene	Accession	Peptide Count	Protein Score	Expectation Value	Coverage (%)
WT1	P28-17	ABD93657	7	111	1.4 x e-5	31
WT2a	P28-17	ABD9365	7	204	6.8 x e-15	29
WT2b	P28-19	ADB93659	6	174	6.8 x e-12	28
DW1	P28-19[U12]	ABD93659	8	261	1.4 x e-20	35
DW4	P28-19	ABD93659	7	320	1.7 x e-26	35
DW11	P28-19	ABD93659	7	332	1.1 x e-27	35

Table 3 – MALDI-TOF/TOF analysis of proteins excised from two dimensional SDS-PAGE of wild-type *Ehrlichia muris* grown in DH82 cells. Each DW spot represents a separate experiment. The WT spots were collected from a single experiment, and one spot, WT2, yielded unique peptides corresponding to two P28 proteins. The peptide coverage is graphically presented in Figure 16. The protein score is calculated as $LOG_{10}(P)*-10$, where P is the absolute probability the match is a random event. Consequently, if we consider a value of $p < 0.05$ as significant, a protein score of greater than 75 is considered a significant match. The expectation value is the number of matches expected to occur with equal or better scores by chance alone, and is directly equivalent to the E-score in a BLAST search. A p value of 0.05 would correspond to an expectation value of 0.05.

WT1 P28-17 Coverage

1 MNCKKFFITT ALISLMSFLP GVSFSDPVQD SSANGNFYIS GK**YMPSASHF**
51 **GVFSAKEEKN PTVALYGLK**Q DWTGVSSTAH NDNEFNNKGY SFK**YENNPFL**
101 **GFAGAIGYSM GGPRVEFEVS YETFDVK**NQG NNYK**NDAHKY CALDQQATSS**
151 **SSATKDKYVL LK**NEGLLDIS FMLNACYDII TDGIPFSPYV CAGIGTDLVS
201 MFEATNPKIS YQGKLGLSYS ISPEASVFVG GHFHKVIGNE FKDIPTLKAF
251 AASTATPDMA IVTLSVCHFG IELGGRFSF

WT2a P28-17 Coverage

1 MNCKKFFITT ALISLMSFLP GVSFSDPVQD SSANGNFYIS GK**YMPSASHF**
51 **GVFSAKEEKN PTVALYGLK**Q DWTGVSSTAH NDNEFNNKGY SFK**YENNPFL**
101 **GFAGAIGYSM GGPRVEFEVS YETFDVK**NQG NNYK**NDAHKY CALDQQATSS**
151 **SSATK**DKYVL LKNEGLLDIS FMLNACYDII TDGIPFSPYV CAGIGTDLVS
201 MFEATNPKIS YQGKLGLSYS ISPEASVFVG GHFHKVIGNE FKDIPTLKAF
251 AASTATPDMA IVTLSVCHFG IELGGRFSF

WT2b P28-19 Coverage

1 MNCKRIFIKS ALISLISFLP GISFSDPIQD SNVSGNFYIS GK**YMPSASHF**
51 **GVFSAKEEK**N ATAKTFGLKQ DWDGAAISNT STDVFTISNY SFK**YENNPFL**
101 **GFAGAIGYSM GGPRIEFEVS YETFDVKNQG NNYKNDAHRY YALSQDTTIA**
151 **QNK**FVVLKNE GLADISFMLN ACYDVTTEGI PFSPYICAGI GTDLVSMFEA
201 TSPKISYQGK LGLSYSISPE TSVFVGGHFH KVVGNEFKDV PAIVPSGSTL
251 AGNHFAIVTL NVCHFGIELG GRFAF

DW1 P28-19 Coverage
DW4 P28-19 Coverage
DW11 P28-19 Coverage

1 MNCKRIFIKS ALISLISFLP GISFSDPIQD SNVSGNFYIS GK**YMPSASHF**
51 **GVFSAKEEK**N ATAKTFGLKQ DWDGAAISNT STDVFTISNY SFK**YENNPFL**
101 **GFAGAIGYSM GGPRIEFEVS YETFDVKNQG NNYKNDAHRY YALSQDTTIA**
151 **QNK**FVVLKNE GLADISFMLN ACYDVTTEGI PFSPYICAGI GTDLVSMFEA
201 TSPKISYQGK **LGLSYSISPE TSVFVGGHFH K**VVGNEFKDV PAIVPSGSTL
251 AGNHFAIVTL NVCHFGIELG GRFAF

Figure 16 – Peptide coverage data for the wild-type *E. muris* samples analyzed by MALDI-TOF/TOF. The protein sequence covered by the detected peptides is in bold red. Due to missed trypsin cleavages, it is possible for peptides to overlap. The coverage of P28-19 in DW1, DW4, and DW11 was identical.

DISCUSSION

The mechanism of ehrlichial transformation

Previously, *Ehrlichia* had proven resistant to attempts at genetic transformation, and they lack naturally occurring plasmids or phage. This situation has posed a significant obstacle to progress in the field, preventing genetic manipulations routinely available in most fields of bacteriology.

The transformation of *Rickettsia prowazekii* was first reported by Rachek *et al* in 1998 (138). The barriers to the transformation of *Ehrlichia* were many. First, only one antibiotic family has been shown to have reliable clinical utility against *E. chaffeensis,* the tetracyclines. Doxycycline is the gold standard drug for treatment of ehrlichial infections, and therefore it would be unwise to create tetracycline-resistant *E. chaffeensis*. Chloramphenicol would be a possible choice for antibiotic selection, based upon retrospective data from one clinical series; however, previous work had shown *E. chaffeensis* to be resistant to chloramphenicol treatment *in vitro* (23).

Because of these issues surrounding *E. chaffeensis*, it was decided to use the murine ehrlichial species *E. muris.* Testing of *E. muris* showed it to be susceptible to chloramphenicol *in vitro*, making it an ideal candidate for antibiotic selection in our experiments.

It is not immediately clear why our efforts have succeeded where others have failed; however, there are several key points.

Inhibition of type one restriction enzymes

First, the use of the TypeOne restriction enzyme inhibitor greatly increased our observed transformation efficiency, resulting in observable transformants at a much earlier time point. Prior to this, it took approximately one month to observe any transformants. Given this time frame, it is likely that prior attempts might have been considered failures before the transformants were given enough time to grow to detectable quantities.

Although no putative type I restriction-modification systems have been identified in the whole genome sequence of *E. chaffeensis*, we have isolated a protein from our two dimensional SDS-PAGE of wild-type *E. muris* which is homologous to the R subunit of the type I restriction-modification system of *Methylococcus capsulatus* strain Bath. This protein has homologs in several species of *Rickettsia*, including *R. conorii, R. sibirica, R. bellii,* and *R. felis*, suggestive of an intact type I restriction-modification system in the order Rickettsiales.

Use of homologous recombination

Furthermore, the use of a homologous promoter allowing for recombination of our antibiotic resistance gene into the host genome was likely critical to propagation of the resistance gene in the transformants. As the ehrlichial origin of replication still remains unknown, despite the availability of several complete genome sequences, it is difficult to design a plasmid which might be propagated in *Ehrlichia*. There are also no known phage or plasmids to harvest for experimental use.

Homologous recombination has been utilized by *Ehrlichia* in the process of genomic reduction and expansion of the P28 locus over the course of evolution, and the origin of replication in *Anaplasma* has been localized near the *p44* locus (51). Exploitation of these recombination mechanisms to generate transgenic *Ehrlichia* opens the field to genetic manipulation long taken for granted in other areas of microbiology. Design of plasmids containing a chloramphenicol resistance gene flanked by homologous ehrlichial sequence from the desired target region of the genome will most likely allow for the generation of a wide variety of recombinant *Ehrlichia* in the future.

Selection of gene targets for transformation

Given the highly reduced nature of ehrlichial genomes, it may be that few targets prove amenable to being completely inactivated. Over time, the genomes have tended to shed those genes not required to exist within their intracellular niche. Since extracellular growth is not possible, mutations or knockouts which result in markedly decreased infectivity or ability to proliferate may be difficult to detect. The most attractive targets, genes which we know from their immunoreactivity or homology to genes of known

function, are typically highly conserved between ehrlichial species and as such may be more likely to serve critical functions for intracellular parasitism.

There are 98 genes found in *E. chaffeensis*, *A. phagocytophilum*, *N. sennetsu,* and *Wolbachia pipientis* but not in *R. prowazekii* (51). These 98 genes may contain members of the host cell proteome which are involved in the unique adaptation to intravacuolar life, since *R. prowazekii* exists in the cytoplasm of host cells. An intense examination of the sequenced genomes in the order Rickettsiales should reveal candidates for specificity to monocytes or granulocytes, as well as morular vs cytoplasmic life.

As[U13] [SWL14]another example, there is one ortholog gene for a class II aldolase present in most of the order Rickettsiales but absent from the tick-borne Anaplasmataceae. One major difference in comparing these two groups is the lack of transovarial transmission in the arthropod vector by the Anaplasmataceae compared to the rest of the order Rickettsiales (51). The transformation method that I have developed would allow for insertion of the rickettsial aldolase gene into *Ehrlichia* to determine if a gain of transovarial transmission occurs.

CHAPTER 3

A MONOCYTE RECEPTOR MOLECULE, P-SELECTIN GLYCOPROTEIN LIGAND 1 (CD162), FOR *EHRLICHIA CHAFFEENSIS* INFECTION

P-selectin glycoprotein ligand 1 was first discovered in 1992 because of its role in the normal inflammatory response, as a ligand for the selectins (116). Later work has shown that PSGL-1 in fact can serve as a receptor for intracellular pathogens, in particular *Anaplasma phagocytophilum* (76). Further investigations demonstrated that the *A. phagocytophilum* outer membrane protein family MSP2 was the pathogen ligand for the PSGL-1 host cell receptor (132).

The *Ehrlichia* and *Anaplasma* are both members of the Family *Anaplasmataceae*, and the P28 outer membrane protein family of *Ehrlichia* is homologous to the MSP2 outer membrane protein family of *Anaplasma*. While *A. phagocytophilum* normally infects granulocytes and *E. chaffeensis* infects monocytes, PSGL-1 is expressed on both these cell types.

We hypothesized a role for PSGL-1 in *Ehrlichia chaffeensis* infection.

METHODS

The organism and cell culture.

The Arkansas isolate of *E. chaffeensis* was cultured in the DH82 canine monocyte cell line. The DH82 cells, infected and uninfected, were cultured in high glucose Dulbecco's Modified Eagle Medium (Gibco, Rockford, IL) with 5% newborn calf serum (NCS) (Gibco, Rockford, IL) and 1 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (Mediatech, Herndon, VA), at 37°C with 5% CO_2. The human monocyte cell-line THP-1 was used for the antibody blocking experiment. The THP-1 cells were cultured in RPMI, followed by 48 hours of culture in serum-free medium prior to the antibody blocking experiment. The B-lymphoblastoid cell line BJAB was obtained from

Dr. Karen Snapp ([U15]University of Illinois – Chicago, Chicago, Il.), along with three transgenic lines which expressed PSGL-1, the fucosyltransferase FucT-VII, or both genes.

KPL-1 antibody blocking.

THP-1 cells in serum-free medium were incubated for 1 hour at 37 °C with either KPL-1 antibody, or mouse IgG1 isotype control (Sigma, St. Louis, MO). After this incubation, the cells were washed twice with sterile PBS, and then exposed to host cell-free *E. chaffeensis* in fresh serum-free medium prepared as described. The cells were then incubated as described for 2 days, and the quantity of *E. chaffeensis* was measured using real-time PCR as described previously (43).

O-sialoglycoprotein endopeptidase treatment.

DH82 cells were harvested from a T75 flask and plated into a 12-well plate containing cell culture-treated #1 coverslips (Corning, Acton, MA). After the DH82 cells had attached (approximately 1 hour), the medium was removed and replaced with fresh DMEM containing the appropriate dose of enzyme. Uninfected DH82 cells were incubated with three concentrations (12 µg/ml, 24 µg/ml, or 48 µg/ml) of purified *Pasteurella* O-sialoglycoprotein endopeptidase (OSGE) (Cedarlane Laboratories, Burlington, NC), with untreated cells as a control. The cells were incubated with enzyme for 1 hour at 37°C with 5% CO_2, according to the manufacturer's instructions. After this, the medium was removed from the cells, and they were washed briefly with PBS. DH82 cell-free *E. chaffeensis* in fresh DMEM were then added to the wells, and incubated with the DH82 cells at 37°C with 5% CO_2 for 1 hour. Then, the medium was removed by aspiration, the cells were washed briefly with PBS, fresh DMEM was added, and the cells were incubated at 37°C with 5% CO_2.

Confocal microscopy.

Cells which were not grown on coverslips were affixed to slides using the Thermo Shandon cytospin at 1,000 x g for 10 min. Otherwise, coverslips and slides were processed in the same manner. The cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. Subsequently, the cells were washed three times in PBS,

for five minutes each wash, at room temperature. The slides were blocked by incubation for 1 hour with a fresh solution of 1% normal goat serum and 3% BSA in PBS. The primary antibody, rabbit anti-*E. chaffeensis* GP120, was diluted in 1% BSA in PBS at a 1:100 dilution, and incubated with the slides for 1 hour at 37°C. After incubation, the slides were washed three times in PBS at room temperature, 20 minutes each wash. The slides were incubated in the dark with the secondary antibody, Alexa Fluor 647-labeled goat anti-rabbit IgG, at a 1:100 dilution in 1% BSA in PBS for 1 hour at room temperature and then washed in PBS at room temperature three times, 10 minutes each wash. The slides were then incubated with DAPI at room temperature for 5 minutes to stain the cell nuclei. Coverslips were mounted with Vectamount and allowed to cure in the dark before visualization on the Olympus Fluoview 1000 confocal microscope.

Statistical analysis.

The percentage of O-sialoglycoprotein endopeptidase (OSGE)-treated infected cells was compared with the percentage in the untreated controls. Standard deviations were calculated, and the significance of the difference between groups was determined using the chi-square test for independence ($\chi 2$).

EFFECT OF A PSGL-1 BLOCKING ANTIBODY, KPL-1, ON *EHRLICHIA CHAFFEENSIS* INFECTION

Previous studies have demonstrated the ability of the mouse monoclonal antibody KPL-1 to bind to PSGL-1, blocking ligand binding (76,150). In our experimental design, THP-1 cells were exposed to either KPL-1 or the isotype control antibody, washed, and then inoculated with host cell-free *E. chaffeensis.* The relative quantities of ehrlichiae were compared at 5 days post-infection, and the KPL-1 treated group showed a 46% reduction in the relative number of ehrlichiae compared to the isotype control as detected by real-time PCR.

GROWTH OF *EHRLICHIA CHAFFEENSIS* IN WILD-TYPE AND TRANSFECTANT LINES OF THE B LYMPHOBLASTOID CELL LINE BJAB

To further investigate the importance of PSGL-1 to *E. chaffeensis* infection, we studied the infection of a PSGL-1 negative B-lymphoblastoid cell line, BJAB, with *E. chaffeensis*. We obtained BJAB cells, along with three transgenic lines, expressing PSGL-1, the fucosylase FucT-VII, or both proteins. The fucosylase is required to properly glycosylate the PSGL-1 molecule, which is important for high affinity binding (86).

We found that at 24 hours the wild-type BJAB cells (PSGL-1 - / FucT-VII -) contained only 49% of the quantity of ehrlichiae compared to the double-transfectant BJAB cells (PSGL-1 + / FucT-VII +). We found that wild-type BJAB cells were capable of infection with *E. chaffeensis*, albeit at a lower level, even in the absence of PSGL-1 expression. Because of the infection of cells lacking PSGL-1, further work with these cell lines was not pursued.

EFFECT OF O-SIALOGLYCOPROTEIN ENDOPEPTIDASE (OSGE) ON INFECTION OF DH82 CELLS BY *EHRLICHIA CHAFFEENSIS*

PSGL-1 is a heavily glycosylated receptor molecule, and the importance of the O-glycan sugars to functional ligation of PSGL-1 has been previously established,(86,93). The OSGE from *Pasteurella* is highly selective for cleaving O-glycan linkages, and has been used previously to investigate glycosylation-dependent interactions with PSGL-1 (76,122).

Treatment of DH82 canine monocytes with OSGE caused a reduction in the binding and infection rates by *E. chaffeensis* (Figure 17). At 24 hours after OSGE treatment and inoculation with *E. chaffeensis*, the number of *E. chaffeensis* morulae in the OSGE-treated DH82 cells was reduced compared with those in the untreated cells (Figure 18).

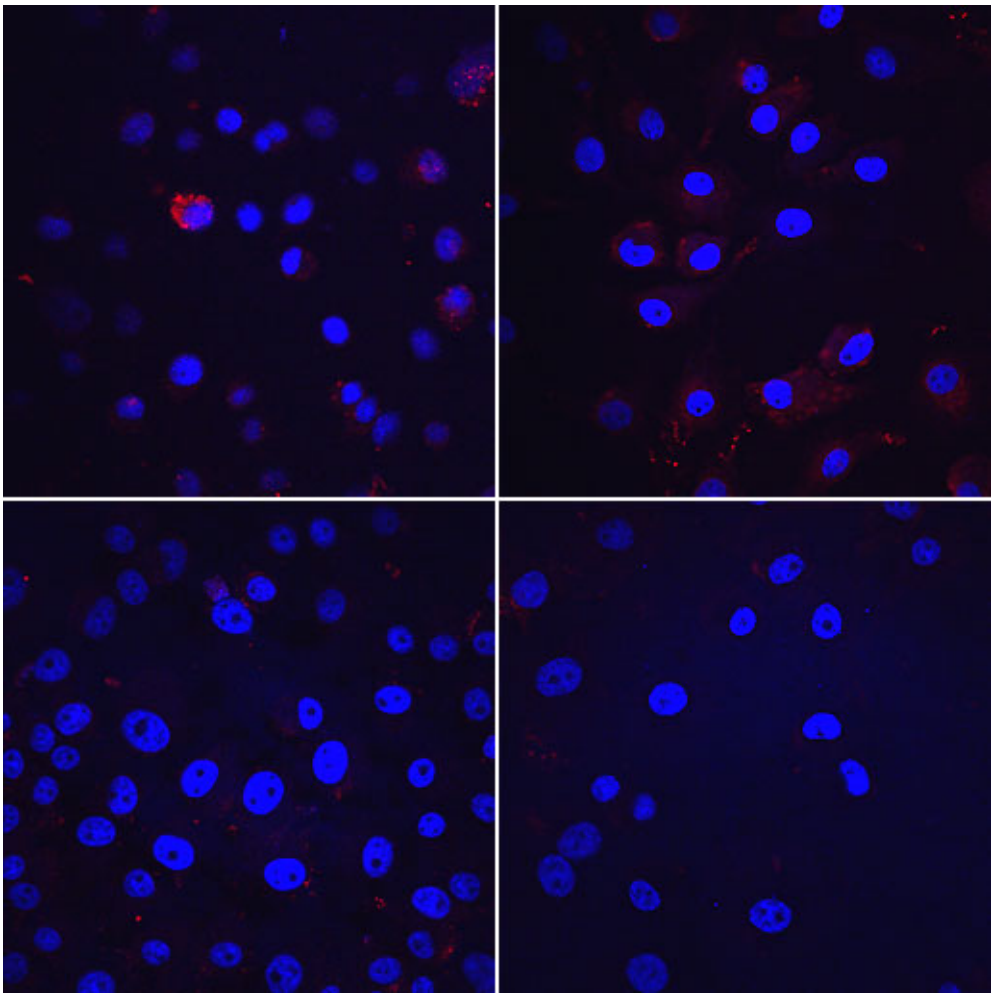


Figure 17 – Confocal microscopy of OSGE-treated DH82 cells and controls, infected with *E. chaffeensis* observed at 24 hours. *E. chaffeensis* was stained using mouse anti-gp120 primary antibody and Alexa Fluor 647-labeled anti-mouse secondary antibody (red), and the cell nuclei were counterstained with DAPI (blue). The groups are untreated DH82 cell controls (upper left), 12 µg/ml OSGE-treated DH82 cells (upper right), 24 µg/ml OSGE treated DH82 cells (lower left), and 48 µg/ml OSGE-treated DH82 cells (lower right).

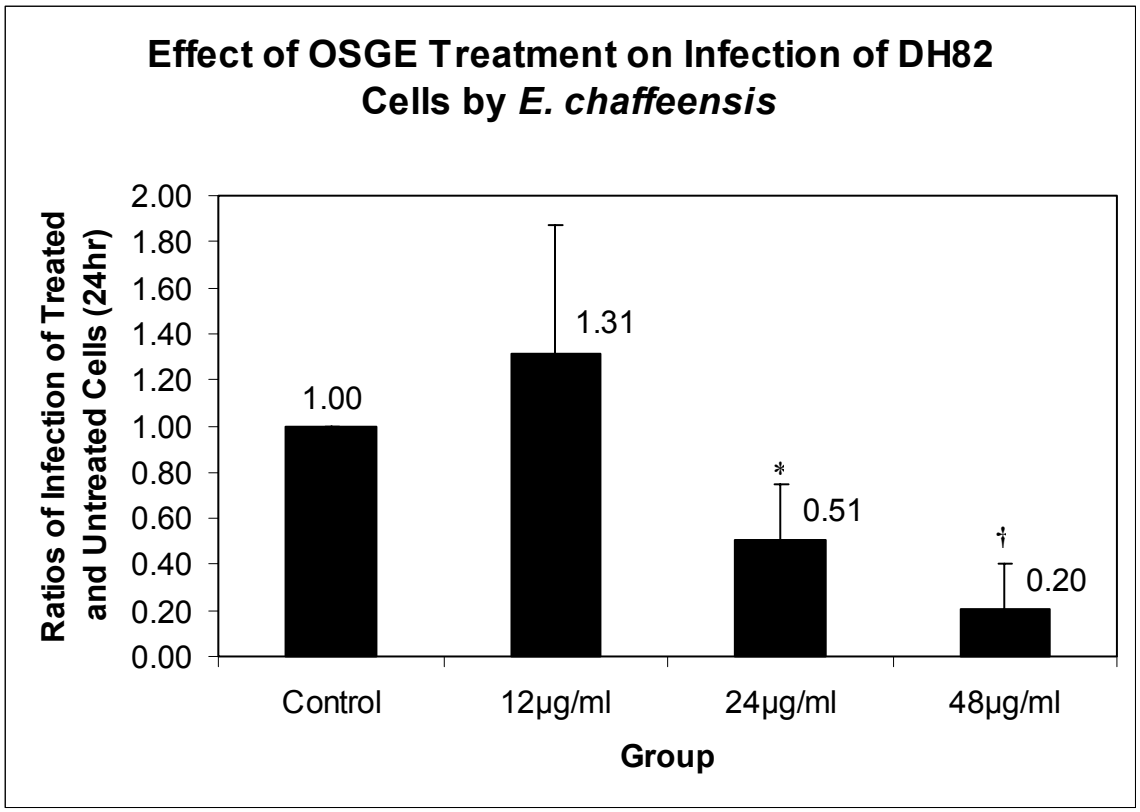


Figure 18 – Effect of OSGE treatment on infection of DH82 cells by *E. chaffeensis*. Treatment of DH82 cells with OSGE prior to infection with *E. chaffeensis* resulted in a significant, dose dependent decrease in observed infected cells at 24 hours for both the 24 µg/ml and 48 µg/ml concentration of OSGE compared to the untreated control cells. Significance was determined using the chi-square test for independence (†: $p = 9.57xE^{-16}$, *: $p = 2.19xE^{-15}$).

DISCUSSION

The dose-dependent effect of treatment of DH82 cells with OSGE prior to infection with *E. chaffeensis* demonstrates the importance of PSGL-1 to successful infection of monocyte host cells by *E. chaffeensis.* However, it is worth noting that even at the highest dose of OSGE treatment, there are a few morulae present, indicating some ehrlichiae do manage to infect the host cell.

To further test the hypothesis that the host cell receptor for *E. chaffeensis* is PSGL-1, we obtained the PSGL-1 negative BJAB cell line to examine the rate of infection in transfectants which produce PSGL-1 and an accompanying fucosylase. While we did find a higher rate of infection in the PSGL-1/FucT-VII transfectants, we also discovered that *E. chaffeensis* is capable of infecting the wild-type BJAB cell line that lacks PSGL-1.

Taken together, these results indicate that while PSGL-1 plays a role in successful infection of monocyte host cells, it is apparently one of a larger number of host cell receptors involved in attachment and invasion of host cells by *Ehrlichia*. It may play a role in monocyte host cell identification and attachment, but is apparently not necessarily an absolute requirement for successful infection, as indicated by the ehrlichial ability to infect BJAB cells that lack PSGL-1.

A role for PSGL-1 in concert with L-selectin and E-selectin

This conclusion is supported by previous work demonstrating a role for L-selectin and E-selectin mediating the attachment of *E. chaffeensis* to monocytes (170). Zhang *et al*. determined that antibodies to E-selectin and L-selectin blocked attachment of *E. chaffeensis* to THP-1 cells, while antibodies to P-selectin, integrin αm, or integrin αx had no effect on attachment and subsequent growth. Furthermore, E-selectin and L-selectin blocking antibodies did not completely prevent ehrlichial attachment and infection of the THP-1 cells, but reduced it in a manner similar to our observations of PSGL-1 antibody blocking in THP-1 cells or deglycosylation of DH82 cells.

Our results concur with Zhang *et al*. by demonstrating another receptor for *E. chaffeensis* on monocytes, PSGL-1, which could explain attachment and entry in cells with blocked E-selectin and L-selectin (170). Furthermore, the expression of L-selectin on lymphocytes, including B cells, may provide a partial explanation for our observations of infection in BJAB cells which did not express PSGL-1. It is interesting to note the expanding family of host cell membrane glycoproteins utilized by *E. chaffeensis*, including selectins as well as a selectin ligand, to recognize and attach to host cells, and possibly facilitate entry. Further studies are needed to fully elucidate the complete set of receptors involved in host cell invasion by these intracellular pathogens.

PSGL-1 in the family Anaplasmataceae

Herron *et al.* first discovered the link between PSGL-1 and growth of the intracellular parasite *Anaplasma phagocytophilum* (76). Subsequently, Park *et al.* determined that the major surface protein 2 (Msp2) outer membrane protein of *A. phagocytophilum* mediates the interaction with PSGL-1 (132). In this work, we have established a role for PSGL-1 in the infection of a different hematopoietic cell type, monocytes, by another member of the family Anaplasmataceae, namely *E. chaffeensis.* It is worth considering what other intracellular parasites may make use of this ubiquitous membrane protein as a means of cellular recognition or entry into the host cell.

PSGL-1 is one of the best characterized selectin ligands and can be found expressed on the surface of almost all known leukocytes (83,116). Most known members of the family *Anaplasmataceae* infect leukocytes as well, and given the role of PSGL-1 in infections of organisms of two separate genera within this family, it is worth considering if binding PSGL-1 has arisen from convergent evolution in these differing species of intracellular parasites or results from the conserved activity of orthologous protein families arising in a common rickettsial ancestor. The P28 multigene family of *E. chaffeensis* is homologous to the MSP2 family of *A. phagocytophilum.* If the P28 protein is found to bind to PSGL-1, an orthologous functional relationship for these protein families may be more likely.

Within the family Anaplasmataceae, the *Neorickettsia* infect monocytes and macrophages. Investigation into their potential utilization of PSGL-1 during infection would shed more light on these questions of parallel evolution versus orthologous protein families.

Cell signaling associated with ehrlichial internalization

As discussed previously, *E. chaffeensis* binds to lipid rafts on the host cell surface which contain caveolin-1, cholesterol, and GPI-anchored proteins, entering the cell via a clathrin-independent receptor-mediated endocytosis typical of caveolae. After ehrlichial infection, PLC-γ2 and tyrosine phosphorylated proteins are also found in the lipid raft fraction, and ehrlichial proteins may be embedded in the morular membrane as well (96). Binding results in activation of transglutaminase (TGase), protein tyrosine kinases (PTK) and PLC-γ2, resulting in release of intracellular stored calcium (97). There is also a unique cytokine response to *E. chaffeensis* infection, with a suppression of some pro-inflammatory cytokines but not others (88,89).

Recently, ligation of PSGL-1 by P-selectin or the monoclonal antibody KPL-1 has been linked to intracellular signaling events including tyrosine phosphorylation and c-abl induced alterations in the F-actin cytoskeleton of neutrophils (163). Ligation of PSGL-1 activates the GTPase Ras as well as the MAP kinase cascade, and stimulates the release of IL-8 from neutrophils (36,77). It has further been demonstrated that PSGL-1 ligation leads to tyrosine kinase-dependent activation of CD11b/CD18 activation in neutrophils, with resulting beta2-integrin dependent cell aggregation (36,55). PSGL-1 ligation results in polarization of PSGL-1 on the cell membrane, with concurrent cytoskeletal changes including F-actin polymerization (163).

There is a great intersection between the observed phenomena related to tyrosine phosphorylation, PLC-γ2 activation and intracellular calcium release after *E. chaffeensis* binding to host cells, and the observed signaling induced by ligation of PSGL-1. Furthermore, IL-8 release is stimulated by both *E. chaffeensis* infection and ligation of PSGL-1 (88,163). The activity of PKA has been shown to block the rickettsicidal effect of IFN-γ on *E. chaffeensis*, and PKC has been implicated in the downstream activation of

c-abl by PSGL-1 and the resulting changes in the cytoskeleton (90,163). Genistein has been shown to block ehrlichial entry and proliferation, ostensibly by interfering with protein tyrosine kinases (97). Genistein has also been shown to block signaling induced by the ligation of PSGL-1 (163). The links between the intracellular signaling events triggered by *E. chaffeensis* infection and PSGL-1 ligation need to be investigated more closely, since PSGL-1 serves as a receptor for *E. chaffeensis*.

PSGL-1 had been neglected as a receptor for *E. chaffeensis*, possibly due to the fact that it was not found in the lipid raft fractions of *A. phagocytophilum*-infected HL-60 cells. However, PSGL exists in the membrane as a dimer of two 120 kDa proteins, which Lin *et al.* admit may result in its detachment from the light lipid raft fraction during density gradient purification. Furthermore, the location of PSGL-1 was not investigated in *E. chaffeensis*-infected THP-1 cells (96). In support of a role for PSGL-1 in the morular membrane, we have noticed a colocalization of PSGL-1 and *E. chaffeensis* morulae in our confocal microscopy of infected PSGL-1 containing BJAB cells (Figure 19).

Other host cell membrane proteins implicated in intracellular parasitism

Utilization of host cell membrane proteins during entry and infection by intracellular parasites is a constantly developing area of study. Most recently, *Listeria monocytogenes* utilization of the insulin-like growth factor II receptor (IGFIIR) was described by Gasanov *et al.* (67). Pathogenic *Neisseria* species have been shown to use a variety of host cell proteins during invasion, including CD46, complement receptor 3, and the I-domain-containing alpha-integrins (52). Determining the mechanisms which intracellular parasites use to gain access and subvert the normal functions of the host cells should yield new insights as well as potential new therapeutics against these infections.

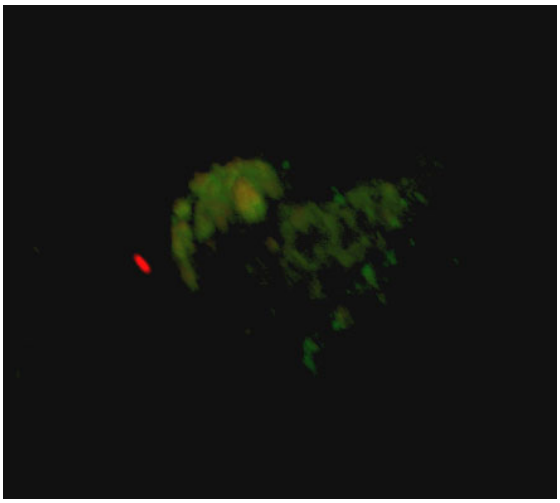


Figure 19 – Three dimensional reconstruction of *E. chaffeensis*-infected PSGL-1 containing BJAB cell. The cell is viewed at a transverse angle from above the plane of the cell, allowing an appreciation of the spherical morulae, staining for both PSGL-1 (green) and *E. chaffeensis* gp120 (red). The majority of PSGL-1 staining seems associated with these spherical morulae, perhaps indicating the segregation of the molecule into the morular membrane.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, we have demonstrated that the observed host cell specific expression of P28 proteins by *E. chaffeensis* is directly related to membrane stability in the host cell, as well as overall fitness and survival of the ehrlichiae within the host. The mechanism controlling the expression of the individual P28 alleles is still unknown; however, temperature does not appear to be involved in altering P28 expression (148).

Although the ligand of the P28 molecule on monocytes remains unknown, increasing amounts of evidence point towards a glycosylated host cell receptor molecule. It may well be that PSGL-1 is the host molecule which recognizes and interacts with P28. Ligation of PSGL-1 has been shown to trigger signaling events within host cells, so the ultimate consequence of the interaction between *E. chaffeensis* and PSGL-1 has yet to be demonstrated.

The genus *Ehrlichia*, indeed, the entire order Rickettsiales is full of opportunities for future research. The full complement of host cell receptors for *E. chaffeensis* should be defined for both monocytes and tick cells. A better understanding of the life cycle of *E. chaffeensis* within the tick is needed. As more genomes are sequenced, an increasing number of proteins of unknown function are being identified.

One tool for identifying protein function in *Ehrlichia* will be genetic recombination, which prior to this work was unavailable in the field, and generally accepted as not possible with the current state of the art. We have advanced the state of the art and provided a protocol for the genetic modification and recombination of *Ehrlichia*.

This achievement will allow for gene knockout studies. In the case of the significantly reduced genomes of intracellular pathogens, it may be that few genes can be knocked out without resulting in a lethal phenotype. However, our experience with P28-19 demonstrates the necessity of testing transformed ehrlichiae in several of their

environmental niches to determine if a mutation is detrimental in one environment, but not another. Using these methods, it may be possible to determine what genes are important to survival in the monocyte but not the tick cell. If a recombinant *E. chaffeensis* which cannot replicate effectively in monocytes but could be grown in another cell culture environment could be developed, this could provide for a highly effective live attenuated vaccine strain.

It may also prove interesting to cross genes from different *Ehrlichia* species, to determine if certain genes or loci confer the ability to infect different cell types. It would be interesting to replace the P28 locus of *E. chaffeensis* with the P28 of *E. ruminantium* to see how the host range or infectivity of the species is affected.

Use of flp recombinase to create conditional knockouts or remove the selection genes in the process may provide another method for highly specific genetic knockouts. Use of transposon systems to generate random transformants should also prove effective, especially since many targeted knockouts may prove lethal.

I would propose that one possible approach would be to knock out genes using a promoter-less resistance gene in a transposon. In this manner, the resistance gene would only be expressed if it inserts in front of another gene, hijacking its promoter and knocking it out in the process. Consequently, only sublethal transformants would be generated. Using a rescue cloning technique and high throughput methods, one could screen a large number of clones using high throughput methods. This approach would identify non-lethal knockouts for further study, and if sufficient numbers of clones were studied, a pattern of potentially lethal genetic knockouts could be discerned from those genes that are underrepresented in the pool.

PSGL-1 has been incriminated now as a receptor for both *Anaplasma* and *Ehrlichia*. Given the degree of homology within the family Anaplasmataceae, it is worth investigating the potential role of PSGL-1 in other ehrlichial infections. To further discern the role of PSGL-1 in ehrlichial infection, a follow up experiment utilizing silencing RNA (siRNA) techniques to knock down the expression of PSGL-1 in DH82 cells should be conducted. Also, PSGL-1 knockout mice are available on a C57/BL6

background, which would provide for an *in vivo* confirmation of the importance of PSGL-1 to *Ehrlichia* infection utilizing the *E. muris* mouse model. Since E-selectin and L-selectin have also been identified as receptors for *Ehrlichia*, an experiment to block E-selectin, L-selectin, and PSGL-1 should be conducted to determine if any other receptors are involved in ehrlichial binding and entry.

APPENDIX A

Detailed MALDI-TOF/TOF Instrument and Software Settings

DATA ACQUISITION

Data was acquired with an Applied Biosystems 4700 MALDI TOF/TOF Proteomics Analyzer. Applied Biosystems software package 4000 Series Explorer (v. 3.0 RC1) with Oracle Database Schema Version (v. 3.19.0), Data Version (3.80.0) to acquire both MS and MS/MS spectral data. The following parameters were used:

MS ACQUISITION

Instrument	
Mode:	Reflectron, Positive Ion
Mass Range:	850.000 to 3000.000 Da
Focus Mass:	1500.000 Da
Spectrum	
Acquisition mode:	Accumulate every n-shot sub-spectrum that passes acceptance
Shots/sub-spectrum:	100
Total Shots/Spectrum:	2000
Stop Conditions:	After 20 sub-spectra are acquired (pass or fail)
Acceptance Criteria:	Accept every sub-spectrum

Automatic Control

Laser Settings:	Move before every sub-spectrum (pass or fail)
	Raster Pattern: Uniform
	Fixed Laser Intensity: (approx.) 3500

Digitizer

Bin Size:	0.5 ns
Input bandwidth:	500 MHz
Detector Voltage Multiplier:	1.00
Final Detector Voltage:	2.100 kV

Calibration

Calibration Type: Internal

Automatic calibration using peptide mixture with reference masses 904.468, 1296.685, 1570.677, 2465.199

Internal Calibration – Peak Matching: Minimum S/N: 25

Mass Tolerance: +/- 3 Da

Minimum Peaks to Match: 3

Use monoisotopic peaks only

MS/MS ACQUISITION

Instrument

Mode:	MS/MS 1kV Positive
CID Control:	Off (PSD only)
Acquisition Control:	Automatic
Precursor Mass Window:	Absolute, +/- 3 Da
Metastable Suppressor:	ON

Spectrum

Accumulate every n-shot sub-spectrum that passes acceptance

Shots/sub-spectrum: 100

Total Shots/Spectrum: 4000

Stop Conditions: After 40 sub-spectra are acquired (pass or fail)

Acceptance Criteria: Accept every sub-spectrum

Automatic Control

Laser Settings: Move before every sub-spectrum (pass or fail)

Raster Pattern: Uniform

Fixed Laser Intensity: (approx.) 4500

Digitizer

Bin size: 0.5 ns

Input Bandwidth: 500 MHz

Detector Voltage Multiplier: 1.00

Final Detector Voltage: 2.200 kV

Calibration:

Calibration Type: Internal

Automatic calibration using peptide with reference mass m/z = 1570.700 (fragment peaks m/z = 175.120, 480.257, 684.347, 1056.475, 1441.635)

Internal Calibration – Peak Matching: Minimum S/N: 15

Mass Tolerance: +/- 3 Da

Minimum Peaks to Match: 3

Use monoisotopic peaks only

INTERPRETATION METHOD

Monoisotopic Precursor Selection for MS/MS
Minimum S/N Filter: 20

Exclusion List (0.50 Da tolerance): 830.400, 842.510, 856.500, 870.450, 1045.560, 1179.600, 1277.710, 1475.790, 2211.100, 2225.100, 2239.128

Adduct Exclusion List (0.03 Da tolerance): 21.982, 37.956

Precursor Final Selection Criteria
Precursor Sorting Order/Spot: Strongest Precursors First
MS/MS Acquistion Order/Spot: Weakest Precursors First
First Precursors to Skip/Spot: 0
Maximum Precursors/Spot: approx. 6 (depends on data)

MS/MS Method
MSMS positive ion mode

DATABASE SEARCH

Applied Biosystems software package GPS Explorer TM Software (v. 3.0) was used in conjunction with Mascot to search the NCBI database utilizing both MS and MS/MS spectral data. The following parameters were used:

MS Peak Filtering
Mass range: 800 Da to 4000 Da
Minimum S/N filter: 10

Mass exclusion list tolerance: 0.5 Da

Mass exclusion list: 842.51, 870.45, 1045.56, 1179.60, 1277.71, 1475.79, 2211.1

MS/MS Peak Filtering

Minimum S/N filter: 10

Database Search

Taxonomy:	Bacteria
Database:	NCBI
Enzyme:	Trypsin
Max. Missed Cleavages:	1
Fixed Modifications:	Carbamidomethyl (C)
Variable Modifications:	Oxidation (M)
Precursor Tolerance:	0.2 Da
MS/MS Fragment Tolerance:	0.3 Da
Mass:	Monoisotopic
Peptide Charges:	+1

BIBLIOGRAPHY

1. **Aguirre, E., A. Sainz, S. Dunner, I. Amusategui, L. Lopez, F. Rodriguez-Franco, I. Luaces, O. Cortes, and M. A. Tesouro**. 2004. First isolation and molecular characterization of *Ehrlichia canis* in Spain. Vet. Parasitol. **125**:365-372.

2. **Aguirre, E., M. A. Tesouro, I. Amusategui, F. Rodriguez-Franco, and A. Sainz**. 2004. Assessment of feline ehrlichiosis in central Spain using serology and a polymerase chain reaction technique. Ann. N. Y. Acad. Sci. **1026**:103-105.

3. **Aivaliotis, M., E. Neofotistou, H. W. Rémigy, G. Tsimpinos, A. Lustig, F. Lottspeich, and G. Tsiotis**. 2004. Isolation and characterization of an outer membrane protein of *Chlorobium tepidum*. Photosynth. Res. **79**:161-166.

4. **Alekseev, A. N., H. V. Dubinina, D. P. Van, I, and L. M. Schouls**. 2001. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic Regions of Russia. J. Clin. Microbiol. **39**:2237-2242.

5. **Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson**. 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29**:2838-2842.

6. **Anderson, B. E., C. E. Greene, D. C. Jones, and J. E. Dawson**. 1992. *Ehrlichia ewingii* sp. nov., the etiologic agent of canine granulocytic ehrlichiosis. Int. J. Syst. Evol. Microbiol. **42**:299-302.

7. **Anderson, B. E., J. W. Sumner, J. E. Dawson, T. Tzianabos, C. R. Greene, J. G. Olson, D. B. Fishbein, M. Olsen-Rasmussen, B. P. Holloway, E. H. George, and A. F. Azad**. 1992. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J. Clin. Microbiol. **30**:775-780.

8. **Asanovich, K. M., J. S. Bakken, J. E. Madigan, M. Aguero-Rosenfeld, G. P. Wormser, and J. S. Dumler**. 1997. Antigenic diversityof granulocytic *Ehrlichia* isolates from humans in Wisconsin and New York and a horse in California. J. Infect. Dis. **176**:1029-1034.

9. **Bakken, J. S., J. S. Dumler, S.-M. Chen, M. R. Eckman, L. L. VanEtta, and D. H. Walker**. 1994. Human granulocytic ehrlichiosis in the upper midwest United States. A new species emerging? JAMA **272**:212-218.

10. **Barbet, A. F., P. F. M. Meeus, M. Belanger, M. V. Bowie, J. Yi, A. M. Lundgren, A. R. Alleman, S. J. Wong, F. K. Chu, U. G. Munderloh, and S. D. Jauron**. 2003. Expression of multiple outer membrane protein sequence variants from a single genomic locus of *Anaplasma phagocytophilum*. Infect. Immun. **71**:1706-1718.

11. **Barnewall, R. E., N. Ohashi, and Y. Rikihisa**. 1999. *Ehrlichia chaffeensis* and *E.sennetsu,* but not the human granulocytic ehrlichiosis agent, colocalize with transferrin receptor and up-regulate transferrin receptor mRNA by activating iron-responsive protein 1. Infect. Immun. **67**:2258-2265.

12. **Barnewall, R. E. and Y. Rikihisa**. 1994. Abrogation of gamma interferon-induced inhibition of *Ehrlichia chaffeensis* infection in human monocytes with iron transferrin. Infect. Immun. **62**:4804-4810.

13. **Barnewall, R. E., Y. Rikihisa, and E. H. Lee**. 1997. *Ehrlichia chaffeensis* inclusions are early endosomes which selectively accumulate transferrin receptor. Infect. Immun. **65**:1455-1461.

14. **Bateman, A., L. Coin, R. Durbin, R. D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, M. Marshall, S. Moxon, E. L. L. Sonnhammer, D. J. Studholme, C. Yeats, and S. R. Eddy**. 2004. The Pfam protein families database. Nucl. Acids Res. **32**:D138-D141.

15. **Bekker, C. P., M. Postigo, A. Taoufik, L. Bell-Sakyi, C. Ferraz, D. Martinez, and F. Jongejan**. 2005. Transcription analysis of the major antigenic protein 1 multigene family of three *in vitro*-cultured *Ehrlichia ruminantium* isolates. J. Bacteriol. **187**:4782-4791.

16. **Bell-Sakyi, L., E. A. Paxton, U. G. Munderloh, and K. J. Sumption**. 2000. Growth of *Cowdria ruminantium*, the causative agent of heartwater, in a tick cell line. J. Clin. Microbiol. **38**:1238-1240.

17. **Bellah, J. R., R. M. Shull, and E. V. S. Selcer**. 1986. *Ehrlichia canis*-related polyarthritis in a dog. J. Am. Vet. Med. Assoc. **189**:922-923.

18. **Berry, D. S., R. S. Miller, J. A. Hooke, R. F. Massung, J. Bennett, and M. G. Ottolini**. 1999. Ehrlichial meningitis with cerebrospinal fluid morulae. Pediatr. Infect. Dis. J. **18**:552-555.

19. **Bishopp, F. C. and H. L. Trembley**. 1945. Distribution and hosts of certain North American ticks. J. Parasitol. **31**:1-54.

20. **Bouloy, R. P., M. R. Lappin, C. H. Holland, M. A. Thrall, D. Baker, and S. O'Neil**. 1994. Clinical ehrlichiosis in a cat. JAMA **204**:1475-1478.

21. **Branger, S., J. M. Rolain, and D. Raoult**. 2004. Evaluation of antibiotic susceptibilities of *Ehrlichia canis, Ehrlichia chaffeensis,* and *Anaplasma phagocytophilum* by real-time PCR. Antimicrob. Agents Chemother. **48**:4822-4828.

22. **Breitschwerdt, E. B., A. C. brams-Ogg, M. R. Lappin, D. Bienzle, S. I. Hancock, S. M. Cowan, J. K. Clooten, B. C. Hegarty, and E. C. Hawkins**. 2002. Molecular evidence supporting *Ehrlichia canis*-like infection in cats. J. Vet. Intern. Med. **16**:642-649.

23. **Brouqui, P. and D. Raoult**. 1992. *In vitro* antibiotic susceptibility of the newly recognized agent of ehrlichiosis in humans, *Ehrlichia chaffeensis*. Antimicrob. Agents Chemother. **36**:2799-2803.

24. **Buhles, W. C., Jr., D. L. Huxsoll, and M. Ristic**. 1974. Tropical canine pancytopenia: clinical, hematologic, and serologic response of dogs to *Ehrlichia canis* infection, tetracycline therapy, and challenge inoculation. J. Infect. Dis. **130**:357-367.

25. **Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikihisa, A. Unver, M. Gaudreault-Keener, F. A. Manian, A. M. Liddell, N. Schmulewitz, and G. A. Storch**. 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. N. Engl. J. Med. **341**:148-155.

26. **Centers for Disease Control and Prevention**. 2002. Summary of notifiable diseases, United States, 2000. MMWR **49**:3.

27. **Centers for Disease Control and Prevention**. 2005. Summary of notifiable diseases-United States, 2003. MMWR **52**:16.

28. **Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker**. 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32**:589-595.

29. **Childs, J. E. and C. D. Paddock**. 2003. The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. Annu. Rev. Entomol. **48**:307-337.

30. **Clauser, K. R., P. Baker, and A. L. Burlingame**. 1999. Role of accurate mass measurement (±10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Anal. Chem. **71**:2871-2882.

31. **Clymer, B. C.** 1970. Animal hosts of economically important ticks (Acarina) in East - Central Oklahoma., p. 612-614. *In* D.E.Howell and J.A.Hair (ed.), Annals of the Entomological Society of America, vol. 63.

32. **Codner, E. C. and L. L. Farris-Smith**. 1986. Characterization of the subclinical phase of ehrlichiosis in dogs. J. Am. Vet. Med. Assoc. **189**:47-50.

33. **Cohn, L. A.** 2003. Ehrlichiosis and related infections. Vet. Clin. North Am. Small Anim. Pract. **33**:863-884.

34. **Cowell, R. L., R. D. Tyler, K. D. Clinkenbeard, and J. H. Meinkoth**. 1988. Ehrlichiosis and polyarthritis in three dogs. J. Am. Vet. Med. Assoc. **192**:1093-1095.

35. **Crocquet-Valdes, P. A., J. W. McBride, H.-M. Feng, N. Ismail, M. Small, X. J. Yu, and D. H. Walker**. 2005. Analysis of ehrlichial *p28* gene expression in a murine model of persistent infection. Ann. N. Y. Acad. Sci. **1063**:420-424.

36. **Cummings, R. D.** 1999. Structure and function of the selectin ligand PSGL-1. Braz. J. Med. Biol. Res. **32**:519-528.

37. **David, N. P.** 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20**:3551-3567.

38. **Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. Goldsmith, K. H. Wilson, and C. W. Duntley**. 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. **29**:2741-2745.

39. **Dawson, J. E., K. L. Biggie, C. K. Warner, K. Cookson, S. Jenkins, J. F. Levine, and J. G. Olson**. 1996. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. Am. J. Vet. Res. **57**:1175-1179.

40. **Dawson, J. E., C. D. Paddock, C. K. Warner, P. W. Greer, J. H. Bartlett, S. A. Ewing, U. G. Munderloh, and S. R. Zaki**. 2001. Tissue diagnosis of *Ehrlichia chaffeensis* in patients with fatal ehrlichiosis by use of immunohistochemistry, *in situ* hybridization, and polymerase chain reactions. Am. J. Trop. Med. Hyg. **65**:603-609.

41. **Dawson, J. E., D. E. Stallknecht, E. W. Howerth, C. Warner, K. Biggie, W. R. Davidson, J. M. Lockhart, V. F. Nettles, J. G. Olson, and J. E. Childs**. 1994. Susceptibility of white-tailed deer (*Odocoileus virginianus*) to infection with *Ehrlichia chaffeensis*, the etiologic agent of human ehrlichiosis. J. Clin. Microbiol. **32**:2725-2728.

42. **Donatien, A. and F. Lestoquard**. 1935. Existence en Algerie d'une *Rickettsia* du chien. Bull. Soc. Pathol. Exot. **28**:418-419.

43. **Doyle, C. K., M. B. Labruna, E. B. Breitschwerdt, Y. W. Tang, R. E. Corstvet, B. C. Hegarty, K. C. Bloch, P. Li, D. H. Walker, and J. W. McBride**. 2005. Detection of medically important *Ehrlichia* by quantitative multicolor TaqMan real-time polymerase chain reaction of the dsb gene. J. Mol. Diagn. **7**:504-510.

44. **Doyle, C. K., X. Zhang, V. L. Popov, and J. W. McBride**. 2005. An immunoreactive 38-kilodalton protein of *Ehrlichia canis* shares structural homology and iron-binding capacity with the ferric ion-binding protein family. Infect. Immun. **73**:62-69.

45. **Dugan, V. G., S. E. Little, D. E. Stallknecht, and A. D. Beall**. 2000. Natural infection of domestic goats with *Ehrlichia chaffeensis*. J. Clin. Microbiol. **38**:448-449.

46. **Dumler, J. S.** 2005. Rocky Mountain spotted fever - changing ecology and persisting virulence. N. Engl. J. Med. **353**:551-553.

47. **Dumler, J. S., K. M. Asanovich, J. S. Bakken, P. Richter, R. Kimsey, and J. E. Madigan**. 1995. Serologic cross-reactions among *Ehrlichia equi, Ehrlichia phagocytophila,* and human granulocytic *Ehrlichia*. J. Clin. Microbiol. **33**:1098-1103.

48. **Dumler, J. S., A. F. Barbet, C. P. Bekker, G. A. Dasch, G. H. Palmer, S. C. Ray, Y. Rikihisa, and F. R. Rurangirwa**. 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designations of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. **6**:2145-2165.

49. **Dumler, J. S. and D. H. Walker**. 1999. Ehrlichioses, p. 598-604. *In* R. L. Guerrant, D. H. Walker, and P. F. Weller (ed.), Tropical Infectious Diseases, Principals, Pathogens, and Practice. Churchill Livingstone, Philadelphia.

50. **Dumler, M. D. and M. D. Bakken**. 1998. Human Ehrlichioses: Newly recognized infections transmitted by ticks. Annu. Rev. Med. **49**:201-213.

51. **Dunning Hotopp, J. C., M. Lin, R. Madupu, J. Crabtree, S. V. Angiuoli, J. Eisen, R. Seshadri, Q. Ren, M. Wu, T. R. Utterback, S. Smith, M. Lewis, H. Khouri, C. Zhang, H. Niu, Q. Lin, N. Ohashi, N. Zhi, W. Nelson, L. M. Brinkac, R. J. Dodson, M. J. Rosovitz, J. Sundaram, S. C. Daugherty, T. Davidsen, A. S. Durkin, M. Gwinn, D. H. Haft, J. D. Selengut, S. A. Sullivan, N. Zafar, L. Zhou, F. Benahmed, H. Forberger, R. Halpin, S. Mulligan, J. Robinson, O. White, Y. Rikihisa, and H. Tettelin**. 2006. Comparative genomics of emerging human ehrlichiosis agents. PLoS Genetics **2**:e21.

52. **Edwards, J. L. and M. A. Apicella**. 2005. I-domain-containing integrins serve as pilus receptors for *Neisseria gonorrhoeae* adherence to human epithelial cells. Cell Microbiol. **7**:1197-1211.

53. **Edwards, M. S., J. E. Jones, D. L. Leass, J. W. Whitmore, J. E. Dawson, and D. B. Fishbein**. 1988. Childhood infection caused by *Ehrlichia canis* or a closely related organism. Pediatr. Infect. Dis. J. **7**:651-654.

54. **Eng, T. R., J. R. Harkess, D. B. Fishbein, J. E. Dawson, C. N. Greene, M. A. Redus, and F. T. Satalowich**. 1990. Epidemiologic, clinical, and laboratory findings of human ehrlichiosis in the United States, 1988. JAMA **264**:2251-2258.

55. **Evangelista, V., S. Manarini, R. Sideri, S. Rotondo, N. Martelli, A. Piccoli, L. Totani, P. Piccardoni, D. Vestweber, G. de Gaetano, and C. Cerletti**. 1999. Platelet/Polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: Role of PSGL-1 as a signaling molecule. Blood **93**:876-885.

56. **Everett, E. D., K. A. Evans, R. B. Henry, and G. McDonald**. 1994. Human ehrlichiosis in adults after tick exposure. Ann. Intern. Med. **120**:730-735.

57. **Ewing, S. A., J. E. Dawson, A. A. Kocan, R. W. Barker, C. K. Warner, R. J. Panciera, J. C. Fox, K. M. Kocan, and E. F. Blouin**. 1995. Experimental transmission of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) among white-tailed deer by *Amblyomma americanum* (Acari: Ixodidae). J. Med. Entomol. **32**:368-374.

58. **Ewing, S. A., W. R. Roberson, R. G. Buckner, and C. S. Hayat**. 1971. A new strain of *Ehrlichia canis*. J. Am. Vet. Med. Assoc. **159**:1771-1774.

59. **Fichtenbaum, C. J., L. R. Peterson, and G. J. Weil**. 1993. Ehrlichiosis presenting as a life-threatening illness with features of the toxic shock syndrome. Am. J. Med. **95**:351-357.

60. **Fishbein, D. B., J. E. Dawson, and L. E. Robinson**. 1994. Human ehrlichiosis in the United States, 1985 to 1990. Ann. Intern. Med. **120**:736-743.

61. **Fishbein, D. B., A. Kemp, J. E. Dawson, N. R. Greene, M. A. Redus, and D. H. Fields**. 1989. Human ehrlichiosis: prospective active surveillance in febrile hospitalized patients. J. Infect. Dis. **160**:803-809.

62. **Fleckenstein, J. M., D. J. Kopecko, R. L. Warren, and E. A. Elsinghorst**. 1996. Molecular characterization of the *tia* invasion locus from enterotoxigenic *Escherichia coli*. Infect. Immun. **64**:2256-2265.

63. **Foggie, A.** 1951. Studies on the infectious agent of tick-borne fever in sheep. J. Pathol. Bacteriol. **63**:1-15.

64. **Fordham, L. A., C. J. Chung, B. B. Specter, D. F. Merten, and D. L. Ingram**. 1998. Ehrlichiosis: Findings on chest radiographs in three pediatric patients. Am. J. Roentgenol. **171**:1421-1424.

65. **Frank, J. and E. B. Breitschwerdt**. 1999. A retrospective study of ehrlichiosis in 62 dogs from North Carolina and Virginia. J. Vet. Intern. Med. **13**:194-201.

66. **Frutos, R., A. Viari, C. Ferraz, A. Morgat, S. Eychenie, Y. Kandassamy, I. Chantal, A. Bensaid, E. Coissac, N. Vachiery, J. Demaille, and D. Martinez**. 2006. Comparative genomic analysis of three strains of *Ehrlichia ruminantium* reveals an active process of genome size plasticity. J. Bacteriol. **188**:2533-2542.

67. **Gasanov, U., C. Koina, K. W. Beagley, R. J. Aitken, and P. M. Hansbro**. 2006. Identification of the insulin-like growth factor II receptor as a novel receptor for binding and invasion by *Listeria monocytogenes*. Infect. Immun. **74**:566-577.

68. **Godzeski, C. W., G.Brier, and D.E.Pavey**. 1962. L-phase growth induction as a general characteristic of antibiotic-bacterial interaction in the presence of serum. Antimicrob. Agents Chemother. 843-853.

69. **Goodman, J. L., C. Nelson, B. Vitale, J. E. Madigan, J. S. Dumler, T. J. Kurtti, and U. G. Munderloh**. 1996. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N. Engl. J. Med. **334**:209-215.

70. **Gordon, W. S.** 1932. `Tick-borne fever '. (A hitherto undescribed disease of sheep). J. Comp. Pathol. Therap. **65**:301-307.

71. **Greig, B., K. M. Asanovich, P. J. Armstrong, and J. S. Dumler**. 1996. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. J. Clin. Microbiol. **34**:44-48.

72. **Grindem, C. B., E. B. Breitschwerdt, P. C. Perkins, L. D. Cullins, T. J. Thomas, and B. C. Hegarty**. 1999. Platelet-associated immunoglobulin (antiplatelet antibody) in canine Rocky Mountain spotted fever and ehrlichiosis. J. Am. Anim Hosp. Assoc. **35**:56-61.

73. **Harrus, S., P. H. Kass, E. Klement, and T. Waner**. 1997. Canine monocytic ehrlichiosis: a retrospective study of 100 cases, and an epidemiological investigation of prognostic indicators for the disease. Vet Rec **141**:360-363.

74. **Harrus, S., T. Waner, H. Bark, F. Jongejan, and A. W. C. A. Cornelissen**. 1999. Recent advances in determining the pathogenesis of canine monocytic ehrlichiosis. J. Clin. Microbiol. **37**:2745-2749.

75. **Harrus, S., T. Waner, A. Keysary, I. Aroch, H. Voet, and H. Bark**. 1998. Investigation of splenic functions in canine moncytic ehrlichiosis. Vet. Immunol. Immunopathol. **62**:15-27.

76. **Herron, M. J., C. M. Nelson, J. Larson, K. R. Snapp, G. S. Kansas, and J. L. Goodman**. 2000. Intracellular parasitism by the human granulocytic ehrlichiosis bacterium through the P-selectin ligand, PSGL-1. Science **288**:1653-1656.

77. **Hidari, K. I. P., A. S. Weyrich, G. A. Zimmerman, and R. P. McEver**. 1997. Engagement of P-selectin glycoprotein ligand-1 enhances tyrosine phosphorylation and activates mitogen-activated protein kinases in human neutrophils. J. Biol. Chem. **272**:28750-28756.

78. **Hongo, I. and K. C. Bloch**. 2006. *Ehrlichia* infection of the central nervous system. Curr. Treat. Options. Neurol. **8**:179-184.

79. **Hudson, J. R.** 1950. The recognition of tick-borne fever as a disease of cattle. Br. Vet. J. **106**:3-17.

80. **Huxsoll, D. L.** 1976. Canine ehrlichiosis (tropical canine pancytopenia): a review. Vet. Parasitol. **2**:49-60.

81. **Kawahara, M., T. Ito, C. Suto, S. Shibata, Y. Rikihisa, K. Hata, and K. Hirai**. 1999. Comparison of *Ehrlichia muris* strains isolated from wild mice and ticks and serologic survey of humans and animals with *E.muris* as Antigen. J. Clin. Microbiol. **37**:1123-1129.

82. **Kawahara, M., C. Suto, Y. Rikihisa, S. Yamamoto, and Y. Tsuboi**. 1993. Characterization of ehrlichial organisms isolated from a wild mouse. J. Clin. Microbiol. **31**:89-96.

83. **Kieffer, J. D., R. C. Fuhlbrigge, D. Armerding, C. Robert, K. Ferenczi, R. T. Camphausen, and T. S. Kupper**. 2001. Neutrophils, monocytes, and dendritic cells express the same specialized form of PSGL-1 as do skin-homing memory T cells: cutaneous lymphocyte antigen. Biochem. Biophys. Res. Commun. **285**:577-587.

84. **Kocan, A. A., G. C. Levesque, L. C. Whitworth, G. L. Murphy, S. A. Ewing, and R. W. Barker**. 2000. Naturally occurring *Ehrlichia chaffeensis* infection in coyotes from Oklahoma. Emerg. Infect. Dis. **6**:477-480.

85. **Kollars, T. M., Jr., J. H. Oliver, Jr., L. A. Durden, and P. G. Kollars**. 2000. Host association and seasonal activity of *Amblyomma americanum* (Acari: Ixodidae) in Missouri. J. Parasitol. **86**:1156-1159.

86. **Kumar, R., R. T. Camphausen, F. X. Sullivan, and D. A. Cumming**. 1996. Core2 beta-1,6-N-acetylglucosaminyltransferase enzyme activity is critical for P-selectin glycoprotein ligand-1 binding to P-selectin. Blood **88**:3872-3879.

87. **Laszik, Z., P. J. Jansen, R. D. Cummings, T. F. Tedder, R. P. McEver, and K. L. Moore**. 1996. P-selectin glycoprotein ligand-1 is broadly expressed in cells of myeloid, lymphoid, and dendritic lineage and in some nonhematopoietic cells. Blood **88**:3010-3021.

88. **Lee, E. H. and Y. Rikihisa**. 1996. Absence of tumor necrosis factor alpha, interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor expression but presence of IL-1b, IL-8, and IL-10 expression of human monocytes exposed to viable or killed *Ehrlichia chaffeensis*. Infect. Immun. **64**:4211-4219.

89. **Lee, E. H. and Y. Rikihisa**. 1997. Anti-*Ehrlichia chaffeensis* antibody complexed with *E. chaffeensis* induces potent proinflammatory cytokine mRNA expression in human monocytes through sustained reduction of IkB-a and activation of NF-kB. Infect. Immun. **65**:2890-2897.

90. **Lee, E. H. and Y. Rikihisa**. 1998. Protein kinase A-mediated inhibition of gamma interferon-induced tyrosine phosphorylation of janus kinases and latent cytoplasmic transcription factors in human monocytes by *Ehrlichia chaffeensis*. Infect. Immun. **66**:2514-2619.

91. **Legendre, A. M.** Ehrlichiosis in cats.[comment]. J. Vet. Intern. Med. **16**:641-Dec.

92. **Lewis, G. E., Jr., D. L. Huxsoll, M. Ristic, and A. J. Johnson**. 1975. Experimentally induced infection of dogs, cats, and nonhuman primates with *Ehrlichia equi*, etiologic agent of equine ehrlichiosis. Am. J. Vet. Res. **36**:85-88.

93. **Li, F., P. P. Wilkins, S. Crawley, J. Weinstein, R. D. Cummings, and R. P. McEver**. 1996. Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. J. Biol. Chem. **271**:3255-3264.

94. **Li, J. S., E. Yager, M. Reilly, C. Freeman, G. R. Reddy, A. A. Reilly, F. K. Chu, and G. M. Winslow**. 2001. Outer membrane protein-specific monoclonal antibodies protect SCID mice from fatal infection by the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis*. J. Immunol. **166**:1855-1862.

95. **Lin, M. and Y. Rikihisa**. 2003. *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. Infect. Immun. **71**:5324-5331.

96. **Lin, M. and Y. Rikihisa**. 2003. Obligatory intracellular parasitism by *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* involves caveolae and glycosylphosphatidylinositil-anchored proteins. Cell Microbiol. **5**:809-820.

97. **Lin, M., M. X. Zhu, and Y. Rikihisa**. 2002. Rapid activation of protein tyrosine kinase and phospholipase C-g2 and increase in cytosolic free calcium are required by ehrlichia chaffeensis for internalization and growth in THP-1 cells. Infect. Immun. **70**:889-898.

98. **Lockhart, J. M., W. R. Davidson, D. E. Stallknecht, and J. E. Dawson**. 1996. Site-specific geographic association between *Amblyomma americanum* (Acari: Ixodidae) infestations and *Ehrlichia chaffeensis*-reactive (Rickettsiales: Ehrlichieae) antibodies in white-tailed deer. J. Med. Entomol. **33**:153-158.

99. **Lockhart, J. M., W. R. Davidson, D. E. Stallknecht, J. E. Dawson, and E. W. Howerth**. 1997. Isolation of *Ehrlichia chaffeensis* from wild white-tailed deer (*Odocoileus virginianus*) confirms their role as natural reservoir hosts. J. Clin. Microbiol. **35**:1681-1686.

100. **Lockhart, J. M., W. R. Davidson, D. E. Stallknecht, J. E. Dawson, and S. E. Little**. 1997. Natural history of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) in the piedmont physiographic province of Georgia. J. Parasitol. **83**:887-894.

101. **Lohr, C. V., K. A. Brayton, A. F. Barbet, and G. H. Palmer**. 2004. Characterization of the *Anaplasma marginale msp2* locus and its synteny with the *omp1/p30* loci of *Ehrlichia chaffeensis* and *E. canis*. Gene **325**:115-121.

102. **Long, J. Z., C. S. Lackan, and A. K. Hadjantonakis**. 2005. Genetic and spectrally distinct in vivo imaging: embryonic stem cells and mice with widespread expression of a monomeric red fluorescent protein. BMC. Biotechnol. **5**:20.

103. **Long, S. W., J. M. Pound, and X. J. Yu**. 2004. *Ehrlichia* prevalence in *Amblyomma americanum*, Central Texas. Emerg. Infect. Dis. **10**:1342-1343.

104. **Long, S. W., T. J. Whitworth, D. H. Walker, and X.-J. Yu**. 2005. Overcoming barriers to the transformation of the genus *Ehrlichia*. Ann. N. Y. Acad. Sci. **1063**:403-410.

105. **Long, S. W., X.-F. Zhang, H. Qi, S. Standaert, D. H. Walker, and X.-J. Yu**. 2002. Antigenic variation of *Ehrlichia chaffeensis* resulting from differential expression of the 28-kilodalton protein gene family. Infect. Immun. **70**:1824-1831.

106. **Long, S. W., X. Zhang, J. Zhang, R. P. Ruble, P. D. Teel, and X.-J. Yu**. 2003. Evaluation of transovarial transmission and transmissibility of *Ehrlichia chaffeensis* (Rickettsiales: Anaplasmataceae) in *Amblyomma americanum* (Acari: Ixodidae). J. Med. Entomol. **40**:1000-1004.

107. **Madigan, J. E.** 1993. Equine Ehrlichiosis, p. 209-214. *In* Z. Woldehiwet and M. Ristic (ed.), Rickettsial and chlamydial diseases of domestic animals. Pergamon Press, Oxford.

108. **Maeda, K., N. Markowitz, R. C. Hawley, M. Ristic, D. Cox, and J. E. McDade**. 1987. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. N. Engl. J. Med. **316**:853-856.

109. **Martin, G. S., B. W. Christman, and S. M. Standaert**. 1999. Rapidly fatal infection with *Ehrlichia chaffeensis*. N. Engl. J. Med. **341**:763-764.

110. **Martinez, M., M. Joffraud, S. Giraud, B. Baisse, M. P. Bernimoulin, M. Schapira, and O. Spertini**. 2005. Regulation of PSGL-1 Interactions with L-selectin, P-selectin, and E-selectin: Role of human fucosyltransferase-IV and -VII. J. Biol. Chem. **280**:5378-5390.

111. **Mathisen, G. E., P. J. Weiss, and C. A. Kennedy**. 1993. Pneumonia, aseptic meningitis, and leukopenia in a 28-year-old man. Clin. Infect. Dis. **16**:809-815.

112. **Mavromatis, K., C. K. Doyle, A. Lykidis, N. Ivanova, M. P. Francino, P. Chain, M. Shin, S. Malfatti, F. Larimer, A. Copeland, J. C. Detter, M. Land, P. M. Richardson, X. J. Yu, D. H. Walker, J. W. McBride, and N. C. Kyrpides**. 2006. The genome of the obligately intracellular bacterium *Ehrlichia canis* reveals themes of complex membrane structure and immune evasion strategies. The Journal of Bacteriology **188**:4015-4023.

113. **McBride, J. W., R. E. Corstvet, S. D. Gaunt, C. Boudreaux, T. Guedry, and D. H. Walker**. 2003. Kinetics of antibdy response to *Ehrlichia canis* immunoreactive proteins. Infect. Immun. **71**:2516-2524.

114. **McBride, J. W., X.-J. Yu, and D. H. Walker**. 1999. Molecular cloning of the gene for a conserved major immunoreactive 28-kilodalton protein of *Ehrlichia canis*: a potential serodiagnostic antigen. Clin. Diagn. Lab. Immunol. **6**:392-399.

115. **McKechnie, D. B., K. S. Slater, J. E. Childs, R. F. Massung, and C. D. Paddock**. 2000. Survival of Ehrlichia chaffeensis in refrigerated, ADSOL-treated RBCs. Transfusion (Paris). **40**:1041-1047.

116. **Moore, K. L., N. L. Stults, S. Diaz, D. F. Smith, R. D. Cummings, A. Varki, and R. P. McEver**. 1992. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J. Cell Biol. **118**:445-456.

117. **Moran, N. A. and G. R. Plague**. 2004. Genomic changes following host restriction in bacteria. Curr. Opin. Genet. Dev. **14**:627-633.

118. **Moshkovski, S. D.** 1945. Cytotropic inducers of infection and the classification of the *Rickettsiae* with *Chlamydozoa*. Adv. Mod. Biol. (Moscow) **19**:1-44.

119. **Mott, J., R. E. Barnewall, and Y. Rikihisa**. 1999. Human granulocytic ehrlichiosis agent and *Ehrlichia chaffeensis* reside in different cytoplasmic compartments in HL-60 cells. Infect. Immun. **67**:1368-1378.

120. **Murphy, C. I., J. R. Storey, J. Recchia, L. A. Doros-Richert, C. Gingrich-Baker, K. Munroe, J. S. Bakken, R. T. Coughlin, and G. Beltz**. 1998. Major antigenic proteins of the agent of human granulocytic ehrlichiosis are encoded by members of a multigene family. Infect. Immun. **66**:3711-3718.

121. **Murphy, G. L., S. A. Ewing, L. C. Whitworth, J. C. Fox, and A. A. Kocan**. 1998. A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E.ewingii* in dogs and ticks from Oklahoma. Vet. Parasitol. **79**:325-339.

122. **Norgard, K. E., K. L. Moore, S. Diaz, N. L. Stults, S. Ushiyama, R. P. McEver, R. D. Cummings, and A. Varki**. 1993. Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated O-linked oligosaccharides. J. Biol. Chem. **268**:12764-12774.

123. **Ohashi, N., Y. Rikihisa, and A. Unver**. 2001. Analysis of transcriptionally active gene clusters of major outer membrane protein mutligene family in *Ehrlichia canis* and *E. chaffeensis*. Infect. Immun. **69**:2083-2091.

124. **Ohashi, N., A. Unver, N. Zhi, and Y. Rikihisa**. 1998. Cloning and characterization of multigenes encoding the immunodominant 30-kilodalton major outer membrane proteins of *Ehrlichia canis* and application of the recombinant protein for serodiagnosis. J. Clin. Microbiol. **36**:2671-2680.

125. **Olano, J. P., E. Masters, L. Cullman, W. Hogrefe, X.-J. Yu, and D. H. Walker**. 1999. Human monocytotrophic ehrlichiosis (HME): epidemiological, clinical and laboratory diagnosis of a newly emergent infection in the United States, p. 262-268. *In* D. Raoult and P. Brouqui (ed.), Rickettsiae and Rickettsial Diseases at the Turn of the Third Millenium. Elsevier, Paris.

126. **Olano, J. P., G. Wen, H.-M. Feng, J. W. McBride, and D. H. Walker**. 2004. Histologic, serologic, and molecular analysis of persistent ehrlichiosis in a murine model. Am. J. Pathol. **165**:997-1006.

127. **Pace, J., M. J. Hayman, and J. E. Galan**. 1993. Signal transduction and invasion of epithelial cells by *S. typhimurium*. Cell **72**:505-514.

128. **Paddock, C. D. and J. E. Childs**. 2003. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clin. Microbiol. Rev. **16**:37-64.

129. **Paddock, C. D., S. M. Folk, G. M. Shore, L. J. Machado, M. M. Huycke, L. N. Slater, A. M. Liddell, R. S. Buller, G. A. Storch, T. P. Monson, D. Rimland, J. W. Sumner, J. Singleton, K. C. Bloch, Y.-W. Tang, S. M. Standaert, and J. E. Childs**. 2001. Infections wih *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in persons coinfected with human immunodeficiency virus. Clin. Infect. Dis. **33**:1586-1594.

130. **Panciera, R. J., S. A. Ewing, and A. W. Confer**. 2001. Ocular Histopathology of Ehrlichial Infections in the Dog. Vet. Pathol. **38**:43-46.

131. **Pappin, D.** 1993. Rapid identification of proteins by peptide-mass fingerprinting. Curr. Biol. **3**:327-332.

132. **Park, J., K. S. Choi, and J. S. Dumler**. 2003. Major surface protein 2 of *Anaplasma phagocytophilum* facilitates adherence to granulocytes. Infect. Immun. **71**:4018-4025.

133. **Peter, T. F., E. C. Anderson, M. J. Burridge, and S. M. Mahan**. 1998. Demonstration of a carrier state for *Cowdria ruminantium* in wild ruminants from Africa. J. Wildl. Dis. **34**:567-575.

134. **Peter, T. F., N. R. Bryson, B. D. Perry, C. J. O'Callaghan, G. F. Medley, G. E. Smith, G. Mlambo, I. G. Horak, M. J. Burridge, and S. M. Mahan**. 1999. *Cowdria ruminantium* infection in ticks in the Kruger National Park. Vet. Rec. **145**:304-307.

135. **Popov, V., V. C. Han, S.-M. Chen, J. S. Dumler, H.-M. Feng, T. G. Andreadis, R. B. Tesh, and D. H. Walker**. 1998. Ultrastructural differentiation of the genogroups in the genus *Ehrlichia*. J. Med. Microbiol. **47**:235-251.

136. **Popov, V. L., X.-J. Yu, and D. H. Walker**. 2000. The 120kDa outer membrane protein of *Ehrlichia chaffeensis:* preferential expression on dense-core cells and gene expression in *Escherichia coli* associated with attachment and entry. Microb. Pathog. **28**:71-80.

137. **Pusterla, N., J. Huder, C. Wolfensberger, B. Litschi, A. Parvis, and H. Lutz**. 1997. Granulocytic ehrlichiosis in two dogs in Switzerland. J. Clin. Microbiol. **35**:2307-2309.

138. **Rachek, L. I., A. M. Tucker, H. H. Winkler, and D. O. Wood**. 1998. Transformation of *Rickettsia prowazekii* to rifampin resistance. J. Bacteriol. **180**:2118-2124.

139. **Rar VA**. 2005. Tickborne pathogen detection, Western Siberia, Russia. Emerg. Infect. Dis. **11**:1708-1715.

140. **Rawlings, J.** 1996. Human ehrlichiosis in Texas. J. Spirochetal Tick-borne Dis. **3**:94-96.

141. **Reddy, G. R., Cr. R. Sulsona, A. F. Barbet, S. M. Mahan, M. J. Burridge, and A. R. Alleman**. 1998. Molecular characterization of a 28 kDa surface antigen gene family of the tribe Ehrlichiae. Biochem. Biophys. Res. Commun. **247**:636-643.

142. **Rikihisa, Y., Y. Zhang, and J. Park**. 1995. Role of Ca^{2+} and calmodulin in ehrlichial infection in macrophages. Infect. Immun. **63**:2310-2316.

143. **Rosenshine, I., M. S. Donnenberg, J. B. Kaper, and B. B. Finlay**. 1992. Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. EMBO J. **11**:3551-3560.

144. **Rosenshine, I., V. Duronio, and B. B. Finlay**. 1992. Tyrosine protein kinase inhibitors block invasin-promoted bacterial uptake by epithelial cells. Infect. Immun. **60**:2211-2217.

145. **Schutze, G. E. and R. F. Jacobs**. 1997. Human monocytic ehrlichiosis in children. Pediatrics **100**:e10.

146. **Shpynov, S. N., N. V. Rudakov, V. K. Iastrebov, G. N. Leonova, T. G. Khazova, N. V. Egorova, O. N. Borisova, V. P. Preider, G. V. Bezrukov, E. G. Fedorov, A. P. Fedianin, M. B. Sherstneva, A. G. Turyshev, A. P. Gavrilov, M. A. Tankibaev, P. E. Fournier, and D. Raoult**. 2004. New evidence for the detection of *Ehrlichia* and *Anaplasma* in *Ixodes* ticks in Russia and Kazakhstan. Med. Parazitol. (Moscow) **2**:10-14.

147. **Silverstein, A. M.** 2005. Paul Ehrlich, archives and the history of immunology. Nat. Immun. **6**:639.

148. **Singu, V., H. Liu, C. Cheng, and R. R. Ganta**. 2005. *Ehrlichia chaffeensis* expresses macrophage- and tick cell-specific 28-kilodalton outer membrane proteins. Infect. Immun. **73**:79-87.

149. **Singu, V., L. Peddireddi, K. R. Sirigireddy, C. Cheng, U. Munderloh, and R. R. Ganta**. 2006. Unique macrophage and tick cell-specific protein expression from the p28/p30-outer membrane protein multigene locus in *Ehrlichia chaffeensis* and *Ehrlichia canis*. Cell. Microbiol. **8**:1475-1487.

150. **Snapp, K. R., H. Ding, K. Atkins, R. Warnke, F. W. Luscinskas, and G. S. Kansas**. 1998. A novel P-selectin glycoprotein ligand-1 monoclonal antibody recognizes an epitope within the tyrosine sulfate motif of human PSGL-1 and blocks recognition of both P- and L-selectin. Blood **91**:154-164.

151. **Standaert, S. M., J. E. Dawson, W. Schaffner, J. E. Childs, K. L. Biggie, J. Singleton, Jr., R. R. Gerhardt, M. L. Knight, and R. H. Hutcheson**. 1995. Ehrlichiosis in a golf-oriented retirement community. N. Engl. J. Med. **333**:420-425.

152. **Stannard, A. A., D. H. Gribble, and R. S. Smith**. 1969. Equine ehrlichiosis: a disease with similarities to tick-borne fever and bovine petechial fever. Vet. Rec. **84**:149-150.

153. **Stockham SL**. 1985. Canine granulocytic ehrlichiosis in dogs from central Missouri: A possible cause of polyarthritis. Vet. Med. Rev. **6**:3-5.

154. **Troy GC**. 1980. Canine ehrlichiosis: a retrospective study of 30 naturally occurring cases. J. Am. Anim. Hosp. Assoc. **16**:181-187.

155. **Unver, A., Y. Rikihisa, K. Borku, Y. Ozkanlar, and B. Hanedan**. 2005. Molecular detection and characterization of *Ehrlichia canis* from dogs in Turkey. Berl. Munch. Tierarztl. Wochenschr. **118**:300-304.

156. **van Heerden, H., N. E. Collins, K. A. Brayton, C. Rademeyer, and B. A. Allsopp**. 2004. Characterization of a major outer membrane protein multigene family in *Ehrlichia ruminantium*. Gene **330**:159-168.

157. **van Vliet, A. H., F. Jongejan, and B. A. M. van der Zeijst**. 1992. Phylogenetic position of *Cowdria ruminantium* (Rickettsiales) determined by analysis of amplified 16S ribosomal DNA sequences. Int. J. Syst. Bacteriol. **42**:494-498.

158. **Velge, P., E. Bottreau, B. Kaeffer, N. Yurdusev, P. Pardon, and N. Van Langendonck**. 1994. Protein tyrosine kinase inhibitors block the entries of *Listeria monocytogenes* and *Listeria ivanovii* into epithelial cells. Microb. Pathog. **17**:37-50.

159. **Waddle, J. R. and M. P. Littman**. 1988. A retrospective study of 27 cases of naturally occurring canine ehrlichiosis. J. Am. Anim. Hosp. Assoc. **24**:615-620.

160. **Waner, T., S. Harrus, D. J. Weiss, H. Bark, and A. Keysary**. 1995. Demonstration of serum antiplatelet antibodies in experimental acute canine ehrlichiosis. Vet. Immunol. Immunopathol. **48**:177-182.

161. **Wen, B., Y. Rikihisa, J. Mott, P. A. Fuerst, M. Kawahara, and C. Suto**. 1995. *Ehrlichia muris* sp. nov., identified on the basis of 16S rRNA base sequences and serological, morphological, and biological characteristics. Int. J. Syst. Bacteriol. **45**:250-254.

162. **Wooldridge, K. G., P. H. Williams, and J. M. Ketley**. 1996. Host signal transduction and endocytosis of *Campylobacter jejuni*. Microb. Pathog. **21**:299-305.

163. **Xueqing Ba, Cuixia Chen, Yanguang Gao, and Xianlu Zeng**. 2005. Signaling function of PSGL-1 in neutrophils: tyrosine-phosphorylation-dependent and c-Abl-involved alteration in the F-actin-based cytoskeleton. J. Cell. Biochem. **94**:365-373.

164. **Yu, X.-J., P. A. Crocquet-Valdes, L. C. Cullman, V. L. Popov, and D. H. Walker**. 1999. Comparison of *Ehrlichia chaffeensis* recombinant proteins for serologic diagnosis of human monocytotropic ehrlichiosis. J. Clin. Microbiol. **37**:2568-2575.

165. **Yu, X.-J., J. W. McBride, and D. H. Walker**. 1999. Characterization of the genus-common outer membrane proteins in *Ehrlichia*, p. 103-107. *In* D. Raoult and P. Brouqui (ed.), Rickettsiae and Rickettsial Diseases at the Turn of the Third Millenium. Elsevier, Paris.

166. **Yu, X.-J., J. W. McBride, and D. H. Walker**. 1999. Genetic diversity of the 28-kilodalton outer membrane protein gene in human isolates of *Ehrlichia chaffeensis*. J. Clin. Microbiol. **37**:1137-1143.

167. **Yu, X.-J., X.-F. Zhang, J. W. McBride, Y. Zhang, and D. H. Walker**. 2001. Phylogenetic relationships of *Anaplasma marginale* and '*Ehrlichia platys*' to other *Ehrlichia* species determined by GroEL amino acid sequences. Int. J. Syst. Evol. Microbiol. **51**:1143-1146.

168. **Yu, X. j., J. W. McBride, X. f. Zhang, and D. H. WALKER**. 2000. Characterization of the complete transcriptionally active Ehrlichia chaffeensis 28 kDa outer membrane protein multigene family. Gene **248**:59-68.

169. **Zhang, J.-Z., H. Guo, G. M. Winslow, and X. j. Yu**. 2004. Expression of members of the 28-kilodalton major outer membrane protein family of *Ehrlichia chaffeensis* during persistent infection. Infect. Immun. **72**:4336-4343.

170. **Zhang, J.-Z., J. W. McBride, and X.-J. Yu**. 2003. L-selectin and E-selectin expressed on monocytes mediating *Ehrlichia chaffeensis* attachment onto host cells. FEMS Microbiol. Lett. **227**:303-309.

171. **Zhang, J.-Z., M. Sinha, B. A. Luxon, and X. Yu**. 2004. Survival strategy of obligately intracellular *Ehrlichia chaffeensis*: novel modulation of immune response and host cell cycles. Infect. Immun. **72**:498-507.

172. **Zhang, Y. and Y. Rikihisa**. 1997. Tyrosine phosphorylation is required for ehrlichial internalization and replication in P388D1 cells. Infect. Immun. **65**:2959-2964.

173. **Zhi, N., Y. Rikihisa, H. Y. Kim, G. Wormser, and H. W. Horowitz**. 1997. Comparison of major antigenic proteins of six strains of the human granulocytic ehrlichiosis agent by Western immunoblot analysis. J. Clin. Microbiol. **35**:2606-2611.

174. **Zhi, N., N. Ohashi, Y. Rikihisa, H. W. Horowitz, G. P. Wormser, and K. E. Hechemy**. 1998. Cloning and expression of the 44-kilodalton major outer membrane protein gene of the human granulocytic ehrlichiosis agent and application of the recombinant protein to serodiagnosis. J. Clin. Microbiol. **36**:1666-1673.

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VITA

Scott Wesley Long was born on August 6, 1976 to James Scott and Priscilla Long. While at graduate school, Wesley received numerous honors, starting with the Truman Graves Blocker, Jr. Research Scholars Minnie and Ward Savage Fellowship for Outstanding Academic Achievement in 2001, followed by the Edward S. Reynolds, M.D. Experimental Pathology Graduate Scholarship Award for Outstanding Research in Experimental Pathology in 2004, and the Center for Tropical Diseases Medical Student Award in 2005.

He received the GSBS Associates Christina Fleischmann Travel Award as well as the International Congress on Rickettsia and Rickettsial Diseases Travel Award in 2005.

His work was funded by the Sealy Center for Vaccine Development Predoctoral Fellowship in Vaccine Development in 2002. He has been funded by the McLaughlin Fellowship for Immunity and Infectious Disease Predoctoral Fellowship since 2003.

Wesley gained significant teaching experience while at the University of Texas Medical Branch. He was an instructor in the Topics in Parasitology course in the GSBS, as well as a teaching assistant in Pathobiology & Host Defense course in the School of Medicine in 2003. He was also a USMLE Step 1 Mentor/Tutor in 2003.

Wesley can be contacted through his parents at 17 Gettysburg, Richardson, Texas 75080.

Education

B.S., December 1998, The University of Texas, Austin, TX

Publications

Long, S.W., X. Zhang, H. Qi, S. Standaert, D.H. Walker, and X.J. Yu. 2002. Antigenic variation of *Ehrlichia chaffeensis* resulting from differential expression of the 28-kDa protein gene family. Infect. Immun. **70(4):**1824-1831.

Long, S.W., X. Zhang, J. Zhang, R.P. Ruble, P. Teel, and X.J. Yu. 2003. Evaluation of transovarial transmission and transmissibility of *Ehrlichia chaffeensis* (Rickettsiales: Anaplasmataceae) in *Amblyomma americanum* (Acari: Ixodidae). J. Med. Entomol. **40(6)**:1000-1004.

Zhang, X.F., **S.W. Long**, J. Zhang, R.P. Ruble, and X.J. Yu. 2003. Experimental *Ehrlichia chaffeensis* infection in beagles. J. Med. Microbiol. **52(11)**:1021-1026.

Long, S.W., J.M. Pound, and X.J. Yu. 2004. The prevalence of *Ehrlichia* species in *Amblyomma americanum* in central Texas. Emerg. Infect. Dis. **10(7)**:1342-1343.

Long, S.W. 2005. Essay. In J. Papaconstantinou (Eds.), Training Students for Changing Demands in Research: Student Perspectives on Meeting this Challenge at the University of Texas Medical Branch. Galveston, Texas: UTMB.

Long, S.W., T.J. Whitworth, X.J. Yu, and D.H. Walker. 2005. Overcoming barriers to the transformation of the genus *Ehrlichia*. Ann. N.Y. Acad. Sci. **1063:**403–410.