TRANSCRIPTIONAL REGULATORS OF \textit{Ehrlichia chaffeensis} DURING INTRACELLULAR DEVELOPMENT AND THE ROLES OF OMPA IN THE BACTERIAL INFECTION AND SURVIVAL

DISSERTATION

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by

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**ABSTRACT**

*Ehrlichia chaffeensis*, an obligatory intracellular bacterium of human monocytes-macrophages, causes human monocytic ehrlichiosis (HME). The bacterium has a biphasic developmental cycle in mammalian cells that alternates between a small ‘dense-cored cell’ (DC) and a large ‘reticulate cell’ (RC), as defined by morphological features, differential surface protein expression, and infectivity. However, how *E. chaffeensis* intracellular development is regulated is poorly understood.

The type IV secretion (T4S) system is critical for the virulence of several pathogens. *E. chaffeensis* T4S apparatus, *virB/D* genes are split into two operons: *virB3–virB6* (preceded by *sodB*) and *virB8–virD4*. Between these two operons, there are duplications of *virB4*, *virB8*, and *virB9*. We found that the transcription of all five loci was down-regulated prior to the release of *E. chaffeensis* from host THP-1 cells and up-regulated at the initiation of the exponential growth. An *E. chaffeensis* 12.3-kDa hypothetical protein, EcxR, specifically bound to the promoter regions upstream of *virB/D* loci. EcxR also activated transcription of all five *virB/D* loci in lacZ reporter constructs. The expression of *ecxR* was positively auto-regulated by EcxR. These results suggest that the five *virB/D* loci are coordinately regulated by EcxR to allow developmental stage–specific expression of the T4S system in *E. chaffeensis*.
The two-component regulatory system (TCS) composed of a sensor histidine kinase and a response regulator, allows bacteria to sense signals and respond to changes in their environment through the activation or repression of specific genes. The genomes of *E. chaffeensis* and *A. phagocytophilum* were each predicted to encode three pairs of TCSs. All six genes encoding three histidine kinases and three response regulators were expressed in both *E. chaffeensis* and *A. phagocytophilum* cultured in human leukocytes. Pretreatment of host-cell-free *E. chaffeensis* or *A. phagocytophilum* with closantel, an inhibitor of histidine kinases, completely blocked the infection of host cells. Treatment of infected cells one day post infection with closantel cleared the infection in dose-dependent manner. Autokinase activities of the three recombinant histidine kinases from *E. chaffeensis* were inhibited by closantel *in vitro*. A number of *E. chaffeensis* genes, including the six TCS genes, were down-regulated within 5-60 min post closantel treatment. These results suggest that these TCSs play an essential role in the infection and survival of *E. chaffeensis* and *A. phagocytophilum* in human leukocytes.

*Caulobacter crescentus* CtrA (cell cycle transcription regulator A) is a transcriptional regulator that allows the coordination of cell cycle progression and morphogenesis by controlling the expression levels of ~100 genes. In *E. chaffeensis*, CtrA mRNA and protein levels were down-regulated within 6 h post infection in synchronously infected cell culture, and significantly up-regulated at the late stage of infection prior to the bacterial release. *C. crescentus* CtrA-binding motif (CtrA box) was
predicted in the upstream regions of more than 30 *E. chaffeensis* genes. Temporal mRNA expressions of these genes were divided into four patterns: 1) up-regulated at the late exponential phase, similar to CtrA, 2) up-regulated at the lag phase and down-regulated during the exponential phase, 3) up-regulated at the lag phase but not down-regulated during the exponential phase, 4) constitutively expressed. *E. chaffeensis* CtrA specifically bound to the promoter regions of *E. chaffeensis ctrA* and *surE*, and rCtrA transactivated *surE* promoter-*lacZ* reporter constructs. These results suggest that CtrA is a global regulator controlling the intracellular development of *E. chaffeensis*.

In *E. chaffeensis*, lipoproteins are required for bacterial infection to the host cells. OmpA (outer membrane protein A, also known as a peptidoglycan-associated lipoprotein) was *E. chaffeensis* surface exposed. *E. chaffeensis* binding and infection to the host cells were inhibited by rabbit anti-OmpA IgG. OmpA mRNA and protein expressions were down-regulated during the lag phase and the early exponential phase, but highly up-regulated at the late exponential phase. CtrA-binding site was predicted in the promoter region of *ompA* gene. CtrA bound to this region and activated OmpA expression. The results suggest that CtrA regulates *ompA* expression, consequently, *E. chaffeensis* infection of host cells.
Dedicated to my family
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CHAPTER 1

INTRODUCTION

Family *Anaplasmataceae*

the ovaries of many species of arthropods and nematodes (Dumler et al., 2001). Only one species, *A. pullorum* is so far known for Genus *Aegyptianella* (Carpano, 1929), which has recently been characterized, displaying closest phylogenetic relationship to *Anaplasma* sp. (Rikihisa et al., 2003).

Many species in family *Anaplasmataceae* cause disease in animals and/or humans. Once only considered as veterinary pathogens, several *Ehrlichia*, *Anaplasma*, and *Neorickettsia* species have been recently recognized as emerging human pathogens in the United States and other parts of the world. *N. sennetsu* (formerly *E. sennetsu*), a monocytotropic sp., was the first human pathogen discovered in Japan in the 1950s (Misao and Kobayashi, 1956), and has also been found in Malaysia, Thailand, and Laos (Newton et al., 2008; Rapmund, 1984). *E. chaffeensis*, a monocyte-specific species that causes human monocytic ehrlichiosis (HME), was discovered in 1986 (Maeda et al., 1987); and *A. phagocytophilum* (formerly the human granulocytic ehrlichiosis [HGE] agent), a granulocyte-specific species was discovered in 1994 (Dumler and Bakken, 1998; Rikihisa et al., 1997). Originally thought to be only canine pathogens, a granulocytic ehrlichial species *E. ewingii* was recognized as a human pathogen in 1998 (Buller et al., 1999); and in 1995 a monocytic ehrlichial species *E. canis* was isolated from a human patient in Venezuela (Perez et al., 1996). Several *E. canis* infected human patients showed clinical signs compatible with HME (Perez et al., 2006). *A. platys* (formerly *E. platys*) causes canine infectious cyclic thrombocytopenia (French and Harvey, 1983). There are reports that *A. platys*-like organisms were found in human platelets by microscopic observations in Venezuela (Arraga-Alvarado et al., 1999), but no molecular or serologic confirmation for these observations.
Transmission of family *Anaplasmataceae*

*Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Aegyptianella* species infect mammals or birds, and replicate in membrane-bound vacuoles (morulae) in the cytoplasm of cells, mainly of hematopoietic origin (Rikihisa, 2006; Rikihisa *et al.*, 2003; Rikihisa, 1991). Little is known about transmission of *Aegyptianella* species. Both mammals (or birds) and ticks are involved in life cycles of *Ehrlichia* and *Anaplasma* species. Horizontal and bidirectional transmission between mammals (or birds) (reservoir) and ticks (vector) are required because transovarial transmission of these bacteria is ineffective if not rare or not occurring. *Ehrlichia* and *Anaplasma* species are maintained through an enzootic cycle between wild animals and ticks, and humans can be infected through the bite of infected ticks. The tick vector for *E. canis* is brown dog tick *Rhipicephalus sanguineus* (Groves *et al.*, 1975) and mammalian reservoirs are domestic and wild canids. *E. chaffeensis* has been commonly found in the Lone Star tick (*Amblyomma americanum*), and white-tailed deer is considered to be the major reservoir for *E. chaffeensis* (Ewing *et al.*, 1995; Lockhart *et al.*, 1995). *A. phagocytophilum* has been found in ticks of *Ixodes* species, and wild rodents, such as white-footed mice (*Peromyscus leucopus*) and dusky-footed wood rats (*Neotoma fuscipes*) are believed to be the major reservoirs of *A. phagocytophilum* in the eastern and western states, respectively (Nicholson *et al.*, 1998; Des Vignes and Fish, 1997; Walls *et al.*, 1997). *Neorickettsia* species (formerly *Ehrlichia*) are maintained through transovarial and transstadial passage in the trematode species, which appear to be specific to each *Neorickettsia* species (Gibson and Rikihisa, 2008). *Wolbachia* is transovarially transmitted in specific invertebrate hosts and is known to alter reproduction...
of the invertebrate host. They are found to infect insects, arachnids, crustaceans, and nematodes (Dumler et al., 2001), but infection in vertebrate has not been found. *A. platys* has been detected in *R. sanguineus* ticks (Sanogo et al., 2003), but successful transmission has not been demonstrated. The mammalian reservoirs for *A. platys* are domestic or wild canids (Woody and Hoskins, 1991).

**Human monocytic ehrlichiosis (HME) and human granulocytic anaplasmosis (HGA)**

HME and HGA are caused by *E. chaffeensis* and *A. phagocytophilum*, respectively. Both HME and HGA are febrile systemic Rocky Mountain Spotted fever-like illness and not distinguishable from each other. Symptoms include fever, headache, myalgia, anorexia and chills, and are frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevations in serum hepatic aminotransferases (Dumler and Bakken, 1998). The severity of the disease varies from asymptomatic seroconversion to fatal, and severe morbidity is frequently seen in immunocompromised patients (Dumler, 2005; Paddock and Childs, 2003; Dumler and Bakken, 1998). In 2005, more cases of ehrlichiosis were reported than ever before, including 471 cases of HME (2396 cases since 1986) and 700 cases of HGA (2963 cases since 1994) (Dumler et al., 2007). Most of HME cases were in the southcentral and southeastern United States (McQuiston et al., 1999). Cases of HGA are correlated with the distribution of its vector *Ixodes* spp. being identified in New England, the mid-Atlantic region, the upper Midwest, and northern California in the United States, as well as in parts of Europe.
An overview of the ultrastructure and physiology of *A. phagocytophilum* and *E. chaffeensis*

*A. phagocytophilum* and *E. chaffeensis* are small coccoid (0.4 μm to 1.5 μm) and stained as dark blue to purple with Romanowsky stain. They often form mulberry-like microcolony, called morulae. By transmission electron microscopy, these bacteria are generally round but sometime pleomorphic, especially in tissue culture (Rikihisa, 1991). They replicate in membrane-bound vacuoles in the cytoplasm of eukaryotic host cells. *Anaplasma* and *Ehrlichia* species have thin bileaflets of outer and inner membranes. *A. phagocytophilum* and *E. chaffeensis* have lost most genes required for the biosynthesis of peptidoglycan and all genes required for the biosynthesis of LPS (Hotopp et al., 2006; Lin and Rikihisa, 2003b). However, *A. phagocytophilum* and *E. chaffeensis* acquire unique capability to take up host cell cholesterol and incorporate cholesterol into bacterial membrane (Lin and Rikihisa, 2003b). Cholesterol has become indispensable for their survival and successful establishment of infection (Lin and Rikihisa, 2003b). These bacteria have distinct ribosomes and a fine meshwork of DNA strands. Clumps of ribosomes are homogenously distributed in the cytoplasm instead of marginated beneath inner membrane (Rikihisa, 1991). The genome sizes of family *Anaplasmataceae* are small; for example, those of *A. phagocytophilum* and *E. chaffeensis* are 1.47 Mb and 1.17 Mb, respectively, which are about one fourth of *Escherichia coli* (Hotopp et al., 2006).

*A. phagocytophilum* and *E. chaffeensis* have the ability to synthesize all nucleotides (Hotopp et al., 2006). This differs from *Rickettsia prowazekii*, which cannot make purines or pyrimidines and therefore, must rely on nucleotide translocases and interconversion of the bases to obtain the full complement of nucleotides (Andersson et al., 1998). *A. phagocytophilum* and *E. chaffeensis* are able to synthesize most vitamins...
and cofactors (Hotopp et al., 2006). The presence of nucleotide, vitamin and cofactor biosynthetic pathway in *A. phagocytophilum* and *E. chaffeensis* suggests that they do not need to compete with the host cells for essential vitamins and nucleotides (Hotopp et al., 2006). However, the members of family *Anaplasmataceae* have a very limited ability to synthesize amino acids and must rely on transporting them from host (Hotopp et al., 2006). They can make glycine, glutamine, glutamate, and aspartate (Hotopp et al., 2006). Additionally, *E. chaffeensis* is predicted to be able to synthesize arginine and lysine (Hotopp et al., 2006). They can utilize glutamine and glutamate to generate adenosine triphosphate (ATP) as genus *Rickettsia*, and they can not utilize glucose-6-phosphate or glucose (Rikihisa, 1991). For the greatest metabolic activity for these bacteria, the optimal pH is at pH 7.2 to 8.0 (Rikihisa, 1991).

**Internalization and intracellular trafficking of *E. chaffeensis***

The entry and establishment of infection of *E. chaffeensis* involve caveolae, host GPI-anchored proteins and the incorporation of cholesterol into bacterial membrane (Lin and Rikihisa, 2003a; Lin and Rikihisa, 2003b). An increase in host cytosolic free calcium ([Ca$^{2+}$]$_i$) is essential for *E. chaffeensis* entry into monocytes (Lin et al., 2002). The following sequential signaling events were described to be induced by *E. chaffeensis* infection: host cell protein cross-linking by transglutaminase, tyrosine phosphorylation, PLC-$\gamma$2 activation, IP3 production, and an increase in [Ca$^{2+}$]$_i$ (Lin et al., 2002). After internalization of host cell, *E. chaffeensis* has the ability to exclude its membrane-bound compartment from fusion with lysosomes. Replicative *E. chaffeensis* inclusions are weakly acidic, early endosomal compartments. They are weakly co-localized with
vacuolar-type H\(^+\)-ATPase, and IFA labeling showed the inclusion positive for the TfR, early endosomal antigen 1, and Rab5 (Mott et al., 1999).

Iron acquisition by *E. chaffeensis* is dependent on the liable iron pool in the host cytosol. When monocytes are treated with deferoxamine, an intracellular iron chelator, the infection of *E. chaffeensis* is completely blocked (Barnewall and Rikihisa, 1994). Since *E. chaffeensis* inclusions accumulate TfR, the bacterium may directly acquire iron from cytosolic transferrin. Interferon-\(\gamma\) (IFN- \(\gamma\)) has a protective role in mice infected *E. chaffeensis* (Bitsaktsis et al., 2004; Ismail et al., 2004). The anti-ehrlichia activity induced in human monocytes by exogenous IFN- \(\gamma\) is mediated by the limitation of available cytosolic iron instead of generation of ROS or nitric oxide (Bakken et al., 1994).

*E. chaffeensis* infection up-regulates host cell TfR mRNA expression (Barnewall et al., 1999). Both internalization and continuous proliferation of ehrlichial organisms or the synthesis of ehrlichial proteins are required for the up-regulation of TfR mRNA (Barnewall et al., 1999). The activation of iron-responsive protein 1 (IRP-1) to the iron-responsive element and subsequent stabilization of TfR mRNA comprise the mechanism of TfR mRNA down-regulation by IFN- \(\gamma\) (Barnewall et al., 1999).

**Type IV secretion system in *E. chaffeensis***

How does *E. chaffeensis* down-regulate a number of critical innate immune responses and modify vesicular traffic to create a sheltered niche in host cells? Our hypothesis is that the bacterial type IV secretion (T4S) system has an important role in these processes. Genes encoding T4S apparatus, *virB/D* loci, are found among members of the order *Rickettsiales*, including *E. chaffeensis* and *A. phagocytophilum* (Hotopp et al.,
The T4S apparatus of Gram-negative bacteria is a transmembrane channel composed of multiple conserved proteins that transport macromolecules across the membrane in an ATP-dependent manner into eukaryotic target cells (Christie et al., 2005). The T4S system is a critical determinant for virulence in several Gram-negative pathogens such as *Agrobacterium tumefaciens*, *Legionella pneumophila*, *Helicobacter pylori*, and *Brucella abortus*, because it delivers effector molecules into the host cell cytoplasm or nucleus, which induce tumors, induce inflammatory cytokines, and create an intracellular compartment for bacterial survival and proliferation (Ninio and Roy, 2007; Christie et al., 2005; Cascales and Christie, 2003).

In several bacteria, it has been shown that the T4S apparatus components and substrates are not constitutively expressed but rather are tightly regulated to ensure proper timing of substrate action. In *A. tumefaciens*, a two-component regulatory system, VirA/VirG, regulates the expression of T4S system (Krishnamohan et al., 2001; Jin et al., 1990). VirA, a signal sensor/histidine-kinase transmembrane protein, is autophosphorylated in response to phenolic compounds and a family of sugars released by wounded plant cells, and then phosphorylates VirG, a transcriptional activator. The phosphorylated VirG activates other *vir* operons through binding to the consensus sequence (*vir* box) of the respective promoter regions of T4S system (Winans, 1990). In *L. pneumophila*, the two-component regulatory systems, PmrB/PmrA (Zusman et al., 2007) and CpxA/CpxR (Gal-Mor and Segal, 2003), regulate the expression of the *icm/dot* T4S system. VjbR, a quorum-sensing regulator, Rsh (Delrue et al., 2005), a RelA/SpoT
stringent response protein homolog (Dozot et al., 2006), and integration host factor (IHF) (Sieira et al., 2004) are involved in the regulation of virB/D T4S system of Brucella sp.

**E. chaffeensis development cycle**

*E. chaffeensis* manipulates host monocytes/macrophages throughout its intracellular developmental cycle (Rikihisa, 2006), and the bacterium has a biphasic developmental cycle in mammalian cells, which alternates between a ‘dense-cored cell’ (DC) and a larger ‘reticulate cell’ (RC). DC is smaller than RC and have a dense nucleoid. In contrast, RC has uniformly dispersed nucleoid filaments and ribosomes, sometimes forming long projections of the cell wall, protrusions of the cytoplasmic membrane into the periplasmic space, or budding protoplast fragments into the periplasmic space (Popov et al., 1995). In DH82 cells (canine histiocytoma cell line), at 1 day post infection (p.i.), *E. chaffeensis* is transformed from small DC (0.4–0.6 μm) to large RC (0.7–1.9 μm) and starts to multiply. After 3 days of culture, the bacterial population converts back to DC to initiate new infection of host cells. The infectivity of the DC-rich population has been reported to be 3.5 million times greater than that of the RC-rich population (Zhang et al., 2007). The differential expressions of surface proteins, P28, gp120 and OMP-1F were demonstrated in *E. chaffeensis* (Kumagai et al., 2008; Zhang et al., 2007). The ability to perform this phenotypic switch is likely important for the adaptation of the bacterium to harsh extracellular conditions and to initiation of infection, survival and replication in a new host cell.
Two-component regulatory system in *E. chaffeensis*

How does *E. chaffeensis* regulate the intracellular development to adapt to the harsh extracellular conditions and to initiation of infection, survival and replication in a new host cell? Our hypothesis is that two-component regulatory system (TCS) has an important role in these processes. In *E. chaffeensis* and *A. phagocytophilum*, three pairs of TCSs were identified (Cheng *et al.*, 2006; Kumagai *et al.*, 2006). The TCS, a family of signal sensor, transduction, and response regulatory systems, allows bacteria to sense wide varieties of signals and respond rapidly to changes in their environment through specific gene activation or repression (Dorman *et al.*, 2001). TCSs are integral in the ability of certain pathogenic bacteria to mount and establish a successful infection within the hosts as emphasized by the many examples of attenuated virulence observed with pathogenic strains in which one or more TCSs have been deleted (Groisman, 2001; Throup *et al.*, 2000).

In addition, many pathogenic bacteria contain TCSs that are essential for viability. For example, *Bacillus subtilis* and *Staphylococcus aureus* contain one TCS, YycG/YycF, essential for viability, whereas only the YycF response regulator is essential for viability in *Streptococcus pneumoniae* (Stephenson and Hoch, 2002; Beier and Frank, 2000; Throup *et al.*, 2000; Lange *et al.*, 1999; Martin *et al.*, 1999). In members of the order *Rickettsiales*, several genes homologous to bacterial TCSs have been recognized (Brayton *et al.*, 2005; Collins *et al.*, 2005; Foster *et al.*, 2005; Ogata *et al.*, 2001; Andersson *et al.*, 1998), but so far expression, biochemical activities, and functions of these genes have not been elucidated.
*Caulobacter crescentus* is a free-living aquatic α-proteobacterium, which is used as a model to study cell development and division. In *C. crescentus*, CckA/CtrA was identified as a pair of TCS. CtrA is a regulatory protein found only in α-proteobacteria (Hallez *et al.*, 2004) that coordinates multiple cell cycle events at the transcriptional level. CtrA in *C. crescentus* can be phosphorylated in *vivo* and in *vitro* and CckA is involved in CtrA phosphorylation (Jacobs *et al.*, 2003; Quon *et al.*, 1996). At the early stage of cell cycle, CtrA binds and silences the origin of replication (Quon *et al.*, 1998). Then CtrA is degraded by protease ClpXP to free the origin and permitting the initiation of DNA replication (McGrath *et al.*, 2006; Domian *et al.*, 1997). At the late stage, the initial synthesis of CtrA leads to positive transcriptional autoregulation and a burst of CtrA synthesis (Domian *et al.*, 1999). The newly synthesized CtrA drives the expression of more than 95 genes, many of which are required for completing the cell cycle, such as DNA segregation, cellular division, morphogenesis, and DNA methylation (Hallez *et al.*, 2004; Laub *et al.*, 2002; Laub *et al.*, 2000).

**Lipoproteins of *Ehrlichia***

How *E. chaffeensis* transiently survives outside and the bacterial ligand which facilitates its binding, entry, and proliferation has not been identified. gp120, which is a glycoprotein containing glucose, galactose and xylose (McBride *et al.*, 2000), was identified as adhesin using *E. coli* expressing gp120 to adhere to HeLa cells. A few *E. coli* expressing the gp120 were identified inside the HeLa cells, suggesting that the gp120 may play a role in bacterial adhesion or invasion of the host cell (Popov *et al.*, 2000). Immunization with recombinant P28 (one of the major OMP-1/P28 family members)
protected mice from *E. chaffeensis* challenge (Ohashi *et al.*, 1998). Monoclonal antibody against OMP-1g (P28) mediated protection of SCID mice from *E. chaffeensis* fatal infection (Li *et al.*, 2001). These studies suggest that OMP-1/P28 family members could be directly or indirectly involved in *E. chaffeensis* entry and proliferation in the host cells.

*E. chaffeensis* lacks all genes for the biosynthesis of LPS and most genes for the biosynthesis of peptidoglycan (Lin and Rikihisa, 2003b). However, *E. chaffeensis* has 16 predicted lipoproteins (Huang *et al.*, 2008; Hotopp *et al.*, 2006). In *E. chaffeensis*, a globomycin-sensitive lipoprotein biogenesis pathway is present, and globomycin treatment inhibits *E. chaffeensis* infection in *vitro*, suggesting that lipoproteins are required for *E. chaffeensis* infection of host cells (Huang *et al.*, 2008). Lipoproteins are proteins with covalently attached lipids such as N-acyl-diacylglycerol. This lipid is linked to the N-terminal cysteine of the mature lipoproteins. Lipoproteins are anchored to the membrane through attached lipid. This means that lipoproteins are exposed to the surface of membrane. In *E. coli*, lipoproteins are anchored in inner and outer membrane facing periplasm (Inouye, 1979). In *Borrelia burgdorferi*, the etiologic agent of Lyme disease, another Gram-negative that lacks LPS in their outer membrane, more than 130 lipoproteins have been predicted (Fraser *et al.*, 1997). Most of them are outer membrane surface exposed (Rosa, 1997). Some of them play important roles in *B. burgdorferi*’s survival and pathogenesis. Among the 16 lipoproteins in *E. chaffeensis*, OmpA (outer membrane protein A) is also known as a peptidoglycan-associated lipoprotein (PAL). It was reported that in *E. coli*, PAL is necessary for resistance to detergents and antibiotics (Cascales *et al.*, 2002) and participates in maintaining the integrity of the bacterial cell (Rodriguez-Herva *et al.*, 1996). Because *E. chaffeensis* lacks peptidoglycan, OmpA may
associate with other components in the bacterial membrane. The study on OmpA will provide a significant advancement in our understanding of the mechanisms utilized by bacteria to transiently survive outside and interact with host cells.

**Hypotheses and objectives of this study.**

The primary goal of this study was to determine the mechanism utilized by *E. chaffeensis* to detect the environmental stimuli and the adaptation of *E. chaffeensis* to harsh extracellular conditions and to initiation of the bacterial infection, survival and replication in a new host cell, and the membrane protein of *E. chaffeensis* required for its entry and infection.

(1) *E. chaffeensis* has a biphasic developmental cycle in mammalian cells that alternates between a ‘dense-cored cell’ (DC) and a larger ‘reticulate cell’ (RC), as defined by morphological features, differential surface protein expression, and infectivity (Kumagai *et al.*, 2008; Zhang *et al.*, 2007). Genes encoding T4S apparatus, virB/D loci, are found among members of the order *Rickettsiales*, including *E. chaffeensis* and *A. phagocytophilum* (Hotopp *et al.*, 2006; Ohashi *et al.*, 2002). In several bacteria, it has been shown that the T4S apparatus components and substrates are not constitutively expressed but rather are tightly regulated to ensure proper timing of substrate action. The expression of virB/D genes are regulated during *A. phagocytophilum* growth in human peripheral blood neutrophils (Niu *et al.*, 2006). Unlike the single locus of clustered virB/D genes encoding the T4S apparatus in most bacteria (Christie, 1997), the split virB/D operons are unique to *E. chaffeensis*, *A. phagocytophilum*, and other members of the order *Rickettsiales* (Hotopp *et al.*, 2006).
2006; Ohashi et al., 2002). The virB/D genes of *E. chaffeensis* and *A. phagocytophilum* are clustered in two primary loci, one consisting of five tandem genes (*virB8-1, virB9-1, virB10, virB11, and virD4*) and another consisting of six tandem genes (*virB3, virB4-1, and four virB6 paralogs*) that are preceded by *sodB*. Between the primary loci, there are three duplicated genes, *virB4-2, virB8-2*, and *virB9-2* (Hotopp et al., 2006; Ohashi et al., 2002). My hypothesis is that a co-regulator is required to co-activate the transcription of genes encoding the T4S apparatus to ensure that the complete T4S apparatus is assembled in these bacteria when needed. The objectives of this study were to determine the temporal expression pattern of T4S genes during *E. chaffeensis* intracellular development in host cells, and to identify and characterize the protein, which binds to promoter regions upstream of *virB/D* loci and regulates the expression of the dispersed T4S apparatus loci during *E. chaffeensis* intracellular development.

(2) The TCS is integral in the ability of certain pathogenic bacteria to mount and establish a successful infection within the hosts. In *E. chaffeensis* and *A. phagocytophilum*, three pairs of TCSs were identified. My hypothesis is that the three pairs of TCSs are expressed and function in *E. chaffeensis* by regulating sets of downstream genes critical for the bacterial intracellular infection and development. The objective of this study was to determine the role of TCSs in the infection and survival of *E. chaffeensis* and *A. phagocytophilum* in leukocytes. Closantel (N-[5-chloro-4-[(R,S)-(4-chlorophenyl)-cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide) targets the carboxyl-terminal catalytic domain of the sensor kinase from both Gram-negative and Gram-positive bacteria, and inhibits activity by
causing structural alterations of kinases that lead to aggregation (Stephenson et al., 2000). In order to assess the requirement of TCSs in intracellular infection, the effects of closantel on *E. chaffeensis* and *A. phagocytophilum* infection and temporal changes in transcription of genes predicted to be regulated by the TCSs were examined

(3) How *E. chaffeensis* intracellular replication is coordinated with intracellular development and extracellular release is poorly understood. In *C. crescentus* CtrA is an essential response regulator of the cell cycle, which regulates more than 95 genes involved in DNA replication initiation, DNA segregation, cellular division, transcription, morphogenesis, and DNA methylation, and then coordinates multiple cell cycle events (Hallez et al., 2004; Domian et al., 1997; Quon et al., 1996). In *E. chaffeensis*, CtrA homolog has been found, and the phosphorylation of CtrA by its cognate histidine kinase, CckA has been determined (Cheng et al., 2006; Kumagai et al., 2006). My hypothesis is that CtrA is global transcriptional regulator, which regulates different sets of genes to coordinate bacterial life cycle. The objectives of this study were to predict CtrA downstream genes using the CtrA 9-mer (TTAAN\_TTAAC) and 8-mer (TTAACCAT) consensus binding sequences from *C. crescentus*, to determine the temporal expression pattern of *ctrA* and its downstream genes in the cell development cycle, and to determine how they are regulated by CtrA.

(4) Little is known how *E. chaffeensis* transiently survives outside of the host cells, and binds to new host cells and initiates new infection. *E. chaffeensis* lacks all genes for the biosynthesis of LPS and most genes for the biosynthesis of peptidoglycan; thus,
it does not produce LPS or peptidoglycan (Lin and Rikihisa, 2003b). However, *E. chaffeensis* has 16 predicted lipoproteins (Huang *et al.*, 2008; Hotopp *et al.*, 2006). And lipoproteins are required for *E. chaffeensis* infection of host cells (Huang *et al.*, 2008). One of lipoprotein, OmpA (outer membrane protein A), is also known as a peptidoglycan-associated lipoprotein (PAL). It was reported that PAL is necessary for resistance to detergents and antibiotics (Cascales *et al.*, 2002) and for integrity of the cell envelope (Rodriguez-Herva *et al.*, 1996). The expressions of outer membrane proteins, OMP-1F, P28 and gp120 in *E. chaffeensis* were shown to be regulated to associate with the bacterial intracellular development. (Kumagai *et al.*, 2008; Zhang *et al.*, 2007). My hypotheses are that the expression of OmpA, one of the lipoprotein, is associated with the bacterial development, and OmpA is essential for the bacterial infection and survival. The objective of this study was to determine the expression pattern of OmpA, the mechanism regulating its expression and its roles in the bacterial infection and survival.

Thereby, the new knowledge generated in my Ph.D. study is expected to facilitate the understanding of the complex mechanisms used by bacterial pathogens to adapt to their environments and infect, survive and replicate in the host. The results has been gained could result in new therapeutics.
CHAPTER 2

REGULATION OF TYPE IV SECRETION APPARATUS GENES DURING EHRlichia chaffeeNIS InTRACELLULAR DEVELOPMENT BY A PREVIOUSLY UNIDENTIFIED PROTEIN

2.1 Abstract

The type IV secretion (T4S) system is critical for the virulence of several pathogens. In the rickettsial pathogen, Ehrlichia chaffeensis, virB/D genes are split into two operons: virB3–virB6 (preceded by sodB) and virB8–virD4. Between these two operons, there are duplications of virB4, virB8, and virB9. In this study we found that transcription of all five loci was down-regulated prior to the release of E. chaffeensis from host THP-1 cells and up-regulated at the initiation of exponential growth. Electrophoretic mobility shift assays revealed an E. chaffeensis–encoded protein that specifically bound to the promoter regions upstream of virB/D loci. The protein was purified from the bacterial lysate by affinity chromatography using a biotinylated promoter region upstream of sodB. Mass spectrometry identified the protein as an E.
*Ehrlichia chaffeensis* 12.3-kDa hypothetical protein, which was named EcXR. Recombinant EcXR bound to the promoter regions upstream of five individual *virB/D* loci. EcXR also activated transcription of all five *virB/D* loci in *lacZ* reporter constructs. The expression of *ecxR* was positively auto-regulated by EcXR. These results suggest that the five *virB/D* loci are coordinately regulated by EcXR to allow developmental stage–specific expression of the T4S system in *E. chaffeensis*.

### 2.2 Introduction

*Ehrlichia chaffeensis* is a Gram-negative obligatory intracellular bacterium that causes human monocytic ehrlichiosis, an emerging tick-borne zoonosis (Demma *et al.*, 2005; Paddock and Childs, 2003). *E. chaffeensis* manipulates host monocytes/macrophages throughout its intracellular developmental cycle (Rikihisa, 2006), and the bacterium has a biphasic developmental cycle in mammalian cells, which alternates between a small ‘dense-cored cell’ (DC) and a large ‘reticulate cell’ (RC), as defined by morphological features, differential surface protein expression, and infectivity (Zhang *et al.*, 2007). The presence of biphasic developmental stages which differ in cell size, infectivity, and expression of a surface protein VirB9 of type IV secretion (T4S) apparatus was demonstrated in *Anaplasma phagocytophilum*, closely related to *E. chaffeensis* (Niu *et al.*, 2006). The ability to perform this phenotypic switch is likely important for the adaptation of the bacterium to harsh extracellular conditions and to initiation of infection, survival and replication in a new host cell. Little is known, however, about the mechanisms regulating the developmental cycle of members of the *Ehrlichia* and *Anaplasma* genera.
Genes encoding T4S apparatus, \textit{virB/D} loci, are found among members of the order \textit{Rickettsiales}, including \textit{E. chaffeensis} and \textit{A. phagocytophilum} (Hotopp \textit{et al.}, 2006; Ohashi \textit{et al.}, 2002). The T4S apparatus of Gram-negative bacteria is a transmembrane channel composed of multiple conserved proteins and transports macromolecules across the membrane into eukaryotic target cells in an ATP-dependent manner. The T4S system is a critical determinant for virulence in several Gram-negative pathogens such as \textit{Agrobacterium tumefaciens}, \textit{Legionella pneumophila}, \textit{Helicobacter pylori}, and \textit{Brucella abortus}, because it delivers effector molecules into the host cell cytoplasm or nucleus, which induce tumors, induce inflammatory cytokines, and create an intracellular compartment for bacterial survival and proliferation (Economou \textit{et al.}, 2006; Cascales and Christie, 2003). Our recent study demonstrated that AnkA, a protein rich in ankyrin repeats and important for \textit{A. phagocytophilum} infection, is translocated to the host cell cytoplasm in a T4S-dependent manner (Lin \textit{et al.}, 2007).

In several bacteria, it has been shown that the T4S apparatus components and substrates are not constitutively expressed, but rather are tightly regulated to ensure proper timing of substrate action. In \textit{A. tumefaciens}, the expression of the T4S system is regulated by a two-component regulatory system, VirA/VirG, which detects chemical signals such as phenolic compounds and particular monosaccharides released by wounded plant cells and subsequently induces the transcription of \textit{virB/D} T4S system (Winans, 1992). In \textit{L. pneumophila}, the two-component systems, PmrB/PmrA (Zusman \textit{et al.}, 2007) and CpxA/CpxR (Gal-Mor and Segal, 2003), regulate the expression of the \textit{icm/dot} T4S system. VjbR, a quorum-sensing regulator (Delrue \textit{et al.}, 2005), Rsh, a RelA/SpoT stringent response protein homolog (Dozot \textit{et al.}, 2006), and integration host
factor (IHF) (Sieira et al., 2004) are involved in the regulation of virB/D T4S system of Brucella sp. The expression of virB/D genes are also regulated during A. phagocytophilum growth in human peripheral blood neutrophils (Niu et al., 2006). Although genes encoding the IHFα and β subunits and three different pairs of two-component regulatory systems have been identified in the A. phagocytophilum and E. chaffeensis genomes, genes encoding homologs of the quorum-sensing regulator or the stringent response protein have not been found (Cheng et al., 2006; Hotopp et al., 2006).

Unlike the single locus of clustered virB/D genes encoding the T4S apparatus in most bacteria (Christie, 1997), the split virB/D loci are unique to E. chaffeensis, A. phagocytophilum, and other members of the order Rickettsiales (Hotopp et al., 2006; Ohashi et al., 2002). The virB/D genes of E. chaffeensis and A. phagocytophilum are clustered in two primary loci, one consisting of five tandem genes (virB8-1, virB9-1, virB10, virB11, and virD4) and another consisting of six tandem genes (virB3, virB4-1, and four virB6 paralogs) that are preceded by sodB. Between the primary loci, there are three duplicated genes, virB4-2, virB8-2, and virB9-2 (Hotopp et al., 2006; Ohashi et al., 2002). The split virB/D loci suggest that a co-regulator is required to co-activate the transcription of genes encoding the T4S apparatus to ensure that the complete T4S apparatus is assembled in these bacteria when needed.

In the present study, we used quantitative PCR to determine the temporal expression patterns of the two primary loci and the intervening duplicated genes that comprise the other three loci during E. chaffeensis intracellular development in a human acute leukemia cell line, THP-1. We found that all five virB/D loci are regulated in a generally synchronous manner. We further identified a previously unknown E.
chaffeensis DNA binding protein, EcxR, which binds to the promoter regions upstream of virB/D loci. Functional studies suggest that EcxR positively auto-regulates the expression of ecxR. We further found that EcxR coordinately regulates the expression of the dispersed T4S apparatus loci during E. chaffeensis intracellular development.

2.3 Materials and methods

Synchronous culture of E. chaffeensis.

E. chaffeensis Arkansas (Anderson et al., 1991) was propagated in THP-1 cells (ATCC, Manassas, VA) in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% L-glutamine at 37°C under 5% CO2 (Cheng et al., 2006). The heavily infected (>90%) THP-1 cells (8 × 10^7 cells) were harvested by centrifugation at 2,000 × g for 5 min. The pellet was resuspended in 8 ml culture medium and sonicated on ice twice at setting 2 for 10 s using a W-380 Sonicator (Heat systems-Ultrasonics, Farmingdale, NY), and the unbroken cells and cell debris were removed by centrifugation at 4,000 × g for 5 min. Host cell–free bacteria in the medium were sonicated on ice twice at setting 4.5 for 30 s to disrupt the fragile bacterial RCs. The sonication-resistant bacterial DCs were harvested by centrifugation at 18,000 × g for 5 min at 4°C. Determination of bacterial viability was performed with the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR). To perform a synchronous infection, the DC pellet was resuspended in 10 ml culture medium and incubated with 4 × 10^7 uninfected THP-1 cells at 37°C for 1 h, shaking every 10 min. The mixture was then washed with cold 2 × phosphate buffered saline (2 × PBS: 274 mM NaCl, 5.4 mM KCl, 20 mM Na2HPO4, 4 mM KH2PO4, pH 7.4) three times and incubated at 37°C. Samples were collected at this
time point (0 h post infection [p.i.]) and at 24, 48, 72, and 96 h p.i. by centrifugation at $2,000 \times g$ for 5 min.

Quantitative RT-PCR.

THP-1 cells synchronously infected with *E. chaffeensis* were harvested at 0, 24, 48, 72 and 96 h p.i. as described above. One-half of the cells was suspended in RNALater (Qiagen, Valencia, CA) and stored at $-20^\circ C$ for RNA extraction. The remaining cells were kept at $-80^\circ C$ for DNA extraction. Total DNA and RNA were extracted, and the RNA was reverse transcribed as described (Cheng *et al.*, 2006). Samples lacking reverse transcriptase were used to assess DNA contamination for each reaction. Quantitative PCR was performed as described (Cheng *et al.*, 2006). Briefly, gene-specific primers were designed to produce amplicons of 100 to 150 bp (primer sequences are shown in Table 2.1). Serially diluted bacterial chromosomal DNA containing known copy numbers of the target genes was used as a standard. Quantitative PCR was performed using a Mx3000P instrument and the Brilliant SYBR Green QPCR Core Reagent kit (both from Stratagene, La Jolla, CA). For each quantitative PCR assay, the dissociation curve was examined to confirm the absence of primer dimers. Log chromosomal DNA versus cycle threshold ($C_T$) was plotted to establish standard curves for each gene. Means and standard deviations of mRNA copy numbers were determined. Samples were normalized by *E. chaffeensis* 16S rDNA and the amount of *gyrB* transcript at 0 h p.i.
Electrophoretic mobility shift assay (EMSA).

DNA fragments of 351 bp and 327 bp in length, corresponding to sequences upstream of the \textit{sodB} and \textit{virB9-2} start codons, respectively, were amplified by PCR. A DNA fragment of 434 bp, corresponding to sequence upstream of the \textit{virB9-1} start codon, was similarly amplified as a negative control (see Table 2.2 for primer pairs). PCR products were biotinylated, and the \textit{E. chaffeensis} lysate supernatant was prepared as described (Wang et al., 2007a). The lysate supernatant (approximately 5 μg protein) was incubated with each DNA probe (0.1 pmol) for 5 min at room temperature in a 20-μl reaction containing 10 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 2.5% (v/v) glycerol, 0.1% (v/v) NP-40, and 50 ng/μl salmon sperm DNA. As a control for binding specificity, a separate reaction containing the above components plus a 50-fold excess of the corresponding unlabeled DNA probe was prepared. Samples were loaded onto a 6% native polyacrylamide gel in 0.5 × Tris-borate-EDTA (0.5 × TBE) buffer (0.044 M Tris base, 0.044 M boric acid, and 0.001 M EDTA pH 8.0) that had been pre-run for 1 h, electrophoresed at 100 V for 2.5 h at 4°C and then transferred to a nylon membrane (Amersham Biosciences, Piscataway, NJ) at 380 mA for 1 hour at 4°C. The transferred DNA was cross-linked to the membrane with UV light. The biotinylated DNA was detected using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL).

For EMSAs using purified rEcxR, DNA fragments upstream of the \textit{virB/D} loci and \textit{ecxR} were amplified by PCR (primers and amplicon sizes are shown in Table 2.2) and were biotinylated as described (Wang et al., 2007a). The DNA probes (0.1 pmol each) were incubated in separate reactions with 25 ng rEcxR purified as described below, and EMSAs were performed as described above.
For supershift experiments, the DNA probe derived from sequence upstream of *sodB* (1 pmol) was amplified by PCR, as indicated above, and incubated with 0.2 μg rEcxR at room temperature for 5 min. Then 2 μl mouse monoclonal anti-His-tag antibody (Sigma, St. Louis, MO) was added and the reaction was incubated for another 5 min at room temperature. Sample electrophoresis was performed as described above followed by DNA staining in 0.5 × TBE containing 0.5 μg/ml ethidium bromide. Bands were visualized with a IAS-3000 luminescent image analyzer (Fujifilm, Stamford, CT).

Affinity purification of DNA binding proteins and mass spectrometry.

The promoter region upstream of *sodB* was amplified by PCR using a 5′ biotin-labeled primer as described (Wang *et al.*, 2007a) and purified using a PCR purification kit (Qiagen). Following incubation of the *E. chaffeensis* lysate supernatant with 15 µg salmon sperm DNA at 4°C for 15 min, 50 pmol of the biotinylated promoter probe upstream of *sodB* was added to the reaction and incubated for 30 min at 4°C. The DNA-bound proteins were affinity-purified using a µMACS streptavidin kit (Miltenyi Biotec, Berglsch Gladbach, Germany), solubilized in 10 µl SDS-PAGE loading buffer at 100°C for 5 min and subjected to 15% SDS-PAGE analysis. After electrophoresis, the gel was fixed as described (Wang *et al.*, 2007a), and the resulting bands were excised and digested with trypsin (Promega, Madison, WI). Trypsinized polypeptides were identified by capillary liquid chromatography-nanospray tandem mass spectrometry (LC/MS/MS) as described (Wang *et al.*, 2007a).
Cloning and expression of rEcxR.

Full-length *ecxR* was amplified by PCR and directionally cloned into the Nde I and Xho I sites of the pET29a(+) vector (Novagen, San Diego, CA). The PCR primers are shown in Table 2.3. The resulting plasmid (pEcxR) was amplified in *E. coli* Novabluce cells (Novagen), and the recombinant protein was expressed in *E. coli* BL21(DE3) cells (Novagen) as described (Cheng et al., 2006). rEcxR was purified by Ni$^{2+}$ affinity chromatography using a His-Select cartridge (Sigma) and dialyzed against EMSA binding buffer.

Construction of *lacZ* reporter fusions.

The *lacZ* reporter fusions were constructed as described (Wang et al., 2007a). Briefly, DNA fragments upstream of the *virB/D* loci (Fig. 2.2B) and *ecxR* were amplified by PCR. PCR primers are shown in Table 2.3. The transcriptional fusion was constructed by placing these promoter fragments upstream of a promoterless *lacZ* gene in pACYC184 (New England Biolabs, Ipswitch, MA). BL21 (DE3) cells were co-transformed with pEcxR and each of the *lacZ* reporter constructs individually. pET29a(+) vector alone was used as a negative control. After overnight culture, transformants were subcultured in LB medium supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol at 37°C for 2 h followed by induction with 0.0625 mM IPTG for 2 h. β-galactosidase activity was measured as described (Wang et al., 2007a). rEcxR expression was determined by western blot analysis using anti-His-tag antibody as described (Kumagai et al., 2006).
Statistical analysis.

Statistical analyses were performed using analysis of variance and Tukey Honestly Significant Differences test or Student’s t test, and \( P < 0.01 \) was considered significant.

2.4 Results

Synchronous culture of *E. chaffeensis*.

Host cell–free *E. chaffeensis*, liberated from heavily infected THP-1 cells by mild sonication (two 10 s pulses on setting 2), varied in size from 0.2 to 1.0 \( \mu m \) (Fig. 2.1A). Because the infectious elementary body from the *Chlamydia* genus is resistant to sonication (Warford *et al.*, 1985), we examined whether host cell–free *E. chaffeensis* was resistant to more intense sonication (two 30s pulses on setting 4.5). We determined that approximately 30–40% of the bacterial population remained intact. The sonication-resistant bacteria were smaller in size (<0.5 \( \mu m \)) and more densely stained with the basic dye in the Diff-Quik staining kit than the sonication-sensitive bacteria (Fig. 2.1A and 2.1B). Because the DC and RC forms described previously (Zhang *et al.*, 2007) were morphologically similar to our sonication-resistant and -sensitive forms, these forms were named ‘DC’ and ‘RC’, respectively. Approximately 80% of the DC population was viable after more intense sonication (as described above), based on staining with LIVE/DEAD BacLight bacterial viability kit (Fig. 2.1C). A synchronous culture of *E. chaffeensis* was prepared using the *E. chaffeensis* DC form as inoculum (Fig. 2.1C). At 0 h p.i., only one to a few DCs per host cell were found by Diff-Quik staining (Fig. 2.1D). At 24 h p.i., almost every infected THP-1 cell (\( > 95\% \)) had 1–5 large morulae (2–3 \( \mu m \)); at 48 h p.i., almost every infected THP-1 cell (\( > 95\% \)) had approximately 20 small (\( \sim 1 \))
μm) morulae; at 72 h p.i., almost every infected cell (>99%) had more than 20 large (2–3 μm) and dense morulae; and by 96 h p.i., almost every infected THP-1 cell (>99%) began to lyse, and morulae became loose and swollen (2–5 μm) (Fig. 2.1D and 2.1E). Quantitative PCR using a primer set specific to *E. chaffeensis* 16S rDNA (Cheng *et al.*, 2006) showed a lag phase of *E. chaffeensis* growth for approximately 24 h, followed by an exponential phase of growth from 24 to 72 h p.i. and a short stationary phase from 72 to 96 h p.i. (Fig. 2.1F). These results confirmed synchronous growth of *E. chaffeensis* for up to 4 days p.i., until the onset of host cell lysis.

Temporal expression of five *virB/D* loci.

Five *E. chaffeensis* *virB/D* loci are shown schematically in Fig. 2.2A, 2.2B and Table 2.4. Constitutively expressed *E. chaffeensis* genes have not been identified, and thus expression of the gene *gyrB* was examined as a potential normalization control because previous studies have demonstrated its constitutive expression in *Chlamydia trachomatis* (Hefty and Stephens, 2007). Using quantitative PCR, we determined that the expression of the two major *virB/D* operons that start with *virB8-1* and *sodB*, respectively, were almost equal to that of *E. chaffeensis* *gyrB* at 0 h p.i., indicating that the T4S apparatus transcripts were expressed at a low level. Co-expression of each of the two operons as a single long transcript was shown previously (Ohashi *et al.*, 2002). In contrast, transcript levels of the three duplicated genes (*virB4-2*, *virB8-2*, and *virB9-2*) were much lower than that of *E. chaffeensis* *gyrB* at 0 h p.i. (Fig. 2.3A). The expression of the two major operons peaked at 24 h p.i. (the end of the lag phase) (Fig. 2.3B). The expression of three duplicated genes significantly increased at 24 h p.i. and peaked at 48 h p.i. (the early
exponential phase) (Fig. 2.3B). All five loci were down-regulated at 96 h p.i. (the stationary phase), and the transcript levels were similar to those observed at 0 h p.i. These results suggest that the expression of virB/D genes in *E. chaffeensis* is coordinately regulated during its intracellular development. Unlike in *C. trachomatis* (Hefty and Stephens, 2007), transcription of gyrB in *E. chaffeensis* was also regulated. The *E. chaffeensis* 16S rRNA amounts at different time points were also determined as a measure of the *E. chaffeensis* total RNA by real time RT-PCR. The results showed that the 16S rRNA levels changed much less than the virB/D loci transcript levels during *E. chaffeensis* intracellular development (Fig. 2.3C).

*E. chaffeensis* protein binds to the promoter regions upstream of *sodB* and *virB9-2*.

5′ RACE was previously utilized to determine transcriptional start sites and σ70-like promoter elements upstream of both *sodB* and *virB8-1* (Ohashi *et al.*, 2002). The transcriptional start sites and σ70-like promoter elements of *virB8-2*, *virB9-2*, and *virB4-2* were predicted upstream of these three genes by the BPROM program (Softberry, Inc., Mount Kisco, NY) (Table 2.5). To determine whether an *E. chaffeensis* native protein binds to the promoter regions upstream of *virB/D* loci, a DNA fragment upstream of the 5′-most proximal gene in the *sodB-virB3-virB6* operon (*sodB*) and a DNA fragment upstream of one of the duplicated genes (*virB9-2*) were amplified by PCR and biotinylated. A DNA fragment upstream of the second gene in the *virB8-1-virD4* operon (*virB9-1*) was amplified as a negative control. Electrophoretic mobility shift assays (EMSAs) revealed that the lysate of *E. chaffeensis* isolated from heavily infected THP-1 cells contained proteins that bound to the DNA probes derived from the sequences
upstream of *sodB* and *virB9-2* but not to the DNA probe derived from the sequence upstream of *virB9-1* (Fig. 2.4A). Binding specificity for each biotinylated probe was demonstrated using a 50-fold excess of the corresponding unlabeled probe.

Proteins from the *E. chaffeensis* lysate that bound to the biotinylated probe derived from the sequence upstream of *sodB* were purified using streptavidin affinity chromatography. SDS-PAGE analysis of the affinity-purified proteins revealed one predominant polypeptide of approximately 13 kDa and several other polypeptides of significantly lower yields and varying molecular masses (Fig. 2.4B). Tandem mass spectrometry of the predominant band identified this polypeptide as a 108–amino acid, 12.3-kDa hypothetical protein (*E. chaffeensis* Arkansas ECH0759, GenBank accession number: YP_507593) with 91% coverage of the amino acid sequence (Fig. 2.4C). We named this protein EcxR, for *E. chaffeensis* expression regulator. The identities of the other polypeptides could not be determined by mass spectrometry due to insufficient amounts of the samples.

Cloning and expression of recombinant EcxR (rEcxR) yielded a 116–amino acid, 13 kDa protein that contained eight C-terminal amino acids derived from the pET29a (+) vector, including a His$_6$ tag. The rEcxR was purified to apparent homogeneity as determined by SDS-PAGE analysis followed by coomassie brilliant blue staining and by western blot analysis (Fig. 2.5).

EMSA analysis with rEcxR.

The EMSA analysis showed that the biotinylated DNA probes derived from sequences upstream of all five *virB/D* loci were shifted upon incubation with rEcxR (Fig. 2.4).
The binding specificity of each DNA probe was demonstrated using a 50-fold excess of corresponding unlabeled DNA probe. In addition, BSA did not shift the DNA probe derived from the sequence upstream of *sodB* (Fig. 2.6B). When an anti-His-tag antibody, which recognized rEcXR, was added to the reaction, the probe was supershifted in the presence of rEcXR but not in the presence of BSA (Fig. 2.6B).

rEcXR activates *lacZ* reporter fusions.

Because EcXR bound to all DNA probes derived from sequences upstream of the *virB/D* loci, we examined whether these regions could be transactivated by EcXR. The *lacZ* reporter fusions were constructed by individually inserting the five *virB/D* promoter fragments (Fig. 2.2B, Table 2.3 and Table 2.4) upstream of the translation start site of the promoterless *lacZ* gene in pACYC184. Each one of the *lacZ* reporter constructs was transformed into *E. coli* BL21 (DE3) containing either pEcXR or empty pET29a(+) vector. For each of the five *lacZ* reporter constructs, IPTG induction of the pEcXR vector resulted in a significant increase in β-galactosidase activity compared to samples lacking IPTG or compared to IPTG induction of the empty pET29a(+) vector (Fig. 2.7). The expression of rEcXR protein upon IPTG induction was confirmed by western blot analysis (Fig. 2.7).

**Autoregulation of EcXR.**

The fragments used for EMSA and reporter construct are shown in Fig. 2.8A. The EMSA shown in Fig. 2.8B indicated that the DNA probe derived from sequence upstream of *ecxR* was also shifted upon incubation with rEcXR and that the interaction
was specific. We, therefore, examined whether EcXR expression is auto-regulated. A lacZ reporter construct containing the promoter region upstream of ecxR was made as described above, and the resulting plasmid was transformed into E. coli BL21 (DE3) containing either pEcXR or pET29a(+) vector alone. In the presence of this lacZ reporter construct, IPTG induction of the pEcXR vector resulted in a significant increase in β-galactosidase activity compared to samples lacking IPTG or compared to IPTG induction of the empty pET29a(+) vector (Fig. 2.8C). Expression of rEcXR upon IPTG induction was confirmed by western blot analysis (Fig. 2.8C). Taken together, these data suggest that the expression of ecxR is positively auto-regulated.

2.5 Discussion

In the present study, we developed a method to synchronize E. chaffeensis cultured in the human myelocytic leukemia cell line THP-1 using the sonication-resistant form of the bacteria. In DH82 cells (canine histiocytoma cell line), at 1 day p.i., E. chaffeensis is transformed from a small DC (0.4–0.6 μm) to a large RC (0.7–1.9 μm) and starts to multiply (Zhang et al., 2007). After 3 days of culture, the bacterial population converts back to DCs to initiate new infection of host cells. The infectivity of the DC-rich population has been reported to be 3.5 million times greater than that of the RC-rich population (Zhang et al., 2007). To establish a synchronized culture in THP-1 cells, we used controlled high-intensity sonication to rupture the RC population and enrich the infectious DC population. Morphological observation and real-time PCR analysis demonstrated the reliability of this method for synchronization. It was previously reported in DH82 cells that each E. chaffeensis bacterium progresses through its cycle in
a discrete membrane-bound inclusion and is released upon host cell lysis (Zhang et al., 2007). In contrast, in human THP-1 cells, we have found that the large inclusion is formed in the host cell by 24 h p.i., which then appears to disperse into multiple small inclusions at 48 h p.i. These small inclusions continued to expand until 72 h p.i., suggesting that a critical signaling event takes place between 24 and 48 h p.i. to form multiple inclusions in each THP-1 cell. The formation of multiple inclusions at 48 h p.i. coincided with the upregulation of the virB/D genes. Future analysis of the molecular basis for this phenomenon may provide important insights into inclusion morphogenesis and development.

The synchronized culture allowed us to characterize the expression pattern of all five virB/D loci during intracellular development of E. chaffeensis. At 0 h p.i., transcripts from the two major operons were expressed, suggesting that the T4S apparatus is present in E. chaffeensis at a low level prior to infection. A. phagocytophilum has virB/D genes homologous to those of E. chaffeensis (Hotopp et al., 2006; Ohashi et al., 2002). In A. phagocytophilum, translocation of AnkA into host cells was shown to commence in a virB/D-dependent manner within a few minutes p.i. and was shown to play an important role in facilitating intracellular infection by activating the Abl-1 signaling pathway (Lin et al., 2007). Although the T4S substrates in E. chaffeensis have not been reported, some T4S substrates may also be delivered via the low levels of preformed T4S apparatus available at the early stage of infection.

The expression of virB9 and virB6 genes as well as VirB9 protein is up-regulated in A. phagocytophilum after internalization into neutrophils, whereas the VirB9 expression is down-regulated in the human promyelocytic leukemia cell line HL-60 prior
to release from the host cells (Niu et al., 2006). Similarly, we have now demonstrated that the expression of the two major *E. chaffeensis* operons peaked at 24 h p.i. The expression of the three duplicated genes also significantly increased at 24 h p.i. and peaked at 48 h p.i.. *Legionella* has been shown to inhibit phagosome-lysosome fusion in host macrophages (Horwitz, 1983), and *Legionella*-containing vacuoles mature into acidic, late endosomes for bacterial replication (Sturgill-Koszycki and Swanson, 2000), which require a functional T4S system (Coers et al., 2000). *E. chaffeensis* replicates in a slightly acidic early endosome that does not mature into a late endosome (Barnewall et al., 1997). Here, we have shown that expression of the *virB/D* genes was up-regulated during the early exponential phase, suggesting that the T4S system contributes to the survival of *E. chaffeensis* and to the establishment of replicative inclusion. Additionally, our results suggest that two operons and three scattered duplicated *virB/D* genes may be utilized by *E. chaffeensis* at slightly different stages of its developmental cycle.

In the present study, EcXR, a previously unidentified DNA binding protein, was identified and shown to activate the expression of the *virB/D* genes in *E. chaffeensis* during intracellular development. The expression of *ecxR* was auto-regulated, like many other transcription factors (Wang et al., 2007a; Maamar and Dubnau, 2005; Tramonti et al., 2002), in response to an unknown signal. The mechanism of the down-regulation of the *virB/D* genes is yet to be determined. The rapid degradation of mRNA at the stationary phase might contribute to it. These results suggest that the expression of the *virB/D* genes is tightly regulated during the intracellular life cycle of *E. chaffeensis* and that EcXR plays an important role in this regulation. In mouse macrophages, the T4S system of *B. abortus* is essential for inhibiting lysosomal fusion of the bacteria-
containing inclusion and for transformation of the initial inclusion into the replicative niche by acquiring endoplasmic reticulum membranes (Celli et al., 2003). Although the binding of a transcriptional regulator or a cis-acting element has not been described for the regulation of the virB operon in Brucella, several reports have suggested that signaling events regulate virB expression during intracellular development. In Brucella suis, the virB promoter is induced in macrophages within 3 h p.i. (after the bacteria enter cells and phagosome acidification occurs) (Boschirol et al., 2002). The stringent response mediator Rsh is required for virB expression, suggesting that a nutrient-poor intracellular environment triggers Brucella melitensis virB expression (Dozot et al., 2006). On the other hand, in B. melitensis, a quorum-sensing pheromone down-regulates virB transcription (Delrue et al., 2005; Taminiau et al., 2002). However, an ecxR homolog has not been detected in Brucella, and genes encoding the RelA/SpoT homologs or the genes required for biosynthesis of a quorum-sensing pheromone have not been found in the E. chaffeensis genome. Thus in E. chaffeensis, the temporal regulation of the virB/D loci during intracellular development is similar to that found in Brucella, but the signaling events that lead to temporal regulation are different.

In Legionella, the icm/dot T4S system is organized into at least 11 transcriptional units that contain monocistronic as well as polycistronic transcripts, several of which contain a conserved sequence (TATAYT) that serves as their putative RpoD (σ70) recognition element and is essential for their expression (Gal-Mor et al., 2002). L. pneumophila contains homologs to at least six sigma factors (RpoD, RpoH, RpoF, RpoE, RpoS, and RpoN) (Gal-Mor et al., 2002). In contrast, E. chaffeensis encodes only two sigma factor homologs: a constitutive σ70 and a single alternative σ factor, σ32 (RpoH)
(Hotopp et al., 2006). The paucity of alternative sigma factors suggests that the intracellular development of *E. chaffeensis* requires transcription factor regulation of the constitutive $\sigma^{70}$-type promoters. The five *virB/D* loci contain putative $\sigma^{70}$-type promoters (Ohashi et al., 2002) and present study), suggesting that EcXR is a common factor regulating T4S system genes. Interestingly the expression of *gyrB*, which encodes the $\beta$-subunit of DNA gyrase, also peaked at 24 h p.i. DNA gyrase is the bacterial type II topoisomerase responsible for introducing negative supercoiling into DNA (Reece and Maxwell, 1991) and is needed to maintain the supercoiling required for bacterial DNA replication, transcription, and recombination.

EcXR homologs have been found in *A. phagocytophilum* strains, in *Anaplasma marginale* St. Maries, in *Ehrlichia canis* Jake, and in *Ehrlichia ruminantium* Welgevonden and Gardel (Wang et al., 2007a). All of these bacterial genomes have split *virB/D* loci (Hotopp et al., 2006). Therefore, the regulation of the *virB/D* genes in these bacteria may be similar to the EcXR regulation of *virB/D* genes in *E. chaffeensis*. In *A. phagocytophilum*, the EcXR homolog, ApxR, regulates the expression of a putative transcription factor *tr1* (Wang et al., 2007a) and the downstream *p44E* locus (Wang et al., 2007b). *p44E* encodes the immunodominant pleomorphic 44-kDa major surface protein (Wang et al., 2007a), suggesting that in *E. chaffeensis* the expression of *tr1* and the downstream *omp-1/p28* locus, which encodes the major outer membrane proteins (Ohashi et al., 2002), might be also regulated by EcXR.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’→3’)</th>
<th>Target (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrB</td>
<td>F: CTGGATTACACCACATGGTA</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>R: CTACAGGTATACCTCTACCA</td>
<td></td>
</tr>
<tr>
<td>sodB</td>
<td>F: ATTTTGATAGCGGATGGGA</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>R: AAGCATGCTCCCATAACATCC</td>
<td></td>
</tr>
<tr>
<td>virB8-1</td>
<td>F: TTTACAGCATTTGCGTGCCAG</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>R: TTGCCGTTCTTGTTTCAATTCA</td>
<td></td>
</tr>
<tr>
<td>virB4-2</td>
<td>F: CAGGTGTGGATTTGAGCATGG</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>R: ACAACGAAAATGCACAAGCA</td>
<td></td>
</tr>
<tr>
<td>virB8-2</td>
<td>F: AAAACAAATTGCGATAAAATGGTG</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>R: AAACTCTAATGGCATAATTCCTGA</td>
<td></td>
</tr>
<tr>
<td>virB9-2</td>
<td>F: ACGATGGAGAGATATCGACA</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>R: GTAGTATACACGCTTTGTTGTA</td>
<td></td>
</tr>
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Table 2.1 Oligonucleotide primers used for quantitative RT-PCR.

* F, forward; R, reverse
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<th>Gene</th>
<th>Primer (5’→3’)</th>
<th>Target (bp)</th>
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<tr>
<td>virB8-1</td>
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<td></td>
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</tr>
<tr>
<td>virB8-2</td>
<td>F: GTAAAAAGTAGTGCTAATCCTCA</td>
<td>403</td>
</tr>
<tr>
<td></td>
<td>R: CATAAAATTCTACATAATACATAATA</td>
<td></td>
</tr>
<tr>
<td>virB4-2</td>
<td>F: AGACGGTGAAATATTTATTTGGCTAA</td>
<td>365</td>
</tr>
<tr>
<td></td>
<td>R: CAGGAAACCCTTTATTTAACCCTA</td>
<td></td>
</tr>
<tr>
<td>virB9-1</td>
<td>F: AGTAAACCCTGTATCAGTCA</td>
<td>434</td>
</tr>
<tr>
<td></td>
<td>R: CATCTGCCCTATAGTAAGTTA</td>
<td></td>
</tr>
<tr>
<td>virB9-2</td>
<td>F: CAGTAGTGAACCTGATGCTA</td>
<td>327</td>
</tr>
<tr>
<td></td>
<td>R: GAACCTTGCAACAAATCTATGC</td>
<td></td>
</tr>
<tr>
<td>sodB</td>
<td>F: TTGATAAGATAACGATCCTTCC</td>
<td>351</td>
</tr>
<tr>
<td></td>
<td>R: TGTTGATATGGAAGTTCCCGT</td>
<td></td>
</tr>
<tr>
<td>ecxR</td>
<td>F: CAGCTTAGTTGTTGTACAGTAC</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td>R: AGTTATGTACCGTAATCTAGTAA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 Oligonucleotide primers used to amplify promoter regions upstream of virB/D loci.
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<thead>
<tr>
<th>ID</th>
<th>Primer</th>
<th>Target DNA (bp)</th>
<th>Enzyme</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>ecxR</td>
<td>F: TATAACATATGACAACACAAATAA GTAAACCACAAATG&lt;sup&gt;a&lt;/sup&gt; R: GTGCTCGAGATCTTCTTTTGT TATTACAAAGA</td>
<td>333</td>
<td>Nde I</td>
<td>pET29a(+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ecxR</td>
<td>F: GGGAAGCTTCAGCCTAGTGTG TTGTACAGTAC</td>
<td>460</td>
<td>Hind III</td>
<td>pACYC184 lacZ</td>
</tr>
<tr>
<td>upstream</td>
<td>R: GGGGATCCAGGTATGTACC GGAATCTAGTAA</td>
<td></td>
<td></td>
<td>(31)</td>
</tr>
<tr>
<td>region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>virB8-1</td>
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<td>450</td>
<td>Hind III</td>
<td>pACYC184 lacZ</td>
</tr>
<tr>
<td>upstream</td>
<td>R: CCGGATCCAAACCTTATAGT TTTAAGCTATA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>virB8-2</td>
<td>F: GGGAAGCTTCCAGGATGGT AATAATGAGTA</td>
<td>454</td>
<td>Hind III</td>
<td>pACYC184 lacZ</td>
</tr>
<tr>
<td>upstream</td>
<td>R: CCGGATCCAAAATCTACA TAATACATAAATACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>virB4-2</td>
<td>F: GGGAAGCTTAGAGCGGTAAT ATTATTGGCTAAA</td>
<td>365</td>
<td>Hind III</td>
<td>pACYC184 lacZ</td>
</tr>
<tr>
<td>upstream</td>
<td>R: CCGGATCCAGGAAAACCCT TATTAAACCCCTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>virB9-2</td>
<td>F: GGTCTAGACAGTAGAAT CTGATGCTA</td>
<td>343</td>
<td>Xba I</td>
<td>pACYC184 lacZ</td>
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<tr>
<td>upstream</td>
<td>R: CCGGATCCCTCAACATCAAT ATGGGAACTTG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sodB</td>
<td>F: GGGAAGCTTCTAGGAAACT AACAAGTGCA</td>
<td>353</td>
<td>Hind III</td>
<td>pACYC184 lacZ</td>
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<tr>
<td>upstream</td>
<td>R: CCGGATCCATGTCTCTATT AAATAAACACA</td>
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<tr>
<td>region</td>
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</tr>
</tbody>
</table>

Table 2.3 Oligonucleotide primers used to construct plasmids.

<sup>a</sup> restriction site is underlined.
<table>
<thead>
<tr>
<th>Chromosomal coordinates</th>
<th>operon or gene</th>
<th>Coordinates of promoter regions used for:</th>
<th>EMSA</th>
<th>Reporter construct</th>
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</thead>
<tbody>
<tr>
<td>42178-36100</td>
<td>virB8-1→virB9-1→virB10→virB11→virD4</td>
<td>42418-42161</td>
<td>42628-42179</td>
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<tr>
<td>480037-501957</td>
<td>sodB→virB3→virB4-1→virB6-1→virB6-2→virB6-3→virB6-4</td>
<td>479718-480068</td>
<td>479684-480036</td>
<td></td>
</tr>
<tr>
<td>1073487-1071112</td>
<td>virB4-2</td>
<td>1073853-1073489</td>
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</tr>
<tr>
<td>588503-589192</td>
<td>virB8-2</td>
<td>588103-588505</td>
<td>588049-588502</td>
<td></td>
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<tr>
<td>207062-206250</td>
<td>virB9-2</td>
<td>207405-207079</td>
<td>207405-207063</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4 *Ehrlichia chaffeensis* virB/D loci.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>-35 box Position</th>
<th>Sequence</th>
<th>-10 box Position</th>
<th>Sequence</th>
<th>Transcriptional start site</th>
</tr>
</thead>
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<tr>
<td>virB4-2</td>
<td>-271→-266</td>
<td>TTAAAT</td>
<td>-246→-238</td>
<td>TTGTATAAT</td>
<td>-231</td>
</tr>
<tr>
<td>virB8-2</td>
<td>-283→-278</td>
<td>TTAACA</td>
<td>-264→-256</td>
<td>AGCTAACTT</td>
<td>-248</td>
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<tr>
<td>virB9-2</td>
<td>-352→-347</td>
<td>TTCACA</td>
<td>-328→-320</td>
<td>TGCTAAAAT</td>
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Table 2.5 Prediction of the transcriptional start sites and Sigma\textsuperscript{70} promoter elements of \textit{virB4-2}, \textit{virB8-2} and \textit{virB9-2}. 
Fig. 2.1 *E. chaffeensis* development in synchronously infected THP-1 cells.

A. Mixed developmental forms of *E. chaffeensis* liberated from infected THP-1 cells. The fragile reticulated cells (RCs) are indicated by arrows. The sonication-resistant dense-cored cells (DCs) are indicated by arrowheads. Cells were stained using Diff-Quik staining. Bars = 1 μm

B. *E. chaffeensis* DCs are enriched after vigorous sonication. Cells were stained using Diff-Quik staining. Bar = 1 μm


D. Synchronously cultured *E. chaffeensis* in THP-1 cells using DCs as inoculum. The bacteria or morulae are indicated by arrowheads. The inset shows a single bacterium associated with the host cell at 0 h p.i. All panels show Diff-Quik staining of cells. Bar = 5 μm.

E. Numbers of small (< 2 μm) and large (>2 μm) inclusions at different times p.i. One hundred infected THP-1 cells were scored at each time point. The values represent the means ± standard deviations from three specimens.

F. Synchronous growth of *E. chaffeensis* determined by quantitative PCR. Genomic DNA extracted from infected THP-1 cells at different times p.i. was subjected to real-time PCR analysis. Data points reflect the numbers of bacteria relative to the amount determined at 0 h p.i. The values represent the means ± standard deviations from three specimens.
Fig. 2.1

A

B

C

D

p. i. (h) 0 24

48 72 96

continued
Fig. 2.1 continued

E

![Graph showing inclusion number per cell over time](image)

- Post infection (h)
  - 24
  - 48
  - 72

- Inclusion number/cell
- Small inclusions
- Big inclusions

F

![Graph showing relative bacterial number over time](image)

- Post infection (h)
  - 0
  - 24
  - 48
  - 72
  - 96

- Relative bacterial number
Fig. 2.2 virB/D gene loci and probe locations.

A. virB/D gene loci on the E. chaffeensis genome. The genome map of E. chaffeensis is shown as a circle. The putative origin of replication is shown as a black box. The virB/D gene loci are shown as gray arrows. Operon 1 includes virB8-1, virB9-1, virB10, virB11 and virD4. Operon 2 includes sodB, virB3, virB4-1, virB6-1, virB6-2, virB6-3 and virB6-4. The length of each arrow is enlarged to 8 times of the size in the genome.

B. Schematic representation of virB/D gene loci and probe locations. virB/D genes are represented by open arrows. The gene names are shown above the arrows, and the numbers of amino acids of the corresponding gene products are shown below the arrows. The lengths of virD4, virB4-1, virB4-2, virB6-1, virB6-2, virB6-3 and virB6-4 were not proportional, as indicated by discontinuous boxes. The upstream regions amplified by PCR to create DNA probes for EMSAs (shaded boxes) and for lacZ reporter constructs (hatched boxes) are indicated. Amino acid sequence identities of the duplicated genes are shown in parentheses. Chromosomal coordinates are underlined above each locus and are shown at the start and end of operons or genes.
Fig. 2.2

E. chaffeensis
(1,176,248 bp)

continued
Fig. 2.2 continued

```
B

operon 1

42178
258bp
virB8-1

450bp
237aa

virB9-1
273aa

virB10
447aa

virB11
332aa

virD4
714aa

36100

operon 2

480037
351bp
sodB

353bp
205aa

virB3
97aa

virB4-1
800aa

virB6-1
826aa

virB6-2
922aa

virB6-3
1468aa

virB6-4
2758aa

501957

365bp
791aa (26% identity with virB4-1)

365bp

1073487
1071112

403bp
virB8-2

588503
589192

454bp
229aa (10% identity with virB8-1)

207062
206250

327bp
virB9-2

343bp
270aa (29% identity with virB9-1)

Fragment used for EMSA

Fragment used for reporter construct
```
Fig. 2.3 Temporal expression of virB/D genes in *E. chaffeensis*.

A. Quantitative RT-PCR to determine the expression of the five virB/D loci at 0 h p.i. Transcript levels are relative to the amount of gyrB transcript at 0 h p.i. The values represent the means ± standard deviations from three specimens.

B. Quantitative RT-PCR to determine the temporal expression of the five virB/D loci. Transcript levels at different developmental stages were normalized by *E. chaffeensis* 16S rDNA. Transcript levels are represented as a log_2 ratio of the transcript at the indicated time point to the amount of gyrB transcript at 0 h p.i. The values represent the means ± standard deviations from three specimens.

C. Quantitative RT-PCR to determine the temporal amounts of 16S rRNA. 16S rRNA levels of *E. chaffeensis* at different developmental stages were normalized by *E. chaffeensis* 16S rDNA. 16S rRNA levels are represented as a log_2 ratio of the amount at the indicated time point to the amount of 16S rRNA at 0 h p.i. The values represent the means ± standard deviations from three specimens.
Fig. 2.4 Identification of an *E. chaffeensis* protein bound to the DNA probes derived from sequences upstream of *virB/D* loci.

A. Electrophoretic mobility shift assay of native *E. chaffeensis* proteins bound to the biotinylated DNA probes derived from sequences upstream of *sodB* and *virB*9-2. Native *E. chaffeensis* proteins bind to the DNA probes (0.1 pmol) upstream of *sodB*, *virB*9-2, but not *virB*9-1. For each panel: lane 1, DNA probe; lane 2, DNA probe incubated with *E. chaffeensis* lysate (5 μg); and lane 3, DNA probe incubated with *E. chaffeensis* lysate in the presence of a 50-fold excess of unlabeled DNA probe. Shifted bands are indicated by arrowheads. Bands were visualized using the LightShift Chemiluminescent EMSA kit.

B. Streptavidin affinity chromatography of *E. chaffeensis* proteins bound to the biotinylated probe derived from sequence upstream of *sodB*. Following chromatography, the purified protein sample was subjected to 15% SDS-PAGE analysis followed by staining. The identity of the protein indicated by the arrow was determined by mass spectrometry. M, Prestained protein size standards.

C. Amino acid sequence identified from the *E. chaffeensis* native protein that bound to the DNA probe derived from sequence upstream of *sodB*. One hypothetical protein (*Ehrlichia chaffeensis* Arkansas Ech0795, GenBank accession number: YP_507593) was identified by nano-LC/MS/MS. 91% of the amino acid sequence was determined (sequenced peptides are shown underlined).
### Fig. 2.4

#### A

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<td>434</td>
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</table>

#### B

- **B**: Allelic variants of the *sodB* gene.
- **M**: Allelic variants of the *virB9* gene.

#### C

- **MTTISNQNDYG**
- **KYKILK**
- **LIKDITYK**
- **KNHWNQVTTA**
- **RILGVDQPK**
- **ISQISNGKT**
- **AGFSLERL**
- **LLIFLRLKCD**
- **VNITIVNPEIALINKDNMAESSLESINLVIIQKED**
Fig. 2.5 Purification of recombinant EcxR.

*E. chaffeensis ecxR* was cloned into the pET29a(+) vector, expressed, and purified using nickel chelate chromatography. Purified protein was subjected to 15% SDS-PAGE analysis followed by coomassie brilliant blue staining (lane 1) and western blot analysis using an anti-His-tag antibody (lane 2). M, Prestained protein size standards. Each lane received 1 µg recombinant protein.
Fig. 2.6 Binding of recombinant EcxR to the DNA probes derived from sequences upstream of \textit{vir}B/D loci.

A. Electrophoretic mobility shift assay. For each panel: lane 1, DNA probe (0.1 pmol); lane 2, DNA probe incubated with rEcxR (25 ng); lane 3, DNA probe incubated with rEcxR in the presence of a 50-fold excess of the corresponding unlabeled DNA probe. Shifted bands are indicated by arrowheads. Bands were visualized using the LightShift Chemiluminescent EMSA kit.

B. Antibody supershift of rEcxR bound to the DNA probe derived from sequence upstream of \textit{sod}B. Lane 1, DNA probe (1 pmol); lane 2, DNA probe incubated with 0.2 μg BSA; lane 3, DNA probe incubated with 0.2 μg rEcxR; lane 4, DNA probe incubated with 0.2 μg BSA in the presence of 2 μl anti–His-tag antibody; lane 5, DNA probe incubated with 0.2 μg rEcxR in the presence of 2 μl anti-His-tag antibody. The gel was stained with ethidium bromide. rEcxR-shifted bands are indicated by arrowheads, and the antibody supershifted band is indicated by an arrow.
Fig. 2.6

A

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B

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</table>

Arrow indicates a specific band shift.
Fig. 2.7 EcxR activates the transcription of *virB/D lacZ* reporter fusions.

β-galactosidase assays were used to measure the transcriptional activities of *lacZ* reporter constructs. The values reflect means ± standard deviations from three specimens. *, significantly different (*P* < 0.01) compared to samples lacking IPTG or compared to IPTG induction of the empty pET29a (+) vector by the Tukey Honestly Significant Differences test. Western blot analyses of samples from the β-galactosidase assays were performed using an anti-His-tag antibody to verify the expression of rEcxR. A representative of three independent experiments is shown in the lower panels. rEcxR is indicated by arrowheads.
Fig. 2.7

**virB8-1 upstream region**

- no IPTG
- IPTG

**sodB upstream region**

- no IPTG
- IPTG

continued
Fig. 2.7 continued

![Graph showing β-galactosidase activity for virB4-2 and virB8-2 upstream regions with and without IPTG](chart.png)

**virB4-2 upstream region**

- **β-galactosidase activity (Miller units)**
  - No IPTG
  - IPTG

**virB8-2 upstream region**

- **β-galactosidase activity (Miller units)**
  - No IPTG
  - IPTG

*continued*
Fig. 2.7 continued

![Graph showing 
\[
\beta\text{-galactosidase activity (Miller units) vs. IPTG concentration}
\]

- virB9-2 upstream region
- pET29a(+) pEcXR

- no IPTG
- IPTG

* indicates a significant difference.
Fig. 2.8 The expression of ecxR is auto-regulated.

A. Schematic representation of ecxR gene and probe locations. The gene name is shown above the arrow, and the number of amino acids is shown below the arrow. The upstream regions amplified by PCR to create DNA probes for EMSA (shaded box) and for lacZ reporter construct (hatched box) are indicated. Chromosomal coordinates are underlined above the locus and are shown at the start and end of the gene.

B. Electrophoretic mobility shift assay of rEcxR bound to the DNA probe derived from sequence upstream of ecxR. Lane 1, DNA probe (0.1 pmol); lane 2, DNA probe incubated with rEcxR (25 ng); lane 3, DNA probe incubated with rEcxR in the presence of a 50-fold excess of unlabeled DNA probe. Shifted bands are indicated by arrowheads. Bands were visualized using the LightShift Chemiluminescent EMSA kit.

C. EcxR activates the transcription of an ecxR lacZ reporter construct.

β-galactosidase assays were used to measure the transcriptional activities of the ecxR-lacZ reporter construct. The values reflect means ± standard deviations from three specimens. *, significantly different ($P < 0.01$) compared to samples lacking IPTG or compared to IPTG induction of the empty pET29a(+) vector by the Tukey Honestly Significant Differences test. Western blot analyses of samples from the β-galactosidase assay were performed using an anti-His-tag antibody to verify the expression of rEcxR. A representative of three independent experiments is shown in the lower panel. rEcxR is indicated by an arrowhead.
Fig. 2.8

A

Fragment used for EMSA
Fragment used for reporter construct

B

ecxR upstream region (460bp)

C

ecxR upstream region

β-galactosidase activity (Miller units)
CHAPTER 3

INTRA-LEUKOCYTE EXPRESSION OF TWO-COMPONENT SYSTEMS IN
EHLRICHIA CHAFFEENSIS AND ANAPLASMA PHAGOCYTOPHILUM AND
EFFECTS OF THE HISTIDINE KINASE INHIBITOR CLOSANTEL

3.1 Abstract

The two-component regulatory system (TCS) composed of a pair of a sensor
histidine kinase and a response regulator, allows bacteria to sense signals and respond to
changes in their environment through specific gene activation or repression. The present
study examined TCS in the obligatory intracellular bacteria Ehrlichia chaffeensis and
Anaplasma phagocytophilum, that cause human monocytic ehrlichiosis (HME) and
human granulocytic anaplasmosis (HGA) respectively. The genomes of E. chaffeensis
And A. phagocytophilum were each predicted to encode three pairs of TCSs. All six
genes encoding three histidine kinases and three response regulators were expressed in
both E. chaffeensis and A. phagocytophilum cultured in human leukocytes. Pretreatment
of host cell-free E. chaffeensis or A. phagocytophilum with closantel, an inhibitor of
histidine kinases, completely blocked the infection of host cells. Treatment of infected
cells 1 day post infection with closantel cleared infection in dose-dependent manner. All six genes in *E. chaffeensis* were cloned, recombinant proteins were expressed, and polyclonal antibodies were produced. Double immunofluorescence labeling and western blot analysis revealed that all six proteins were expressed in cell culture. Autokinase activities of the three recombinant histidine kinases from *E. chaffeensis* were inhibited by closantel in *vitro*. A number of *E. chaffeensis* genes, including the six TCS genes, were down-regulated within 5–60 min post closantel treatment. These results suggest that these TCSs play an essential role in infection and survival of *E. chaffeensis* and *A. phagocytophilum* in human leukocytes.

### 3.2 Introduction

*Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* are emerging pathogens that exclusively parasitize monocytes/macrophages and neutrophils respectively {Rikihisa, 2006 #46}. As a result *E. chaffeensis* and *A. phagocytophilum* cause potentially fatal systemic febrile illnesses called human monocytic ehrlichiosis (HME) and human granulocytic anaplasmosis (HGA) respectively. These Gram-negative obligatory intracellular bacteria belong to the family *Anaplasmataceae* in the order *Rickettsiales* (Dumler et al., 2001).

Bacterial two-component regulatory systems (TCSs), generally composed of a sensor kinase and a response regulator. Sensor kinases are often integral inner membrane proteins with a periplasmic sensor domain and an intracellular catalytic kinase domain. Environment stimuli lead to the autophosphorylation on a conserved histidine residue in the sensor kinase. The phosphoryl group is then transferred to a conserved aspartate
residue in a cognate response regulator. The response regulator is often a transcription factor and its phosphorylation results in changes in the expression of a specific regulon that mediates adaptation of the bacterium to the environmental changes first sensed by the histidine kinase. The importance of TCSs in bacterial pathogenesis is evident in the attenuated virulence observed when one or more TCSs are inactivated (Dorman et al., 2001; Groisman, 2001; Throup et al., 2000). In the intracellular bacterium Brucella abortus, the BvrR/BvrS TCS controls cell invasion and intracellular survival by regulating Brucella surface protein expression (Guzman-Verri et al., 2002; Sola-Landa et al., 1998). In Agrobacterium tumefaciens, the VirA/VirG TCS regulates the type IV secretion system that transfers T-DNA to cause crown gall tumours at plant wound sites (Krishnamohan et al., 2001; Winans, 1992; Jin et al., 1990). Chlamydia trachomatis has a single pair of TCS, CtcB/CtcC, predicted to transcriptionally regulate the obligatory intracellular development of chlamydia (Koo and Stephens, 2003). In addition, many pathogenic bacteria contain TCSs that are essential for their viability. For example, Bacillus subtilis and Staphylococcus aureus each contain a TCS, YycG/YycF, essential for their viability (Stephenson and Hoch, 2002; Martin et al., 1999; Fabret and Hoch, 1998). In members of the order Rickettsiales, several genes homologous to bacterial TCSs have been recognized (Brayton et al., 2005; Collins et al., 2005; Foster et al., 2005; Ogata et al., 2001; Andersson et al., 1998), but so far relationships among them and functions of these genes have not been elucidated.

Recognition of important roles of TCSs has led to development of small molecules that inhibit TCSs. Stephenson et al (2000) reported that closantel (N-[5-chloro-4-[(R,S)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide), and
several other drugs that target to the C-terminal catalytic domain of the histidine kinase, inhibit kinase activity by causing structural alterations and aggregation. Foster et al. (2004) reported that closantel inhibits histidine kinase autophosphorylation activity and the dimerization, which is required for activation.

In the present study, we identified potential pairs of TCSs in the genomic sequences of *E. chaffeensis* and *A. phagocytophilum* and searched for homologues among sequenced members in the order *Rickettsiales*. We then determined the levels of expression of transcripts from these predicted genes in human leukocytes infected with *E. chaffeensis* or *A. phagocytophilum*. There is no useful system for genetic manipulation and no naturally isolated mutants of obligatory intracellular bacteria. Therefore, in order to assess the requirement of TCSs in intracellular infection, the effects of closantel on *E. chaffeensis* and *A. phagocytophilum* infection and temporal changes in transcription of genes predicted to be regulated by the TCSs were examined. We also analysed expression of the TCS proteins by *E. chaffeensis* and in vitro autokinase activities of three recombinant histidine kinases and their sensitivities to closantel.

**3.3 Materials and Methods**

Bacterial strains and culture

*E. chaffeensis* Arkansas (Anderson et al., 1991) and *A. phagocytophilum* HZ strain (Rikihisa et al., 1997) were propagated in THP-1 cells and HL-60 cells, respectively, in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% L-glutamine at 37°C in 5% CO₂ and 95% air. *E. coli* strains NovaBlue (Novagen, San Diego, CA), BL21(DE3) (Novagen) and JM109 (Stratagen, La Jolla, CA) used for DNA cloning and
protein expression were cultured in LB broth. \textit{E. coli} strain DH5\textalpha{} (Invitrogen, Carlsbad, CA), used for MBP-recombinant protein expression, was cultured in Rich medium containing glucose (New England Biolabs, Beverly, MA).

Reverse transcription PCR analysis

Total RNA was extracted from $5 \times 10^6 \ E.\ chaffeensis$-infected THP-1 cells (80% infected cells) or $5 \times 10^6 \ A.\ phagocytophilum$-infected HL-60 cells (80% infected cells) with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. One microgram of RNA was treated with 1 U of DNase I (Invitrogen) in the presence of 40 U of RNaseOUT (Invitrogen), an RNase inhibitor, for 15 min at room temperature. DNase I was inactivated by heating the reaction mixture for 10 min at 65°C in the presence of 2.5 mM EDTA. After purified, the RNA (0.5 μg) was reversely transcribed using 200 U of Superscript II reverse transcriptase (Invitrogen) with 300 ng of random hexamer primer (Invitrogen) and 1 mM dNTP by incubating at 42°C for 50 min. The reaction was terminated by heating the reaction mixture at 70°C for 15 min. To ensure the absence of DNA contamination in the RNA preparations, the assay was duplicated without reverse transcriptase. The PCR conditions were 15–35 cycles consisting of 45 s of denaturation at 94°C, 1 min of annealing at 51–62°C (depending on primer sequence) and 1 min of extension at 72°C. The PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The primers used are shown in Table 3.1 and 3.2.
Construction of plasmids for expression of the HKDs of sensor kinases and the entire lengths of the response regulators

*E. chaffeensis* chromosomal DNA was extracted from infected THP-1 cells using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. The DNA fragments encoding the HKDs of the sensor kinases and the entire lengths of the response regulators were amplified by PCR with primers shown in Table 3.3 using chromosomal DNA as a template. The PCR products were purified with the PCR Purification Kit (Stratagene). The amplified DNA was digested with restriction enzymes and ligated into the restriction enzyme-digested pET33b(+) or pET11a vector (Novagen). After *E. coli* strain NovaBlue cells were transformed, plasmids were extracted from each colony using Strataprep Plasmid Miniprep Kit (Stratagene) and the sequences of the cloned fragments were confirmed by DNA sequencing. *E. coli* strain BL21 (DE3) cells were transformed with the recombinant plasmids and induced to express the recombinant proteins with isopropyl-thio-β-D-galactoside.

For functional studies, the HKD of the CckA fragment was digested and ligated to pMAL-c2X vector (New England Biolabs). The plasmid was amplified in *E. coli* strain JM109 cells. The recombinant protein was expressed in *E. coli* strain DH5α cells.

Recombinant protein purification

The *E. coli* cells were harvested by centrifugation at 8,000 × g for 5 min and suspended in binding buffer (0.1 M sodium phosphate, pH 8.0). The suspensions were sonicated and centrifuged at 18,000 × g for 10 min at 4°C. The pellets were solubilized with binding buffer containing 6 M urea, filtered through a 0.45 μm filter, and loaded
onto a HIS-Select Cartridge (Sigma, Saint Louis, MO) equilibrated with binding buffer containing 6 M urea. The column was washed with wash buffer (0.1 M sodium phosphate, 10 mM imidazole, pH 8.0) containing 6 M urea. The proteins were eluted with elution buffer (0.1 M sodium phosphate, 250 mM imidazole, pH 8.0) containing 6 M urea. The purified proteins were suspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5% 2-mercaptoethanol), boiled for 3 min, and then separated using 12% SDS-PAGE. The bands of proteins were cut from the gel and were quantified using a BCA Protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

Amylose column made of amylose resin (New England Biolabs) was used for purification of MBP-rCckAHKD. After harvest, the *E. coli* cells were sonicated and centrifuged. The supernatant was loaded onto an amylose column equilibrated with amylose column binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% glycerol). The bound protein was eluted with the binding buffer containing 10 mM maltose.

Double-immunofluorescence labelling

Rabbit antibodies were produced against the purified recombinant proteins at ProSci (Poway, CA). All antisera used were preabsorbed with uninfected THP-1 cells. At 2 days post infection (p.i.), the *E. chaffeensis*-infected THP-1 cells (50% infected cells) were fixed by 1% paraformaldehyde in 1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) at room temperature for 20 min, then incubated with rabbit antiserum against each recombinant protein at a 1:50 dilution in PGS (PBS containing 0.1% gelatin and 0.3% saponin) at room temperature for 1 h. After rinsing
with PGS, the cells were incubated with dog antiserum against *E. chaffeensis* at a 1:200 dilution in PGS at 37°C for 30 min. Lastly, the cells were incubated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-dog IgG) at a 1:200 dilution in PGS at 37°C for 30 min. As negative controls, *E. chaffeensis*-infected THP-1 cells were incubated with the preimmune rabbit serum or rabbit antiserum against an unrelated protein (rhvP44-18 of *A. phagocytophilum*) cloned, expressed, and purified in the similar manner (Wang *et al.*, 2006) as the six TCS recombinant proteins and secondary conjugated antibodies or with preimmune dog serum and secondary conjugated antibodies. Cells were cytocentrifuged on a glass slide and analysed by a Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments, Melville, NY).

Inhibition of histidine autokinase activity by closantel

Closantel (N-[5-chloro-4-[(R,S)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl] -2-hydroxy-3,5-diiodobenzamide) was obtained from Wako Pure Chemical Industries (Osaka, Japan) and dissolved in DMSO. Ten micrograms of MBP-rCckAHKD, rNtrYHKD, or rPleCHKD were incubated with 0.1 mM ATP and 1 μCi of [γ-32P]ATP in phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 20 mM MgCl₂, 1 mM DTT) in the presence of 100 μM closantel for 15 min at room temperature. DMSO (1% v/v) was used as control. The reaction was terminated by addition of SDS-PAGE sample buffer. After electrophoresis on SDS-PAGE, the gel was dried and exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA). The screen was scanned with a PhosphorImager 445 Si (Molecular Dynamics).
Closantel treatment of bacteria and evaluation of infection

Host cell-free bacteria were treated with closantel at either 10 or 100 μM at 37°C for 20 min. Bacteria were washed by centrifugation and used to infect THP-1 or HL-60 cells. Alternatively, cells at 1 day p.i. were treated with closantel at 20 or 50 μM at 37°C for 20 min. DMSO solvent (1% v/v) was used as control. The infected cells were further cultured until 3 days p.i., when the numbers of *E. chaffeensis* or *A. phagocytophilum* organisms in 100 cells were estimated in triplicate culture wells as previously described (Lin et al., 2002).

Closantel treatment of bacteria and evaluation of the transcription of TCS genes and genes predicted to be regulated by TCSs

*E. chaffeensis*-infected THP-1 cells at 3 days p.i. (80% infected cells) were treated with closantel (100 μM) at 37°C for varying times. DMSO (1% v/v) was used as control. Ice-cold PBS was added to stop the reaction. The infected cells (1 × 10⁷) were washed three times with ice-cold PBS to remove closantel. One half of the cells were suspended in RNALater (Qiagen) at −20°C for RNA extraction. Remaining cells were kept at −80°C for DNA extraction. Total DNA and RNA were extracted as described above. RNA was reverse transcribed as described above. 16S rDNA was used to normalize across specimens by quantitative PCR as described below. The cDNA, derived from the same number of bacteria in each specimen based on 16S rDNA levels, was used as template for RT-PCR analysis as described above. The primers used are shown in Table 3.1.
Quantitative PCR

cckA, ntrY, pleC and 16S rRNA PCR primers were designed to produce amplicons of 100–150 base pairs (primer sequences are shown in Table 3.4). For each gene, the corresponding target DNA fragment was amplified from the *E. chaffeensis* chromosomal DNA and cloned into the pCR II vector of a TA Cloning kit (Invitrogen) to serve as a standard. The plasmid copy number was calculated based on the concentration of DNA and the molecular size of the plasmid. A 10-fold serial dilution (from $10^2$ to $10^9$ copies) of the plasmid was used to generate a standard curve to calculate the copy number. Assays with linear regression R-values of >99% were accepted. The cDNA copy number in the specimen was calculated based on the standard curve. Quantitative PCR was performed in a quantitative PCR instrument, Mx3000P (Stratagene), using a Brilliant SYBR Green QPCR Core Reagent Kit (Stratagene). Before performing quantitative PCR, the primer and Mg$^{2+}$ concentrations and annealing temperatures were optimized using ordinary PCR to ensure that the primers did not dimerize and that only a single PCR product was synthesized. For each quantitative PCR analysis, the dissociation curve was examined to confirm the absence of primer dimers. The quantitative PCR was carried out in a 25 μl reaction containing appropriate amounts of each primer (0.05–0.25 μM) and MgCl$_2$ (1.5–3.5 mM), 200 nM each of dNTP, 8% (v/v) glycerol, 3% (v/v) DMSO, 30 nM reference dye, 0.5 × SYBR Green I dye, 2.5 U of SureStart Taq DNA polymerase and 5 μl of cDNA template. The cycling conditions were 94°C heating for 10 min, and then 40 or 60 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. The dissociation curve detection began with a 30 s incubation at 55°C followed by 40 successive 30 s plateaus in which the temperature was increased by 1.0°C
for each plateau. For each cDNA sample, an RNA sample without reverse transcriptase was used to assess DNA contamination. \( C_T \) values and the target copy numbers were calculated using the Mx3000P software from Stratagene. Statistical analyses were performed using Student t-test.

### 3.4 Results

Six genes were predicted to encode TCSs in *E. chaffeensis* and *A. phagocytophilum* and were conserved in some members of the order *Rickettsiales*

Through sequence (Hotopp *et al.*, 2006) analysis and domain analysis, and due to overall similarities in protein structures and the coexistence of pairing response regulators, we identified three sensor kinases in each of the genomes of *E. chaffeensis* and *A. phagocytophilum* (Fig. 3.1): a homologue of CckA from *Caulobacter crescentus* (Jacobs *et al.*, 1999), a homologue of NtrY from *Azorhizobium caulinodans* (Pawlowski *et al.*, 1991), and a homologue of PleC from *C. crescentus* (Sommer and Newton, 1989). The CckA, NtrY and PleC homologues from *E. chaffeensis* and *A. phagocytophilum* each contained a histidine autophosphorylation site and an ATP binding motif (NG\(_1\)G\(_2\)G\(_3\) in NtrY and PleC and NG\(_1\)FG\(_2\)G\(_3\) in CckA). With the exception of NtrY, each had transmembrane domains. Only CckA had a receiver domain. We identified in the genomes of *E. chaffeensis* and *A. phagocytophilum* three response regulators each containing a conserved receiver domain with an aspartate phosphorylation site (Fig. 3.1): a homologue of CtrA from *C. crescentus* (Quon *et al.*, 1996), a homologue of NtrX from *A. caulinodans* (Pawlowski *et al.*, 1991), and a homologue of PleD from *C. crescentus* (Hecht and Newton, 1995). The CtrA and NtrX homologues of *E. chaffeensis* and *A.
phagocytophilum had C-terminal DNA-binding domains, and the PleD homologue of E. chaffeensis and A. phagocytophilum had C-terminal GGDEF domains (Fig. 3.1). CckA and CtrA, NtrY and NtrX, and PleC and PleD from E. chaffeensis transmit signals through specific amino acid-dependent phosphotransfer (Kumagai et al., 2006). We searched for homologues of these six TCS proteins in the genome sequences of Neorickettsia sennetsu and other sequenced members of the order Rickettsiales. The six TCS proteins were highly conserved, although many of these genes had not been specifically annotated or were annotated differently. Homologues of NtrY and NtrX were detected in all members with the exception of two Wolbachia species and N. sennetsu (Table 3.5). CckA was not detected in Rickettsia species (Table 3.5), but was present in all other species evaluated. Unlike the other bacteria in Table 3.5, mammalian infection is not part of the life cycles of either Wolbachia species or N. sennetsu.

All six genes of TCSs were expressed by E. chaffeensis and A. phagocytophilum in human leukocyte culture

Using reverse transcription polymerase chain reaction (RT-PCR) analysis, we showed that all six TCS genes were transcribed in E. chaffeensis-infected THP-1 cells and A. phagocytophilum-infected HL-60 cells cultured at 37°C (Fig. 3.2). No amplicon was detected without reverse transcriptase, indicating the absence of contamination by genomic DNA in the RNA preparation. We cloned DNA fragments encoding the histidine kinase domain (HKD) of three sensor kinases and the entire length of three response regulators of E. chaffeensis and then expressed the each protein in E. coli. The six recombinant proteins were purified using an Ni-affinity column. Rabbits were
immunized with each of the recombinant proteins and specific antibodies were raised. Western blot analysis indicated that the rabbit antibodies against each protein specifically recognized the target recombinant proteins and native *E. chaffeensis* proteins and there was no cross reactivity (Kumagai *et al.*, 2006). Double immunofluorescence labeling of *E. chaffeensis* in THP-1 cells with each of the six anti-recombinant TCS protein antibodies and anti-*E. chaffeensis* antibody confirmed that all these six proteins were expressed by replicating *E. chaffeensis* in microcolonies called morulae in THP-1 cells (Fig. 3.3A). As negative controls for immunofluorescence labelling, *E. chaffeensis*-infected THP-1 cells were incubated with the respective preimmune rabbit serum, the rabbit serum against an unrelated recombinant protein and secondary conjugated anti-rabbit IgG, or with the preimmune dog serum and secondary conjugated anti-dog IgG. There was no detectable labelling with these sera, indicating that labelling with both dog anti-*E. chaffeensis* and rabbit antirecombinant TCS protein sera was specific (Fig. 3.3B–D).

Closantel inhibited infection with *E. chaffeensis* and *A. phagocytophilum*

Closantel is a histidine kinase inhibitor (Foster *et al.*, 2004; Stephenson *et al.*, 2000). To determine whether closantel could inhibit autokinase activity of *E. chaffeensis* putative histidine kinases, an in *vitro* autokinase assay was performed using affinity-purified recombinant HKDs. The autokinase activities of rNtrYHKD and rPleCHKD of *E. chaffeensis* were detected in *vitro* and inhibited by closantel (Fig. 3.4). The rCckAHKD used for rabbit antibody production had no activity, presumably due to incorrect refolding of the recombinant protein. Therefore, the DNA fragment encoding the CckAHKD was cloned into a pMAL-c2X vector in order to prepare a fusion with maltose binding protein.
(MBP) (MBP-rCckAHKD). The MBP-rCckAHKD had autokinase activity, and the activity was inhibited by closantel (Fig. 3.4).

To determine the role of TCSs in *E. chaffeensis* infection, host cell-free bacteria or infected cells were treated with closantel, at varying concentrations, for 20 min at 37°C. The extent of infection was determined at 3 days p.i. Pretreatment of host cell-free *E. chaffeensis* or *A. phagocytophilum* with 100 μM closantel completely blocked the infection of host cells (Fig. 3.5A). Treatment of infected cells at 1 day p.i. with closantel cleared infection in dose-dependent manner (Fig. 3.5B). Treatment of host cell-free bacteria or infected cells with 1% v/v DMSO had no effect on infection (Fig. 3.5). These results suggest that histidine kinases are essential for *E. chaffeensis* infection of human leukocytes.

Closantel down-regulated the transcription of TCS genes and genes predicted to be regulated by TCSs

We examined the genome sequences of members of the order *Rickettsiales* and selected the genes listed in Table 3.6 for analysis. *fisK* and *fisZ*, regulated by CtrA in *C. crescentus* (Kelly *et al.*, 1998), were found in all members of the order *Rickettsiales*. NtrX is considered as a functional homologue of NtrC (Ishida *et al.*, 2002; Pawlowski *et al.*, 1991), and found in some members of the order *Rickettsiales* (Table 3.5). In the upstream of *ubiE*, *argD*, and *p5cR* (putative pyrroline-5-carboxylate reductase) of *E. chaffeensis* putative NtrC binding sites were found. *exoD* is involved in extracellular polysaccharide synthesis in *Rhizobium meliloti* (Reed and Walker, 1991), which is thought to be regulated by a GGDEF-domain containing protein (D'Argenio and Miller,
exoD was not found in the two *Rickettsia* sp., but was found in other members of the order. *virB8* is the most upstream gene in one of the two operons of the *virB/D* system of *E. chaffeensis* and *A. phagocytophilum* (Ohashi et al., 2002); *virB8* homologues were found in all members of the order *Rickettsiales*. *sodB* was found in all members of the order *Rickettsiales*, but was found upstream of the remaining operon of the *virB/D* system only in *Anaplasma* and *Ehrlichia* species. It was coexpressed with downstream *virB3*, *virB4* and *virB6* in *E. chaffeensis* and *A. phagocytophilum* (Ohashi et al., 2002). To study the role of TCSs on gene transcription, *E. chaffeensis* infected THP-1 cells were treated with closantel (100 μM) for varying times at 37°C. Levels of transcription of genes predicted to be regulated by TCSs were determined by RT-PCR using gene-specific primers (Table 3.1). Striking reductions were seen in levels of mRNAs of the three sensor kinases, *cckA*, *ntrY* and *pleC*, after 5 min of closantel treatment (Fig. 3.6A). The mRNAs of the three response regulators, *ctrA*, *ntrX*, *pleD* and *virB8* began to decrease after 15 min and became undetectable after 1 h of closantel treatment (Fig. 3.6A). 16S rRNA, a lipoprotein gene (ECH0462), *fisK* and *fisZ*, *ubiE*, *argD*, *p5cR*, *exoD* and *sodB* mRNA levels were reduced after 30 min (Fig. 3.6A and B).

To quantify the inhibition of histidine kinases, we treated the *E. chaffeensis* infected THP-1 cells with 100 μM closantel for 5 min. The copy numbers of mRNA were estimated using quantitative RT-PCR (Fig. 3.6C). The mRNA copy numbers of *cckA*, *ntrY* and *pleC* were normalized to 16S rRNA levels. 16S rRNA levels per bacterium (16S rDNA) were unchanged at this time point (Fig. 3.6C). However, the mRNA copy numbers of *cckA*, *ntrY* and *pleC*, were reduced by 53%, 83% and 84%, respectively,
compared with treatment with DMSO (Fig. 3.6C). Thus these three histidine kinases were rapidly and significantly down-regulated in *E. chaffeensis* upon closantel treatment.

### 3.5 Discussion

We have identified three potential pairs of TCSs in *E. chaffeensis* and *A. phagocytophilum* that may mediate their adaptive responses during infection. Three sensor kinases, CckA, NtrY and PleC, and three response regulators, CtrA, NtrX and PleD, were found. The present study suggests that these TCSs are essential for intracellular infection and survival of *E. chaffeensis* and *A. phagocytophilum*.

CtrA is a regulatory protein found only in α-proteobacteria (Hallez *et al.*, 2004) that coordinates multiple cell cycle events at the transcriptional level. In *C. crescentus* CckA is involved in in *vivo* CtrA phosphorylation (Jacobs *et al.*, 2003; Quon *et al.*, 1996). Only CtrA, but not CckA, is found in the *Rickettsia prowazeki* chromosome (Hallez *et al.*, 2004). The CtrA 9-mer and 8-mer consensus binding sequences (TTAAN₇TTAAC and TTAACCAT) from *C. crescentus* were detected upstream of numbers of genes involved in DNA replication initiation, DNA segregation, cellular division, transcription, morphogenesis and DNA methylation of several members of α-proteobacteria (Hallez *et al.*, 2004). In the present study we found the consensus CtrA binding sites upstream of *ftsK* and *ftsZ* in both *E. chaffeensis* and *A. phagocytophilum* genomes. We also observed downregulation of transcription of these two genes upon closantel treatment, suggesting CtrA regulation of DNA segregation and cell division in *E. chaffeensis* and *A. phagocytophilum*. CtrA also controls its own transcription by positive and negative feedback loops in *C. crescentus* (Domian *et al.*, 1999). We found consensus CtrA-binding
sites in the promoter region of the putative CtrA in *E. chaffeensis* and *A. phagocytophilum*. Closantel treatment down-regulated CtrA transcription, providing evidence for positive feedback regulation.

The approximately 170 amino acid-long protein domain GG(D/E)EF is a conserved domain found in many families of bacteria (Chan *et al.*, 2004; Galperin *et al.*, 2001; Aldridge and Jenal, 1999). The domain name originates from the conserved amino acid motif, Gly-Gly-Asp/Glu-Glu-Phe. This domain is involved in the synthesis and hydrolysis of cyclic-bis (3′→5′) dimeric GMP (c-di-GMP), which functions as a second messenger in several bacterial species (Ryjenkov *et al.*, 2005). The GG(D/E)EF domain was identified in PleD from *C. crescentus*. PleD is a global regulatory protein that controls cell differentiation, a developmental transition in the life cycle of the aquatic bacterium that enables the cell to switch between a sessile, surface-attached form (stalked cell) and a motile, flagellated form (swarmer cell) (Hecht and Newton, 1995). PleD phosphorylation is modulated by PleC and DivJ in *C. crescentus* (Paul *et al.*, 2004). c-di-GMP is known to regulate exopolysaccharide synthesis in several bacteria such as *G. xylinum* and *Rhizobium leguminosarum* (D'Argenio and Miller, 2004). ExoD is involved in exopolysaccharide synthesis (Leigh and Lee, 1988) and we found homologues to ExoD in all sequences available of members of the family *Anaplasmataceae* (Table 3.6). *exoD* was down-regulated by closantel treatment in *E. chaffeensis*, supporting the potential regulation of expression of this gene by TCS. The NtrB and NtrC system regulates genes involved in nitrogen fixation and metabolism. NtrX and NtrY, homologous to NtrB and NtrC, have been identified and characterized in *A. caulinodans* (Pawlowski *et al.*, 1991) and *Azospirillum brasileense* (Ishida *et al.*, 2002)(Ishida *et al.*,
Pawlowski et al. and Ishida et al. suggested that, unlike NtrB, which is a cytosolic sensor kinase, NtrY may be involved in sensing extracellular nitrate levels because of the sequence of putative transmembrane domains (Ishida et al., 2002; Pawlowski et al., 1991). However, in *E. chaffeensis* and *A. phagocytophilum* NtrY does not have cleavable signal peptides, and thus it may sense cytosolic nitrate levels (Fig. 3.1). NtrY was demonstrated to be present in the cytosolic fraction (Kumagai et al., 2006). NtrX protein may regulate alternative nitrogen assimilation pathways (Pawlowski et al., 1991). The roles of NtrX and NtrY in non-nitrogen fixing bacteria such as *C. crescentus* are unknown. However, *ubiE, argD* and *p5cR* were down-regulated in *E. chaffeensis* by closantel treatment, supporting the potential involvement of NtrX and NtrY in nitrogen metabolism in *E. chaffeensis*.

A remarkable finding from the present study was the downregulation of the three histidine kinase genes in *E. chaffeensis* after only 5 min of closantel treatment. This rapid inhibitory effect of closantel on kinase expression had not been reported for other bacteria, suggesting the possibility that closantel may have an additional site of action in these intracellular pathogens. The host cells were viable during the treatment, indicating that closantel was not toxic to host cells. Although the present study was limited to in vitro experiments, rapid and strong inhibitory activity of closantel on *A. phagocytophilum* and *E. chaffeensis* infection suggests that the histidine kinase inhibitor, or its derivatives, will have therapeutic potential. Currently, there are very limited choices of antibiotics available for treatment of rickettsial infections (Rikihisa, 1991).
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<tr>
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<td>R: GTG GAC GAA CTA TGT CAC C</td>
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<td>R: CAT GGA CTC AGC CTT CAT CA</td>
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Table 3.1 Oligonucleotides used for gene-specific RT-PCR in *E. chaffeensis*.

a F, forward; R, reverse.
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<th>Amplicon size (bp)</th>
</tr>
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</table>
| cckA | F: AAC TGG CAC AAT GTT CAG AG  
      | R: ATC TTG GGT TGT AGA GTC TG | 672               |
| ntrY | F: AAT GCA GTA CTG CTG TCA TG  
      | R: TGC ATA CGT TGC TCA CTT AG | 652               |
| pleC | F: GAA ATC GTG CAT GGA ACA AG  
      | R: TCA GCA CTA TTT GTG ATT CTG | 634               |
| ctrA | F: TAA GAG TGA GTT ACT TGC AAG  
      | R: ATC CTC TCC ATC ATC ACA AG | 385               |
| ntrX | F: CTG TTA AGT CAC TGC ACA TAG  
      | R: CTA TAG TAG AGA TCC TCA CAG | 634               |
| pleD | F: TTC CAA GAG ACT ACT ATA CTC  
      | R: CTG CTG CAG TAT TTC ATC TC  | 528               |

Table 3.2 Oligonucleotides used for gene-specific RT-PCR in *A. phagocytophilum.*
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<th>Restriction Enzyme Site</th>
<th>Plasmid</th>
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| **rCckAHKD**             | F: GGG GAT CCG AAC TCA TAT GTA AAA ATT CAT<sup>a</sup>  
R: GGG CCG CCG CTC AAG GGA TAA GGA TAA TAA AAG  
MBP-                      | BamH I/ Not I  
P: GGG CGG CCG CTC AAG GGA TAA GGA TAA TAA AAG | pET33b(+) |
| **rCckAHKD**             | F: CAG GAA TTC ATC ACA TTT AAA GCA CAT ATA TCA C  
R: CCC CTG CAG TTA AGG GAT AAG GAT AAT AAA AGT AG | EcoR I/ Pst I  
P: CAG GAA TTC ATC ACA TTT AAA GCA CAT ATA TCA C  
R: CCC CTG CAG TTA AGG GAT AAG GAT AAT AAA AGT AG | pMAL-c2X |
| **rNtrYHKD**             | F: GGC CAT GGG CCA TCA TCA TCA TCA TCA TGG CAA AGT TCA AAA GGG  
TGA TTT A  
Nco I/ Not I  
P: GGC CAT GGG CCA TCA TCA TCA TCA TCA TGG CAA AGT TCA AAA GGG  
TGA TTT A  
Nco I/ Not I | pET33b(+) |
| **rPleCHKD**             | F: GGC ATA TGG GTC ACC ATC ACC ATC ACC ATG GAA AAA ATG CAA AAA  
TGC TAA GTA AGC  
Nde I/ BamH I  
P: GGC ATA TGG GTC ACC ATC ACC ATC ACC ATG GAA AAA ATG CAA AAA  
TGC TAA GTA AGC  
Nde I/ BamH I | pET11a |
| **rCtrA**                | F: CGG GAT CCG ATG ATG CTT TTA TTA ATA GAA G  
R: CGG CCG CCG CTA CCT CTC AAC TAT ATT ATG C  
BamH I/ Not I  
P: CGG GAT CCG ATG ATG CTT TTA TTA ATA GAA G  
R: CGG CCG CCG CTA CCT CTC AAC TAT ATT ATG C  
BamH I/ Not I | pET33b(+) |
| **rNtrX**                | F: CGG GAT CCA ATG GCA CAG AAT TTT GAA ATG  
R: CGG CCG CCG CGT TAC TCA GAT ATA CTA CAC  
BamH I/ Not I  
P: CGG GAT CCA ATG GCA CAG AAT TTT GAA ATG  
R: CGG CCG CCG CGT TAC TCA GAT ATA CTA CAC  
BamH I/ Not I | pET33b(+) |
| **rPleD**                | F: CGG AGC TCC ATG ACT GCA AAA GTA TTA ATA G  
R: CCC TCG AGC TAT GAT AGA TAT GTG ACA AC  
Xho I/ Sst I  
P: CGG AGC TCC ATG ACT GCA AAA GTA TTA ATA G  
R: CCC TCG AGC TAT GAT AGA TAT GTG ACA AC  
Xho I/ Sst I | pET33b(+) |

Table 3.3 Oligonucleotides used for cloning and expression of TCSs in *E. chaffeensis*.

<sup>a</sup> Restriction site is underlined
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Table 3.4 Oligonucleotides used for gene-specific quantitative RT-PCR in *E. chaffeensis.*
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<th>PleC</th>
<th>PleD</th>
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<td>(0885)</td>
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Table 3.5 TCS homologues found in sequenced genomes of the order *Rickettsiales*.a

a GenBank accession numbers and gene locus tags (in parentheses) are given. Some genes were not annotated as TCS homologues. Percent amino acid sequence identities of the entire open reading frames are relative to the *Ehrlichia chaffeensis* genes. E value of < 10^-15 based on amino acid sequences was used as cut-off. b Percent amino acid sequence identities of the sensor domains. c Percent amino acid sequence identities of the histidine kinase and receiver domains. d Percent amino acid sequence identities of the histidine kinase domains.
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Table 3.6 Presence of genes predicted to be regulated by TCSs in sequenced genomes of the order *Rickettsiales*.\(^a\)

\(^a\) Some genes were not annotated as TCS-regulated homologues. E value of $< 10^{-15}$ based on amino acid sequences was used as cut-off. A plus sign indicates that a homologous gene was found whereas a minus sign indicates that no homologous gene was present.
Fig. 3.1 Structures of TCS proteins predicted to be encoded by *E. chaffeensis* and *A. phagocytophilum* genomes.

Names of bacteria with closely related protein sequences (and E-values) are shown beneath each protein diagram.
Fig. 3.1

**E. chaffeensis**

- **Sensor kinase**
  - CckA ECH0755, 95 kDa
    - (C. crescentus CckA, 3E-55)
  - NtrY ECH0299, 81 kDa
    - (A. caulinodans NtrY, 3E-90)
  - PleC ECH0885, 53 kDa
    - (C. crescentus PleC, 1E-48)

**Response Regulator**

- CtrA ECH1012, 30 kDa
  - (C. crescentus CtrA, 9E-33)
- NtrX ECH0339, 53 kDa
  - (A. caulinodans NtrX, 2E-96)
- PleD ECH0773, 52 kDa
  - (C. crescentus PleD, 2E-94)

**A. phagocytophilum**

- CckA APH0582, 91 kDa
  - (C. crescentus CckA, 2E-57)
- NtrY APH0136, 79 kDa
  - (A. caulinodans NtrY, 2E-66)
- PleC APH0944, 53 kDa
  - (C. crescentus PleC, 7E-43)

**Putative signal peptides**

- Transmembrane domain
- Sensor domain
- His kinase domain
- Phosphorylation site

H: histidine, D: aspartate
Fig. 3.2 Six genes encoding TCSs are transcribed by *E. chaffeensis* and *A. phagocytophilum* during infection of human leukocytes.

Total RNA was prepared from *E. chaffeensis*-infected THP-1 cells (80% infected cells) and *A. phagocytophilum*-infected HL-60 cells (80% infected cells). M, molecular size marker; D, positive control (chromosomal DNA was used as template) for the PCR reaction; + and – indicate the presence and absence of reverse transcriptase respectively. Genes and base pair sizes of amplified products are indicated.

A. Expression of three sensor kinases.

B. Expression of three response regulators.
Fig. 3.2

**E. chaffeensis**

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Fig. 3.3 The six TCS proteins are expressed by *E. chaffeensis* in THP-1 cells. The infected THP-1 cells at 2 days p.i. (50% infected cells) were double immunofluorescence labelled.

A. The following antisera were used: anti-*E. chaffeensis* (anti-dog IgG, red, left panels) and anti-rCckA, anti-rNtrY, anti-rPleC, anti-rCtrA, anti-rNtrX, anti-rPleD (anti-rabbit IgG, green, centre panels). The panels on the right are superimposed images viewed with green and red filters.

B–D. As controls for immunofluorescence labelling, *E. chaffeensis*-infected THP-1 cells were incubated (B) with the respective preimmune rabbit serum, (C) with the rabbit antiserum against an unrelated recombinant protein (rhvP44-18 of *A. phagocytophilum*) and secondary conjugated anti-rabbit IgG, or (D) with the preimmune dog serum and secondary conjugated anti-dog IgG. The scale bar is 5 μm.
Fig. 3.3 continued
Fig. 3.3 continued
Fig. 3.4 Closantel inhibits the autokinase activities of the three *E. chaffeensis* sensor kinases.

The recombinant histidine kinase domains, MBP-rCckAHKD, rNtrYHKD and rPleCHKD, were incubated with [γ-^{32}P]ATP in phosphorylation buffer in the presence of 100 μM of closantel (+) or 1% v/v DMSO (−) for 15 min at room temperature. Protein names and molecular weights are indicated. The phosphorylated proteins are designated by arrows.
Fig. 3.5 Closantel inhibits infection with *E. chaffeensis* and *A. phagocytophilum*.

A. Host cell-free *E. chaffeensis* or *A. phagocytophilum* were pretreated with closantel (10 or 100 μM) at 37°C for 20 min and used to infect THP-1 or HL-60 cells.

B. *E. chaffeensis* or *A. phagocytophilum* infected cells were treated with closantel (20 or 50 μM) at 37°C for 20 min at 1 day p.i.. DMSO (1% v/v) was used as control. Numbers of bacteria present in 100 host cells at 3 days p.i. are shown.
A. Pretreatment of bacteria at 37°C for 20 min

**E. chaffeensis**

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**A. phagocytophilum**

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continued
B. Treatment of infected cells at 1 day p.i.

**E. chaffeensis**

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**A. phagocytophilum**

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Fig. 3.6 Closantel down-regulates the transcription of TCS genes and genes predicted to be regulated by TCSs.

A. *E. chaffeensis*-infected THP-1 cells were treated with closantel (100 μM) for 5, 15, 30 min or with DMSO (1% v/v) for 30 min as a control.

B. *E. chaffeensis*-infected THP-1 cells were treated with closantel (100 μM) for 30, 60, 120 min or with DMSO (1% v/v) for 120 min as a control. The level of gene expression was determined using RT-PCR. D indicates positive control (chromosomal DNA was used as template) for the PCR reaction; + and – indicate the presence and absence of reverse transcriptase respectively. Genes are identified on the left. Base pair sizes of amplified products are indicated on the right.

C. Rapid reduction of mRNAs of *cckA*, *ntrY* and *pleC* was observed following closantel treatment as determined by quantitative RT-PCR. The results are expressed as the percentage of sensor kinase transcript levels after 5 min of closantel treatment relative to those in the DMSO control. The values are the means ± standard deviations (n = 3). The results with an asterisk are significantly (P < 0.01) different compared with DMSO control as shown by the Student’s t-test.
Fig. 3.6 continued
Fig. 3.6 continued

**16S rRNA**

- Closantel 5min
- DMSO control

**cckA**

- Closantel 5min
- DMSO control

**ntrY**

- Closantel 5min
- DMSO control

**pleC**

- Closantel 5min
- DMSO control
CHAPTER 4

ROLES OF CTRA IN EHRlichia Chaffeensis intracellular DEVELOPMENT

4.1 Abstract

*Ehrlichia chaffeensis*, an obligatory intracellular bacterium of human monocytes-macrophages, causes human monocytic ehrlichiosis. How *E. chaffeensis* intracellular replication is coordinated with intracellular development and extracellular release is poorly understood. *Caulobacter crescentus* CtrA (cell cycle transcription regulator A) is a transcriptional regulator that allows the coordination of cell cycle progression and morphogenesis by controlling the expression levels of ~100 genes. We examined temporal expressions of *E. chaffeensis* CtrA homolog transcript and protein during *E. chaffeensis* intracellular development in human acute leukemia THP-1 cells. CtrA mRNA and protein levels were down-regulated within 6 h post infection in synchronously infected cell cultures, and significantly upregulated at the late stage of infection prior to the host cell lysis. *C. crescentus* CtrA binding motif (CtrA box) was predicted in the upstream regions of more than 30 *E. chaffeensis* genes. Temporal mRNA expressions of
these genes were divided into four patterns: 1) up-regulated at the late exponential phase, similar to CtrA, 2) up-regulated at the lag phase and down-regulated during the exponential phase, 3) up-regulated at the lag phase but not down-regulated during the exponential phase, 4) constitutively expressed. Electrophoretic mobility shift assay showed specific binding of recombinant *E. chaffeensis* CtrA to the promoter regions of *E. chaffeensis* *ctrA* and *surE*. rCtrA transactivated *surE* promoter-*lacZ* construct in the *lacZ* reporter assay. These results suggest that CtrA is a global regulator controlling the intracellular development of *E. chaffeensis*.

4.2 Introduction

Human monocytic ehrlichiosis (HME) caused by *Ehrlichia chaffeensis* is an emerging life-threatening infectious disease. HME is a moderate to severe illness with approximately 40–60% of patients requiring hospitalization. The estimated case-fatality rate for HME is approximately 3%. Death is generally attributed to multisystem organ failure, respiratory failure, catastrophic haemorrhage, or secondary bacterial or fungal infections (Paddock and Childs, 2003).

*E. chaffeensis* is an obligatory intracellular bacterium that resides in early endosomes of mononuclear phagocytes (Mott et al., 1999). The life cycle of *E. chaffeensis* involves a tick vector and a mammalian host.

Electron microscopy showed that *E. chaffeensis* has two forms: the dense-cored cells (DCs) (0.4–0.6 μm) and reticulate cells (RCs) (0.4–0.6 μm by 0.7–1.9) residing within the intracellular parasitophorous vacuoles (morulae) in mammalian host cells. DC is smaller than RC and has a dense nucleoid. In contrast, RC has uniformly dispersed
nucleoid filaments and ribosomes, sometimes forms long projections of the cell wall, protrusions of the cytoplasmic membrane into the periplasmic space, or budding protoplast fragments into the periplasmic space (Popov et al., 1995). Both RC and DC were believed to divide through binary fission, which contrasts to *Chlamydia*, in which only the reticulate body replicates. The developmental cycle of *E. chaffeensis* has been determined in DH82 cells (Zhang et al., 2007). How *E. chaffeensis* intracellular replication is coordinated with intracellular development and extracellular release is poorly understood. During the bacterial intracellular life cycle in THP-1 cells, type IV secretion apparatus genes are differentially expressed and regulated by a DNA binding protein, EcXR (Cheng et al., 2008). However, there is no study on the regulation of other genes that might be involved in intracellular development of *E. chaffeensis*.

The bacterium *Caulobacter crescentus* is a model useful for examining cell cycle regulation in bacteria (Skerker and Laub, 2004). Each cell division in *Caulobacter* is asymmetric and produces daughter cells—stalked and swarmer cells—that are committed to different stages of the cell cycle. The master regulator of the *Caulobacter* cell cycle is CtrA, an essential response regulator whose activity as a transcription factor varies as a function of the cell cycle (Domian et al., 1997; Quon et al., 1996). At the early stage of cell cycle, CtrA binds and silences the origin of replication (Quon et al., 1998). Then CtrA is degraded by protease to free the origin, which permits the initiation of DNA replication (McGrath et al., 2006; Domian et al., 1997). At the late stage, the initial synthesis of CtrA leads to positive transcriptional autoregulation and a burst of CtrA synthesis (Domian et al., 1999). The newly synthesized CtrA drives the expression of
more than 95 genes, many of which are required for completing the cell cycle (Laub et al., 2002; Laub et al., 2000).

In *E. chaffeensis*, CtrA homolog has been found, and the phosphorylation of CtrA by its cognate histidine kinase, CekA has been determined (Cheng et al., 2006; Kumagai et al., 2006). Here we examined temporal expressions of *E. chaffeensis* CtrA homolog transcript and protein during *E. chaffeensis* intracellular development in host cells. *C. crescentus* CtrA binding motif (CtrA box) was determined by Excel and pDRAW (Acaclone software) in the upstream regions of more than 30 *E. chaffeensis* genes. Electrophoretic mobility shift assay showed specific binding of recombinant *E. chaffeensis* CtrA to the promoter regions of downstream genes. CtrA was determined as a transactivator by the *lacZ* reporter assay. This work lays the foundation for the identification of the direct interactions in the transcriptional network governing cell cycle progression in an obligatory intracellular bacterium.

4.3 Materials and methods

Bacterial strains and culture

*E. chaffeensis* Arkansas (Anderson et al., 1991) was propagated in THP-1 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% L-glutamine at 37°C in 5% CO₂ and 95% air. *Escherichia coli* strains NovaBlue (Novagen, San Diego, CA), BL21(DE3) (Novagen), JM109 (Stratagen, La Jolla, CA) and DH5α (Invitrogen, Carlsbad, CA) used for DNA cloning and protein expression were cultured in LB broth.
Synchronous culture of *E. chaffeensis*.

The heavily infected (>90%) THP-1 cells (8 × 10^7 cells) were harvested by centrifugation at 2,000 × g for 5 min. The pellet was resuspended in 8 ml culture medium and sonicated on ice twice at setting 2 for 10 s using a W-380 Sonicator (Heat systems-Ultrasonics, Farmingdale, NY), and the unbroken cells and cell debris were removed by centrifugation at 4,000 × g for 5 min. Host cell–free bacteria in the medium were sonicated on ice twice at setting 4.5 for 30 s to disrupt the fragile bacterial RCs. The sonication-resistant bacterial DCs were harvested by centrifugation at 18,000 × g for 5 min at 4°C. To perform a synchronous infection, the DC pellet was resuspended in 10 ml culture medium and incubated with 4 × 10^7 uninfected THP-1 cells at 37°C for 1 h, shaking every 10 min. The mixture was then washed with cold 2 × phosphate buffered saline (2 × PBS: 274 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.4) three times and incubated at 37°C. Samples were collected at this time point (0 h post infection [p.i.]) and at 0, 24, 48 and 72 h p.i. by centrifugation at 2,000 × g for 5 min.

Identification of putative CtrA binding sites in *E. chaffeensis* genomes.

Whole genomic sequence of *E. chaffeensis* and information of the open reading frames (ORFs) in the genome were obtained from NCBI ftp site. The process to identify putative CtrA-binding sites was performed in Microsoft Excel using some formulas and original programs in Visual Basic for Application (VBA). DNA sequence of 400 bp upstream of each ORF was extracted from the genome as a putative promoter region by programs in VBA. Sequence matching CtrA-binding sites, TTAAN_7TTAAC and
TTAACCAT, completely or with one mismatch was searched in each putative promoter sequence using a "search" formula.

Quantitative RT-PCR.

THP-1 cells synchronously infected with *E. chaffeensis* were harvested at 0, 24, 48 and 72 h p.i. as described above. One-half of the cells was suspended in RNAlater (Qiagen, Valencia, CA) and stored at −20°C for RNA extraction. The remaining cells were kept at −80°C for DNA extraction. Total DNA and RNA were extracted, and the RNA was reverse transcribed as described (Cheng *et al.*, 2006). Samples lacking reverse transcriptase were used to assess DNA contamination for each reaction. Quantitative PCR was performed as described (Cheng *et al.*, 2006). Briefly, gene-specific primers were designed to produce amplicons of 100 to 150 bp (primer sequences are shown in Table 4.1). Serially diluted bacterial chromosomal DNA containing known copy numbers of the target genes was used as a standard. Quantitative PCR was performed using a Mx3000P instrument and the Brilliant SYBR Green QPCR Core Reagent kit (both from Stratagene, La Jolla, CA). For each quantitative PCR assay, the dissociation curve was examined to confirm the absence of primer dimers. Log chromosomal DNA versus cycle threshold (C_T) was plotted to establish standard curves for each gene. Means and standard deviations of mRNA copy numbers were determined.

Double-immunofluorescence labeling

Rabbit antibodies were produced against the purified recombinant CtrA at ProSci (Poway, CA). All antisera used were preabsorbed with uninfected THP-1 cells. At 0 and
6 h p.i., and the late infection stage, the *E. chaffeensis*-infected THP-1 cells were cytocentrifuged on a glass slide and fixed by cold methanol at -20°C for 5 min, then incubated with rabbit antiserum against CtrA and dog antiserum against *E. chaffeensis* at a 1: 100 dilution in 1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) at 37°C for 1 h. Lastly, the cells were incubated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-dog IgG) at a 1:200 dilution in 1 × PBS at 37°C for 1 h. Cells were analyzed by a Nikon Eclipse E400 fluorescence microscope with a xenon–mercury light source (Nikon Instruments Inc., Melville, NY).

Western blot analysis

The same amounts of *E. chaffeensis*, normalized according to 16S rDNA copy numbers, from different time points p.i. were suspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5% 2-mercaptoethanol), boiled for 3 min then loaded onto 12% SDS-PAGE gel. Following separation, proteins were transferred to a nitrocellulose membrane. The membrane was incubated with rabbit antiserum against CtrA, preabsorbed with THP-1 cells, at a 1: 1000 dilution at room temperature for 3 h, followed by incubation of secondary antibody, horseradish peroxidase-conjugated goat anti rabbit IgG (KPL, Gaithersburg, MA), at a 1:1000 dilution at room temperature for 3 h. The membrane was incubated with ECL western blotting detection reagents (Amersham, Piscataway, NJ) and bands were visualized with an LAS-3000 luminescent image analyzer (Fujifilm, Stamford, CT).
Construction of plasmids for expression of the full-length CtrA

*E. chaffeensis* chromosomal DNA was extracted from infected THP-1 cells using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. The DNA fragment encoding the entire length of CtrA was amplified by PCR with primers shown in Table 4.2 using chromosomal DNA as a template. The PCR product was purified with the PCR Purification Kit (Stratagene). The amplified DNA was digested with restriction enzymes and ligated into the restriction enzyme-digested pET33b(+) (Novagen) vector. After *E. coli* strain NovaBlue cells were transformed, plasmids were extracted from each colony using Strataprep Plasmid Miniprep Kit (Stratagene) and the sequence of the cloned fragments was confirmed by DNA sequencing. *E. coli* strain BL21 (DE3) cells were transformed with the recombinant plasmids and induced to express the recombinant protein with isopropyl-thio-β-D-galactoside (IPTG).

For MBP fusion protein, the entire length of CtrA fragment was digested and ligated to pMAL-c2X vector (New England Biolabs). The plasmid was amplified in *E. coli* strain JM109 cells. The recombinant protein was expressed in *E. coli* DH5α cells.

Recombinant protein purification

The *E. coli* cells were harvested by centrifugation at 8,000 × g for 5 min and suspended in binding buffer (50 mM sodium phosphate, pH 8.0, 0.3M NaCl). The suspensions were sonicated and centrifuged at 18,000 × g for 10 min at 4°C. The supernatants were loaded onto an amylase column equilibrated with binding buffer. The column was washed with binding buffer twice. The proteins were eluted with elution
buffer (10 mM maltose in binding buffer). The purified proteins were dialyzed in EMSA buffer (10 mM Tris-HCl, pH 7.5, 1 mM DTT).

Electrophoretic mobility shift assay (EMSA).

DNA fragments of 448 bp and 430 bp in length, corresponding to sequences upstream of the start codons of \textit{ctrA} and \textit{surE} respectively, were amplified by PCR. The primers are shown in Table 4.3. PCR product was biotinylated. The purified MBP-rCtrA (2 μg) were incubated with DNA probe (0.1 pmol) for 30 min at 4°C in a 20-μl reaction containing 10 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl2, 1 mM DTT, 2.5% (v/v) glycerol, 0.1% (v/v) NP-40, and 50 ng/μl salmon sperm DNA. As controls for binding specificity, a separate reaction using MBP protein or containing the above components plus a 50-fold excess of the corresponding unlabeled DNA probe was prepared. Samples were loaded onto a 5% native polyacrylamide gel in 0.5 × Tris-borate-EDTA (0.5 × TBE) buffer (0.044 M Tris base, 0.044 M boric acid, and 0.001 M EDTA pH 8.0) that had been pre-run for 1 h, electrophoresed at 100 V for 2.5 h at 4°C and then transferred to a nylon membrane (Amersham Biosciences, Piscataway, NJ) at 380 mA for 1 hour. The transferred DNA was cross-linked to the membrane with UV light. The biotinylated DNA was detected using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL).

Construction of \textit{lacZ} reporter fusions

The \textit{lacZ} fusion was constructed as described (Wang \textit{et al.}, 2007a). Briefly, DNA fragment upstream of \textit{surE} gene was amplified by PCR. PCR Primers are shown in Table 4.2. The transcriptional fusion was constructed by placing the promoter fragment
upstream of the promoterless lacZ gene in pACYC184 (New England Biolabs, Ipswich, MA). BL21 (DE3) was co-transformed with pET33b(+) expressing rCtrA and the lacZ reporter construct. pET33b(+) expressing an unrelated protein, rNtrY, was used as a negative control (Kumagai et al., 2006). After overnight culture, transformants were subcultured in LB medium supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol at 37ºC for 2 h followed by induction with 0.0625 mM IPTG for 2 h. β-galactosidase activity was measured as described (Wang et al., 2007a). Recombinant protein expressions were determined by western blot analysis using anti-His-tag antibody as described (Kumagai et al., 2006).

Statistical analysis.

Statistical analyses were performed using analysis of variance and Tukey Honestly Significant Differences test or Student’s t test, and P < 0.01 was considered significant.

4.4 Results

Temporal expression of CtrA.

By quantitative RT-PCR, we examined the expression level of ctrA gene in synchronized E. chaffeensis-infected THP-1 cells. The expression of ctrA mRNA was down-regulated during the lag phase and up-regulated at the late exponential phase, when bacteria were ready to be released and start the next round of infection (Fig. 4.1 A and B). After being normalized by bacterial numbers as determined by quantitative PCR, western blot analysis also showed that CtrA protein was highly expressed at the late exponential
phase. Also a weak band could be detected at 0 h p.i., which reduced to undetectable level at 24 h p.i. (Fig 4.1 C).

Double immunofluorescence labeling showed that CtrA was degraded rapidly within 6 h p.i. (Fig. 4.2 A). At the late infection stage, CtrA protein was highly expressed in intact inclusion. After the inclusion broke, CtrA level began to reduce. When the bacteria were released from the infected cells, CtrA reduced to an undetectable level (Fig. 4.2 B).

These results suggest that the amount of CtrA protein is controlled in two different levels. After bacteria are released from the host cells and prepared for the next round of infection, the existed CtrA protein is removed. And at the same time, the expression of ctrA mRNA is down-regulated, which will reduce the production of CtrA.

Temporal expression of putative CtrA downstream genes

The consensus sequence of CtrA binding sites (CtrA box) has been identified in C. crescentus (Hallez et al., 2004). After we searched in E. chaffeensis genome calculating by Microsoft Excel and using pDRAW (Acaclone software), we predicted at least 35 genes that are members of the CtrA cell cycle regulon. The functions of 15 genes are unknown. 19 genes are annotated and predicted to function in a wide range of cellular processes involved in essential cell cycle processes and bacterial survival. We selected the 19 annotated genes for further study. The names of these genes and the positions of CtrA box are shown in Table 4.4.

By quantitative RT-PCR, we examined the expression patterns of these genes in synchronized E. chaffeensis infected THP1-cells. All these genes could be divided into
four groups. Group A includes Ech0462 (ompA), Ech0929, Ech0930 (bolA) and surE genes, whose expressions are up-regulated at the late exponential phase and down-regulated at the lag phase and early exponential phase. Group B includes Ech0628, Ech0689, gshA, gshB, secA, secB, secD and trmU, whose expressions are up-regulated at the lag phase and down-regulated during the exponential phase. Group C includes Ech0721, Ech0944, recO and fitsZ, whose expressions are up-regulated at the lag phase, but not down-regulated during the exponential phase. Group D includes prfB and mfd, whose expressions are constitutive (Fig. 4.3).

CtrA binds to the promoter regions of the putative CtrA downstream genes

To perform EMSA to confirm CtrA binding to the CtrA Box upstream of the putative CtrA downstream genes, the plasmid expressing recombinant CtrA was constructed. However rCtrA was insoluble, so we constructed plasmid expressing maltose binding protein (MBP)-fusion CtrA. The protein was purified and the purity of the protein was examined by SDS-PAGE (Fig. 4.4). After the incubation of the biotinylated ctrA promoter region (448 bp) and surE promoter region (430 bp) with the purified protein, the shifted bands were detected (Fig. 4.5 A and B). The specificity of the binding was confirmed by adding 50-fold unlabeled DNA fragment or using MBP protein as negative controls.

rCtrA activates lacZ reporter fusions

Because CtrA bound to DNA probe derived from sequence upstream of surE gene, we examined whether this region could be transactivated by CtrA. The lacZ reporter
fusion was constructed by inserting the surE gene promoter fragment upstream of the translation start site of the promoterless lacZ gene in pACYC184. The lacZ reporter construct was transformed into E. coli BL21 (DE3) containing either pET33b(+) expressing rCtrA or pET33b(+) expressing an unrelated protein, rNtrY, as a negative control. For the reporter constructs, IPTG induction resulted in a significant increase in β-galactosidase activity compared to samples lacking IPTG or compared to IPTG induction of the pET33b(+) expressing rNtrY (Fig. 4.6). The expressions of rCtrA and rNtrY upon IPTG induction were confirmed by western blot analysis (Fig. 4.6).

4.5 Discussion

In the present study, we found 35 putative downstream genes regulated by CtrA, and examined the expression patterns of the 19 genes in synchronized E. chaffeensis-infected THP1-cells and found that the genes could be divided into four groups according to their different expression patterns. E. chaffeensis was shown to have biphasic intracellular growth to adapt its complicated life cycle, and several proteins were shown to be differentially expressed during intracellular growth (Cheng et al., 2008; Kumagai et al., 2008; Zhang et al., 2007). Genes encoding type IV secretion system are up-regulated at the end of lag phase and down-regulated during stationary phase prior to rerelease, which are regulated by a newly indentified global regulator, EcxR (Cheng et al., 2008). However the mechanism to regulate the bacterial intracellular developments has not been illustrated. In this study, for the first time, we demonstrated a global transcriptional regulator, CtrA, in E. chaffeensis. Unlike EcxR, CtrA regulates at least four sets of genes, which are expressed in different patterns. These results suggest that in E. chaffeensis
CtrA regulates the bacterial intracellular life cycle by activating or repressing the expressions of specific sets of genes at different stages of development. How CtrA activates or represses specific genes but not other genes is still unknown. In *C. crescentus*, the mechanisms of CtrA regulation include the position of CtrA box, the concentration of CtrA protein, the level of CtrA phosphorylation by its cognate CckA and DNA methylation (McAdams and Shapiro, 2003). In *E. chaffeensis*, it has been demonstrated that CtrA could be phosphorylated by CckA (Kumagai *et al.*, 2006). In this study, we found that CtrA was quickly removed after internalization and highly expressed at the late exponential phase. In *C. crescentus*, CtrA is digested by protease ClpXP (Jenal and Fuchs, 1998) ClpP and ClpX have been found in the genome of *E. chaffeensis* (Hotopp *et al.*, 2006), which suggests that CtrA degradation maybe involve proteolysis by ClpXP. The position and sequence of CtrA boxes in the promoter regions of the downstream genes may play an important role in CtrA regulation.

In *C. crescentus*, CtrA regulates the expression of at least 95 genes (Laub *et al.*, 2002). Unlike *C. crescentus*, which is a free-living bacterium, *E. chaffeensis* is an obligatory intracellular bacterium, which faces more complicated environments, and then develops unique mechanisms to initiate infection and survive in host cells (Rikihisa, 2006; Rikihisa, 2003). Among the 19 downstream genes predicted in *E. chaffeensis*, only two cell cycle regulation genes, *ftsZ* and *ctrA*, are reported to be controlled by CtrA in *C. crescentus*. The other 17 genes were predicted to be regulated by CtrA for the first time. The expressions of these genes coordinate with the expression of cell cycle regulation genes, such as *ctrA* and *ftsZ*, suggesting that these genes play important roles in the intracellular development associating with the bacterial infection and survival.
Group A includes Ech0462 (*ompA*), Ech0929, Ech0930 (*bolA*) and *surE* genes. All these genes are down-regulated at the lag phase and early exponential phase, but highly up-regulated at the late exponential phase, which suggests all these proteins play important roles in the bacterial survival outside of the host cells and the initiation of the bacterial infection. OmpA protein and the protein encoded by Ech0929 are lipoproteins. Bacterial lipoproteins are known to induce proinflammatory cytokines and to be involved in the pathogenesis of various bacterial infections (Sha *et al.*, 2008; Sander *et al.*, 2004). In *E. chaffeensis*, these two proteins may be essential for the survival in the harsh environment outside of the host cells and the binding to new host cells to start the next round of infection. In *E. coli*, the BolA protein is essential for a shift in cell morphology upon entry to stationary phase in minimal medium or in response to sudden carbon starvation. Furthermore, *E. coli* bolA is induced by several stresses, including low pH, high salinity and high temperature (Santos *et al.*, 1999), and plays a role in *E. coli* biofilm formation (Vieira *et al.*, 2004). In *E. coli*, SurE plays a significant physiological role in stress-response, which is highly expressed in the stationary phase (Mura *et al.*, 2003). These two proteins maybe are important for the bacterial survival in the heavy infected host cells, which are lack of nutrition, and the transformation from reticulate cells into the infectious dense-cored cells.

Group B includes Ech0628, Ech0689, *gshA*, *gshB*, *secA*, *secB*, *secD* and *trmU*. The expressions of these genes are up-regulated at the end of lag phase and down-regulated during the exponential phase, which suggests that these proteins play important roles in the establishment of replication inclusion and the initiation of bacterial replication. Ech0628 encodes iron-sulfur cofactor synthesis protein, and Ech0689
encodes iron-sulfur cluster assembly accessory protein. Iron-sulfur protein plays a key role in energy generation and cell carbon synthesis by anaerobic microbes (Stich et al., 2006). In *E. chaffeensis*, these two genes maybe are involved in the cell rapid growth and division during the exponential phase. The tripeptide glutathione-glutamyl-L-cysteinylglycine (GSH), an antioxidant, is used by many bacteria to protect themselves against oxidant damage. And GshA and GshB are two enzymes to synthesis GSH from glutamate (Harrison et al., 2005). *E. chaffeensis* infects macrophages and monocytes, in which it will face the attack from reactive oxygen species (ROS). *E. chaffeensis* actively blocks $O_2^-$ generation by monocytes and causes the rapid degradation of p22phox, a component of NADPH oxidase (Lin and Rikihisa, 2007). It is possible that *E. chaffeensis* utilizes GSH to protect itself at the beginning of infection before ROS decreases. In *E. coli*, Sec proteins form a translocation pore, along which the preproteins pass the cytoplasmic membrane. SecA is a ATPase, and SecB is cytosolic chaperon, which mediates protein translocation. SecY, SecE, SecG, SecD and SecF are interal membrane proteins (Driessen et al., 1998). In *E. chaffeensis* genome, homologs of all these genes are found. In the promoter regions of secA, secB and secD genes, CtrA boxes are found. Our results suggest that *E. chaffeensis* secrets proteins or integrates proteins into membrane to establish a haven for its survival and replication, and this system is regulated by CtrA. tRNA-methyltransferase, has been shown to be required for optimal growth of the Gram-negative organisms *E. coli* and *S. enterica* serovar Typhimurium (Bjork and Nilsson, 2003; Persson et al., 1995). In *E. chaffeensis*, TrmU is highly up-regulated at the beginning of the exponential phase, which suggests that it plays an important role in the cell rapid growth and division.
Group C includes Ech0721, Ech0944, recO and fisZ. The expressions of these genes are up-regulated at the lag phase, but not down-regulated during the exponential phase, which suggests that these proteins play important roles in the bacterial survival in the host cells and the bacterial replication. Ech0721 also encodes a lipoprotein, which is not homologous to the protein encoded by Ech0929 or Ech0462. The high expression level of Ech0721 maybe associates with the cell replication. Ech0944 encodes Na\(^+\)/H\(^+\) ion antiporter family protein. In *E. coli*, NhaA is a Na\(^+\)/H\(^+\) ion antiporter protein and plays a significant role in the regulation of pH and Na\(^+\) concentrations in cells (Counillon and Pouyssegur, 2000). The replication inclusions of *E. chaffeensis* are early endosomes which are slightly acidic (Mott et al., 1999). The Na\(^+\)/H\(^+\) ion antiporter family protein may contribute the bacterial survival in this inclusion. In *E. coli*, RecO is required for RecA-dependent recombinational repair (Kidane et al., 2004). In *A. phagocytophilum*, which is very close to *E. chaffeensis*, recombinational repair is required for the expression of surface protein P44 (Lin et al., 2006). In *E. coli*, FtsZ is a tubulin-like GTPase that polymerizes and forms a cytokinetic ring associated with the cytoplasmic membrane at the site of cell division in bacteria (Bi and Lutkenhaus, 1991). FtsZ is an essential determinant of the timing and the localization of cell division (Rothfield and Justice, 1997).

Group D includes prfB and mfd, which are constitutively expressed during cell cycle. *prfB* encodes release factor 2 in *E. coli*, which recognizes the stop codons, UGA and UAA (Rengby and Arner, 2007). *mfd* encodes Mfd in *E. coli*, which is an ATP-dependent transcription-repair coupling factor that removes stalled RNA polymerase.
from damaged DNA, and interacts with the nucleotide excision repair protein (Savery, 2007). These two proteins are constantly required during the cell cycle.

In summary, these temporal expression patterns reveal that cell cycle-regulated genes are maximally induced immediately before or coincident with the time of execution of the cell cycle events in which they participate, and genes that encode subunits of molecular complexes or that function in the same cellular process are coexpressed. And this kind of differential expression is regulated by the differential expression level of CtrA.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’→3’)</th>
<th>Target (bp)</th>
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| ctrA   | F: TGCACTGTGCAAAAGGCAGTAG  
R: ACATCATAGTCATCATTTCTTCA  | 117         |
| Ech0462| F: CTAAGTCATGTTCCATTGG  
R: GATACTCTCTGCCTTTTGCA  | 150         |
| Ech0929| F: GAAGTTGTAGTACGCTCAGA  
R: GAGACTAGCCAGAGGTACCA  | 104         |
| Ech0930| F: CCATTCCATCTCCAAATATTTCA  
R: GAGCTTGTTGTACTCTCTCTC  | 172         |
| surE   | F: GCAGCAGGTATGGCATACAGA  
R: TAGGATGAGTACTGTTTACAA | 145         |
| Ech0628| F: GCCATATAGATATAGACGACGA  
R: CAGTAACTCTCTCAACACTGA  | 140         |
| Ech0689| F: GTCAAACCTAAAGAGCTCAGAC  
R: TGAAATCCCTGCACAGATTTCA | 135         |
| gshA   | F: CGGCAGTATTAAAGGTAGTACAGA  
R: GCTACAGTTTCTGAGATTTA  | 145         |
| gshB   | F: ATGGCAGAACGAgCTGAGAAGA  
R: CAAATGAGGTTTTTGACGCA  | 115         |
| secA   | F: AGTACCACTAGCTGCAAGATAG  
R: GTTTGAGGATATACAGCTCA  | 153         |
| secD   | F: AGATCTGCAAGGGTGTAGTTTA  
R: ATCCCTGCTCATCTCAGTTTA  | 145         |

Table 4.1 Oligonucleotide primers used for quantitative RT-PCR.

a F: forward; R: reverse.
Table 4.1 continued

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<th>Target (bp)</th>
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<td>R: ATAATGACCGGTAACTAGGATA</td>
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<tr>
<td>Ech0721</td>
<td>F: GCTTCAGTTGTTAGGATTAGCA</td>
<td>117</td>
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<tr>
<td></td>
<td>R: AGC ACAAGTAATAACTAAGCCA</td>
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<tr>
<td>rNtrY</td>
<td>F: GGCATGGCCCATCTC ATCTCATATGGCAAG TTCAATTGGGTGATTTA</td>
<td>Nco I</td>
</tr>
<tr>
<td></td>
<td>R: GCGGGCTCGATTGA AATATTTATGCGGCGC CCCC</td>
<td>Not I</td>
</tr>
<tr>
<td>MBP-rCtrA</td>
<td>F: CCGGATCCGATGCGTAT ATTATTAATAGAAG</td>
<td>BamH I</td>
</tr>
<tr>
<td></td>
<td>R: GGCTGCAGCTACCCCTCA ACTATATTATGC</td>
<td>Pst I</td>
</tr>
<tr>
<td>surE upstream region</td>
<td>F: GGGAAGCTTATGAGTAG TGATCCTGTGCAA</td>
<td>Hind III</td>
</tr>
<tr>
<td></td>
<td>R: CCGGATCCAGCCTTTC TATCCCTAATAATTAA</td>
<td>BamH I</td>
</tr>
</tbody>
</table>

Table 4.2 Oligonucleotides primers used to construct plasmids.

<sup>a</sup> Restriction site is underlined.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’→3’)</th>
<th>Target (bp)</th>
</tr>
</thead>
</table>
| *ctrA* | F: GAGTGTCATTAGAAGATATGG  
          R: AAAATACCTCTAAAAACCCCAATAA | 448         |
| *surE* | F: ATGAGTAGTGATCCTGTGCAA  
          R: AGCActTTATTCCTAATAATTA | 430         |

Table 4.3 Oligonucleotide primers used to amplify promoter regions of *ctrA* and *surE*. 
<table>
<thead>
<tr>
<th>locus</th>
<th>Gene</th>
<th>Protein name</th>
<th>From ATG (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ech1012</td>
<td><em>ctrA</em></td>
<td>DNA-binding response regulator CtrA</td>
<td>-312</td>
</tr>
<tr>
<td>Ech0462</td>
<td><em>ompA</em></td>
<td>OmpA family protein</td>
<td>-286</td>
</tr>
<tr>
<td>Ech0929</td>
<td></td>
<td>Putative lipoprotein</td>
<td>-113</td>
</tr>
<tr>
<td>Ech0930</td>
<td><em>bolA</em></td>
<td>Putative BolA protein</td>
<td>-55</td>
</tr>
<tr>
<td>Ech0791</td>
<td><em>surE</em></td>
<td>Stationary-phase survival protein SurE</td>
<td>-128</td>
</tr>
<tr>
<td>Ech0721</td>
<td></td>
<td>Putative lipoprotein</td>
<td>-36</td>
</tr>
<tr>
<td>Ech0944</td>
<td></td>
<td>Na+/H+ ion antiporter family protein</td>
<td>-181</td>
</tr>
<tr>
<td>Ech0536</td>
<td><em>recO</em></td>
<td>Putative DNA repair protein RecO</td>
<td>-131</td>
</tr>
<tr>
<td>Ech1153</td>
<td><em>fisZ</em></td>
<td>Cell division protein FtsZ</td>
<td>-231</td>
</tr>
<tr>
<td>Ech0628</td>
<td></td>
<td>Iron-sulfur cofactor synthesis protein</td>
<td>-192</td>
</tr>
<tr>
<td>Ech0689</td>
<td></td>
<td>Iron-sulfur cluster assembly accessory protein</td>
<td>-335</td>
</tr>
<tr>
<td>Ech0125</td>
<td><em>gshA</em></td>
<td>Glutamate-cysteine ligase</td>
<td>-217</td>
</tr>
<tr>
<td>Ech0336</td>
<td><em>gshB</em></td>
<td>Glutathione synthetase</td>
<td>-280</td>
</tr>
<tr>
<td>Ech1149</td>
<td><em>secA</em></td>
<td>Preprotein translocase</td>
<td>-361</td>
</tr>
<tr>
<td>Ech0233</td>
<td><em>secB</em></td>
<td>Protein-export protein</td>
<td>-46</td>
</tr>
<tr>
<td>Ech1106</td>
<td><em>secD</em></td>
<td>Protein-export membrane protein</td>
<td>-263</td>
</tr>
<tr>
<td>Ech0872</td>
<td><em>trmU</em></td>
<td>tRNA-methyltransferase</td>
<td>-292</td>
</tr>
<tr>
<td>Ech0705</td>
<td><em>prfB</em></td>
<td>Peptide chain release factor 2</td>
<td>-169</td>
</tr>
<tr>
<td>Ech0250</td>
<td><em>mfd</em></td>
<td>Transcription-repair coupling factor</td>
<td>-190</td>
</tr>
</tbody>
</table>

Table 4.4. Genes predicted to have CtrA box in the promoter region.
Fig. 4.1 Temporal expressions of \textit{ctrA} and CtrA protein by synchronously cultured \textit{E. chaffeensis}.

(A) DNA extracted from \textit{E. chaffeensis}-infected THP-1 cells at different time p.i. was subjected to quantitative RT-PCR analysis. Data points reflect the numbers of bacteria relative to the amount determined at 0 h p.i. The values represent the means ± standard deviations from three specimens. (B) mRNA prepared from infected THP-1 cells at different time p.i. was subjected to real-time PCR analysis. Transcript levels are relative to the amount of \textit{ctrA} transcript at 0 h p.i. The values represent the means ± standard deviations from three specimens. (C) Western blot analyses of samples from infected THP-1 cells at different time p.i. were performed using an anti-rCtrA rabbit antiserum to determine the expression of CtrA. All the samples are normalized by bacterial number.
Fig. 4.1

A

![Graph showing relative bacterial number over time](image)

B

![Bar graph showing relative ctrA mRNA/bacterium](image)

C

![Image of Western blot with 31kD marker](image)
Fig. 4.2 The differential expression of CtrA by *E. chaffeensis* in THP-1 cells.

The following antisera were used: anti-*E. chaffeensis* (red) and anti-rCtrA (green). The panels on the right are superimposed images viewed with green and red filters. The scale bars are 5 µm.

(A) The infected THP-1 cells at 0 h and 6 h p.i. were double immunofluorescence labeled. Individual bacterium is indicated by arrows. (B) The infected THP-1 cells at the stationary stage of growth were double immunofluorescence labeled. In the upper panel, the ruptured inclusion is indicated by an arrow, and the intact inclusion is indicated by an arrowhead. In the lower panel, cluster of releasing bacteria is indicated by an arrow.
Fig. 4.2

A

p.i. | Anti-Ec | Anti-rCtrA | Merge
---|--------|-----------|------
0 h | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png)
6 h | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png)

B

Anti-Ec | Anti-rCtrA | Merge
---|--------|------
![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png)

124
Fig. 4.3 The temporal expressions of putative CtrA downstream genes.

mRNA prepared from infected THP-1 cells at different time p.i. was subjected to quantitative RT-PCR analysis. All the genes are divided into four groups: group A (A), group B (B), group C (C) and group D (D). Transcript levels are relative to the amount of the same gene’s transcript at 0 h p.i.. The values represent the means ± standard deviations from three specimens.
Fig. 4.3

A

Ech0462

Ech0929

Ech0930

surE

continued
Fig. 4.3 continued
Fig. 4.3 continued

C

Ech0721

Ech0944

0h 24h 48h 72h

0h 24h 48h 72h

recO

ftsZ

D

prfB

mfd

0h 24h 48h 72h

0h 24h 48h 72h
Fig. 4.4 Purification of recombinant MBP-CtrA.

*E. chaffeensis* *ctrA* was cloned into the pMAL-c2X vector, expressed, and purified using amylose column chromatography. Purified protein was subjected to 12% SDS-PAGE analysis followed by Coomassie Brilliant blue staining (lane 1). MBP was also expressed and purified (lane 2). M, Prestained protein size standards. Each lane received 1 μg recombinant protein.
Fig. 4.5 Binding of recombinant MBP-CtrA to the biotinylated DNA probes derived from sequences upstream of \textit{ctrA} (A) and \textit{surE} (B).

For each panel: lane 1, DNA probe (0.1 pmol); lane 2, DNA probe incubated with MBP (25 ng); lane 3, DNA probe incubated with MBP-rCtrA (25 ng); lane 4, DNA probe incubated with MBP-rCtrA in the presence of a 50-fold excess of the corresponding unlabeled DNA probe. Shifted bands are indicated by arrowheads. Bands were visualized using the LightShift Chemiluminescent EMSA kit.
Fig. 4.6 CtrA activates the transcription of surE-lacZ reporter fusions.

β-galactosidase assays were used to measure the transcriptional activities of lacZ reporter constructs. The values reflect means ± standard deviations from three specimens. *, significantly different ($P < 0.01$) compared to samples lacking IPTG or compared to IPTG induction of the pET33b(+) vector expressing an unrelated protein, rNtrY, by the Tukey Honestly Significant Differences test. Western blot analyses of samples from the β-galactosidase assays were performed using an anti-His-tag antibody to verify the expression of rCtrA and rNtrX. A representative of three independent experiments is shown in the lower panels. rCtrA and rNtrY are indicated by arrows.
5.1 Abstract

Human monocytic ehrlichiosis (HME) is an emerging tick-borne zoonosis. The etiologic agent of HME is *Ehrlichia chaffeensis*, a Gram-negative, obligatory intracellular bacterium, which has tropism for monocytes/macrophages. This bacterium lacks pili, lipopolysaccharides, and peptidoglycans, suggesting that the outer membrane proteins play an important role in its interaction with host cells. In *E. chaffeensis*, lipoproteins are required for bacterial infection of host cells. One of the lipoproteins, homologous to OmpA (outer membrane protein A, also known as peptidoglycan-associated lipoprotein) is found in several bacteria. In this study, double-immunofluorescence labeling showed that OmpA protein was *E. chaffeensis* surface exposed. *E. chaffeensis* binding and infection were inhibited by rabbit anti-OmpA IgG. The temporal expression patterns of *ompA* gene and OmpA protein were determined by quantitative RT-PCR and western blot analysis, respectively. OmpA mRNA and protein expression were down-regulated during
the lag phase and early exponential phase, but highly up-regulated at the late exponential phase. A two-component system regulator, CtrA binding site was predicted in the promoter region of \textit{ompA} gene. EMSA and the \textit{lacZ} reporter assay confirmed that CtrA bound to the promoter region of \textit{ompA} gene and activated its expression. The results suggest that CtrA regulates \textit{ompA} expression, consequently, \textit{E. chaffeensis} infection of host cells.

**5.2 Introduction**

Human monocytic ehrlichiosis (HME) is an emerging tick-borne zoonosis (Demma \textit{et al.}, 2005). Since its discovery in 1986 (Paddock and Childs, 2003), HME has been increasingly diagnosed in the United States and other parts of the world (Demma \textit{et al.}, 2005; Paddock and Childs, 2003). HME is a systemic disease characterized by fever, headache, myalgia, anorexia, and chills, and frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevated serum hepatic aminotransferase levels. The etiologic agent of HME is \textit{Ehrlichia chaffeensis}, which belongs to the family \textit{Anaplasmataceae} (Dawson \textit{et al.}, 1991). In North America, the major vector of \textit{E. chaffeensis} is the Lone Star tick, \textit{Amblyomma americanum}, and the white-tailed deer is considered to be the major reservoir of \textit{E. chaffeensis} (Lockhart \textit{et al.}, 1997; Ewing \textit{et al.}, 1995).

\textit{E. chaffeensis} is a Gram-negative, obligatory intracellular bacterium which has tropism for monocytes/macrophages. The entry and proliferation of \textit{E. chaffeensis} involve host caveolae, glycosylphosphatidylinositol-anchored proteins, and incorporation of cholesterol into the bacterial membrane (Lin and Rikihisa, 2003a). However, bacterial
ligand which facilitates its binding, entry, and proliferation has not been identified. gp120, which is predicted to be a glycoprotein and contains glucose, galactose and xylose when expressed in *Escherichia coli* (McBride *et al.*, 2000), was identified as an adhesin to adhere to HeLa cells using *E. coli* expressing the entire gp120. A few *E. coli* expressing the gp120 were identified inside the host cells, suggesting that the gp120 may play a role in bacterial adhesion or invasion of the host cells (Popov *et al.*, 2000). Immunization with recombinant P28 (one of the major OMP-1/P28 family members) protected mice from *E. chaffeensis* challenge (Ohashi *et al.*, 1998). Monoclonal antibody against OMP-1g (P28) mediated protection of SCID mice from *E. chaffeensis* fatal infection (Li *et al.*, 2001; Winslow *et al.*, 2000). These studies suggest that OMP-1/P28 family members could be directly or indirectly involved in *E. chaffeensis* entry and proliferation in the host cells.

*E. chaffeensis* has 16 predicted lipoproteins (Huang *et al.*, 2008; Hotopp *et al.*, 2006). In *E. chaffeensis*, a globomycin-sensitive lipoprotein biogenesis pathway is present, and globomycin treatment inhibits *E. chaffeensis* infection in *vitro*, suggesting that lipoproteins are required for *E. chaffeensis* infection of host cells (Huang *et al.*, 2008). One of the lipoproteins, OmpA (outer membrane protein A) is a peptidoglycan-associated lipoprotein (PAL). PAL is highly conserved across Gram-negative bacterial species and involved in the stabilization of the outer membrane through a strong but non-covalent association with the peptidoglycan layer and interaction of N-terminal fatty acids with the outer membrane in *E. coli* (Cascales *et al.*, 2002). PAL interacts with murein through a C-terminal conserved peptidoglycan binding motif (NX$_2$LSX$_2$RGX$_2$VX$_3$L), which forms an $\alpha$-helix (Koebnik, 1995).
Expression of several *E. chaffeensis* genes encoding membrane proteins were shown to be regulated during bacterial intracellular development. Genes encoding type IV secretion apparatus is regulated by a new global transcriptional regulator, EcxR (Cheng *et al.*, 2008). Type IV secretion system plays important roles in bacterial intracellular survival and replication (Lin *et al.*, 2007; Niu *et al.*, 2006). Outer membrane proteins, OMP-1, P28 in *E. chaffeensis* are also differentially expressed (Kumagai *et al.*, 2008; Zhang *et al.*, 2007), although their regulation is unknown. OMP-1 and P28 have porin activities, which are important for the bacterial nutrient acquisition (Kumagai *et al.*, 2008). The temporal expression pattern of OmpA, and the mechanism regulating its expression have yet been determined.

CtrA is a global transcriptional regulator, which coordinates *E. chaffeensis* intracellular development (chapter 4). In *E. chaffeensis*, 19 genes including OmpA were predicted to be regulated by CtrA based on the promoter presence of the consensus sequence of CtrA binding site identified in *Caulobacter crescentus* (Hallez *et al.*, 2004). However the binding of CtrA to the predicted promoter region of *ompA* and CtrA regulation of *ompA* expression have not been demonstrated.

In the present study, we found the conserved peptidoglycan binding motif in *E. chaffeensis* OmpA, and determined the bacterial surface exposure of OmpA and the role of OmpA in bacterial binding to and infection of THP-1 cells. We further examined the temporal expression pattern of OmpA during *E. chaffeensis* intracellular growth, and the regulation of *ompA* expression by CtrA.
5.3 Materials and methods

Bacterial strains and culture

*E. chaffeensis* Arkansas (Anderson *et al.*, 1991) was propagated in THP-1 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% L-glutamine at 37°C in 5% CO₂ and 95% air. *E. coli* strains NovaBlue (Novagen, San Diego, CA), BL21(DE3) (Novagen) used for DNA cloning and protein expression were cultured in LB broth.

Synchronous culture of *E. chaffeensis*.

The heavily infected (>90%) THP-1 cells ($8 \times 10^7$ cells) were harvested by centrifugation at $2,000 \times g$ for 5 min. The pellet was resuspended in 8 ml culture medium and sonicated on ice twice at setting 2 for 10 s using a W-380 Sonicator (Heat systems-Ultrasonics, Farmingdale, NY), and the unbroken cells and cell debris were removed by centrifugation at $4,000 \times g$ for 5 min. Host cell–free bacteria in the medium were sonicated on ice twice at setting 4.5 for 30 s to disrupt the fragile bacterial RCs. The sonication-resistant bacterial DCs were harvested by centrifugation at $18,000 \times g$ for 5 min at 4°C. To perform a synchronous infection, the DC pellet was resuspended in 10 ml culture medium and incubated with $4 \times 10^7$ uninfected THP-1 cells at 37°C for 1 h, shaking every 10 min. The mixture was then washed with cold 2 × phosphate buffered saline (2 × PBS: 274 mM NaCl, 5.4 mM KCl, 20 mM Na₂HPO₄, 4 mM KH₂PO₄, pH 7.4) three times and incubated at 37°C. Samples were collected at this time point (0 h post infection [p.i.]) and at 0, 24, 48 and 72 h p.i. by centrifugation at $2,000 \times g$ for 5 min.
Quantitative RT-PCR.

THP-1 cells synchronously infected with *E. chaffeensis* were harvested at 0, 24, 48 and 72 h p.i. as described above. One-half of the cells was suspended in RNALater (Qiagen, Valencia, CA) and stored at −20°C for RNA extraction. The remaining cells were kept at −80°C for DNA extraction. Total DNA and RNA were extracted, and the RNA was reverse transcribed as described (Cheng *et al.*, 2006). Samples lacking reverse transcriptase were used to assess DNA contamination for each reaction. Quantitative PCR was performed as described (Cheng *et al.*, 2006). Briefly, gene-specific primers were designed to produce amplicons of 150 bp (F: CTACTGATCATGTGTTCCATTGG, R: GATACTCTTCTGCCTTTTGCA). Serially diluted bacterial chromosomal DNA containing known copy numbers of the target genes was used as a standard. Quantitative PCR was performed using a Mx3000P instrument and the Brilliant SYBR Green QPCR Core Reagent kit (both from Stratagene, La Jolla, CA). For quantitative PCR assay, the dissociation curve was examined to confirm the absence of primer dimers. Log chromosomal DNA versus cycle threshold (*C*ₚ) was plotted to establish standard curves for each gene. Means and standard deviations of mRNA copy numbers were determined.

Double-immunofluorescence labeling

Rabbit antibody was produced against a 14-mer peptide of *E. chaffeensis* OmpA (amino acids 81 to 94, HTDTRGTDEYNLEL; EZBiolab, Westfield, IN) (Huang *et al.*, 2008). The heavily infected (>90%) THP-1 cells (10⁷ cells) were harvested by centrifugation at 2,000 × g for 5 min. The pellet was resuspended in 6 ml culture medium and homogenized on ice, and the unbroken cells and cell debris were removed by
centrifugation at 4,000 × g for 5 min. Host cell–free bacteria in the medium were centrifuged and resuspend in 1 ml. 0.1 ml host cell-free bacteria was cytocentrifuged on glass slides. Organisms were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄; pH 7.2) for 1 h at room temperature and then incubated with rabbit against OmpA IgG, rabbit serum against recombinant *E. chaffeensis* PleD (an unrelated protein as a negative control) (Kumagai *et al.*, 2006), and pre-immune rabbit IgG for 1 h at 37°C. After being washed twice with 1 × PBS, the cells were incubated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG) at a 1:200 dilution in 1 × PBS at 37°C for 1 h. All antisera used were preabsorbed with uninfected THP-1 cells. Cells were analyzed by a Nikon Eclipse E400 fluorescence microscope with a xenon–mercury light source (Nikon Instruments Inc., Melville, NY).

Effects of anti-OmpA IgG on binding and infection in *vitro*.

For the binding analysis, the host cell-free *E. chaffeensis* was freshly prepared from 2.5 × 10⁴ infected THP-1 cells (>90% cells infected) as previously described. The preparation was then incubated with various concentrations (20, 100, 500 μg/ml) of either pre-immune rabbit IgG or rabbit anti-OmpA IgG, or RPMI 1640 medium at room temperature for 30 min with gentle shaking. Each mixture was then added to 2 × 10⁵ uninfected THP-1 cells (final concentration, 1.3 × 10⁶ cells/ml) in RPMI 1640 medium supplemented with 5% FBS and 2 mM L-glutamine. After incubation at room temperature for 15 min with shaking, each mixture was incubated at 37°C in 95% air-5% CO₂ for 45 min. Cells were harvested by centrifugation at 750 × g for 5 min and washed.
with 1 × PBS twice to remove the unbound bacteria. The mixture was cytocentrifuged and fixed with cold methanol for 5 min and sequentially incubated with dog anti-\textit{E. chaffeensis} serum and Texas Red-conjugated goat anti-dog IgG. The number of bound organisms was scored in 100 THP-1 cells in triplicate samples.

For analysis of infection, the host cell-free \textit{E. chaffeensis} freshly prepared from 4 × 10^5 cells (>90% cells infected) as previously described, was incubated with either pre-immune rabbit IgG or rabbit anti-OmpA IgG (500 μg/ml), or RPMI 1640 medium at room temperature for 30 min with gentle shaking. Each of the mixtures was added to 2 × 10^5 uninfected THP-1 cells (final concentration, 10^6 cells/ml) in RPMI 1640 medium supplemented with 5% FBS and 2 mM L-glutamine and incubated at 37°C in 95% air-5% CO_2. After 48 h, the infected cells were harvested by centrifuge. DNA from each sample was extracted. And the bacterial numbers were determined by quantitative PCR.

Construction of plasmid for expression of the full-length of OmpA

\textit{E. chaffeensis} chromosomal DNA was extracted from infected THP-1 cells using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. The DNA fragment encoding the entire length of OmpA was amplified by PCR with primers (F: CCCAAGCTTATGAAACATAAGTTGGTATTTATTA, R: CCCCTCGAGTCATTGTTCTGTGTTTATTAGAGG) using chromosomal DNA as a template. The PCR product was purified with the PCR Purification Kit (Stratagene). The amplified DNA was digested with restriction enzymes (Hind III and Xho I) and ligated into the restriction enzyme-digested pET33b(+) (Novagen) vector. After \textit{E. coli} strain NovaBlue cells were transformed, plasmids were extracted from each colony using
Strataprep Plasmid Miniprep Kit (Stratagene) and the sequence of the cloned fragment was confirmed by DNA sequencing. *E. coli* strain BL21 (DE3) cells were transformed with the recombinant plasmid and induced to express the recombinant protein with isopropyl-thio-β-D-galactoside (IPTG).

Western blot analysis

The same amounts of *E. chaffeensis*, normalized according to 16S rDNA copy numbers, from different time points p.i. were prepared as described in Chapter 4. After IPTG induction, the crude *E. coli* lysate was prepared by sonication. All samples were suspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5% 2-mercaptoethanol), boiled for 3 min then loaded onto 12% SDS-PAGE gel. Following separation, proteins were transferred to a PVDF membrane. (1) The membrane was incubated with rabbit anti-OmpA serum at a 1:1000 dilution at room temperature for 3 h, followed by incubation of secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MA), at a 1:1000 dilution at room temperature for 3 h. (2) The membrane was incubated with mouse monoclonal antibody against T7 tag (Invitrogen) at a 1:5000 dilution at room temperature for 3 h. (3) The membrane was incubated with the sera from HME human patient and experimental *E. chaffeensis* infected dog, at a 1:1000 dilution at room temperature for 3 h, followed by incubation of secondary antibody, horseradish peroxidase-conjugated goat anti-human IgG and goat anti-dog IgG (KPL), at a 1:1000 dilution at room temperature for 3 h. The membrane was incubated with ECL western
blotting detection reagents (Amersham, Piscataway, NJ) and bands were visualized with an LAS-3000 luminescent image analyzer (Fujifilm, Stamford, CT).

Electrophoretic mobility shift assay (EMSA).

DNA fragment of 368 bp in length, corresponding to sequence upstream of the start codon of *ompA*, was amplified by PCR (F: CACAAAAATAACTTGAATGCACG, R: AGTGCCCTCAATTGTGTTTAAACAA). PCR product was biotinylated. The purified MBP-rCtrA (2 μg) were incubated with DNA probe (0.1 pmol) for 30 min at 4°C in a 20-μl reaction containing 10 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 2.5% (v/v) glycerol, 0.1% (v/v) NP-40, and 50 ng/μl salmon sperm DNA. As controls for binding specificity, a separate reaction using MBP protein or containing the above components plus a 50-fold excess of the corresponding unlabeled DNA probe was prepared. Samples were loaded onto a 5% native polyacrylamide gel in 0.5 × Tris-borate-EDTA (0.5 × TBE) buffer (0.044 M Tris base, 0.044 M boric acid, and 0.001 M EDTA pH 8.0) that had been pre-run for 1 h, electrophoresed at 100 V for 2.5 h at 4°C and then transferred to a nylon membrane (Amersham Biosciences, Piscataway, NJ) at 380 mA for 1 hour. The transferred DNA was cross-linked to the membrane with UV light. The biotinylated DNA was detected using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL).

Construction of lacZ reporter fusions

The lacZ fusion was constructed as described (Wang et al., 2007a). Briefly, DNA fragment upstream of *ompA* gene was amplified by PCR (F:
The transcriptional fusion was constructed by placing the promoter fragment upstream of the promoterless lacZ gene in pACYC184 (New England Biolabs, Ipswich, MA). BL21 (DE3) was co-transformed with pET33b(+) expressing rCtrA and the lacZ reporter construct. pET33b(+) expressing an unrelated protein, rNtrY, was used as a negative control. After overnight culture, transformants were subcultured in LB medium supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol at 37°C for 2 h followed by induction with 0.0625 mM IPTG for 2 h. β-galactosidase activity was measured as described (Wang et al., 2007a). Recombinant protein expressions were determined by western blot analysis using anti-His-tag antibody as described (Kumagai et al., 2006).

Statistical analysis.

Statistical analysis was performed by one-way analysis of variance (ANOVA) or Tukey Honestly Significant Differences test, and \( P < 0.01 \) was considered significant.

5.4 Results

Predicted peptidoglycan binding motif in E. chaffeensis OmpA

The peptidoglycan binding motif (NX3LSX2RGX2VX3L) was found in E. chaffeensis OmpA (Fig. 5.1). The sequence of the motif is NLELGKQRANAVKDFIL, which is located between 91 and 107 aa. Compared to the peptidoglycan binding motif in E. coli Pal, (NISLGERRANAVKMYL), which is located between 97 and 112 aa (Cascales and Lloubes, 2004), the sequence is conserved except that there are 4 amino
acids between valine and leucine, instead of 3 amino acids. Amino acid sequences of 
OmpA are highly conserved in *Ehrlichia, Anaplasima, Rickettsia* and *Wolbachia* spp., 
although most of these genes have been annotated as Pal or Pal precursor (Table 5.1).

OmpA were exposed to the surface of host cell-free bacteria.

OmpA (PAL) is predicted to be an outer membrane lipoprotein by PSort (Huang 
*et al.*, 2008). In order to determine whether OmpA are exposed to the bacterial surface, 
host cell-free organisms were fixed with paraformaldehyde to prevent the penetration of 
the antibody into the organisms. Rabbit anti-OmpA IgG preferentially stained the surface 
of individual bacteria, resulting in a ring-like labeling pattern (Fig. 5.2A). Normal rabbit 
IgG did not label *E. chaffeensis* (Fig. 5.2C). A rabbit antibody directed against the 
recombinant *E. chaffeensis* PleD (a cytoplasmic protein) (Cheng *et al.*, 2006) did not 
label prefixed bacteria (Fig. 5.2D).

Temporal expression of OmpA.

By quantitative RT-PCR, the expression level of *ompA* gene by *E. chaffeensis* was 
examined in synchronously infected THP1-cells. The expression of *ompA* mRNA was 
down-regulated during the lag phase and up-regulated at the late exponential phase, when 
bacteria were ready to be released and start the next round of infection (Fig. 5.3 A and B). 
After being normalized by bacterial numbers as determined by quantitative PCR, western 
blot analysis also showed that OmpA protein was highly expressed at the late exponential 
phase. During the lag phase and early exponential phase, the expression of OmpA protein 
was undetectable (Fig. 5.3 C).
Immunogenicity of *E. chaffeensis* OmpA.

In order to determine the immunogenicity of *E. chaffeensis* OmpA, the full-length *ompA* gene was cloned into pET33b(+) vector and expressed in *E. coli* BL21(DE3). After IPTG induction, the expression of rOmpA was confirmed by western blotting using mouse monoclonal antibody against T7 tag derived from vector (Fig. 5.4, lane 1). The human ehrlichiosis patient serum (lane 2) provided by the Centers for Disease Control (Rikihisa *et al.*, 1994), dog 3918815 anti-*E. chaffeensis* Arkansas serum (lane 3), both recognized the recombinant *E. chaffeensis* OmpA (Fig. 5.4). Sera collected from the dogs prior to experimental inoculation with *E. chaffeensis* or human sera from a region where HME is not endemic (Japan) did not react with *E. chaffeensis* OmpA (not shown). The result indicates that OmpA is expressed by *E. chaffeensis* in infected human and dog, and antigenic.

Inhibition of binding and infection of *E. chaffeensis* with anti-OmpA IgG in vitro.

To analyze whether anti-OmpA IgG blocks the binding of *E. chaffeensis* to host cells, host cell-free organisms were preincubated with anti-OmpA IgG and then coincubated with THP-1 cells at room temperature for 15 min with gentle shaking and at 37°C for 45 min to allow binding of *E. chaffeensis* to THP-1 cells. Anti-OmpA IgG inhibited the binding of *E. chaffeensis* to the host cells on a dose-dependent manner, compared to pre-immune rabbit IgG or medium (Fig. 5.5 A).

To analyze whether anti-OmpA IgG blocks infection of *E. chaffeensis*, host cell-free organisms were preincubated with anti-OmpA IgG and then coincubated with THP-1...
cells at 37°C for 48 h to allow the growth of the internalized *E. chaffeensis*. As shown in Fig. 5.5 B, anti-OmpA IgG blocked infection, whereas pre-immune rabbit IgG, or medium had no effect on *E. chaffeensis* infection.

**CtrA binds to the promoter regions of ompA gene**

Binding site consensus sequence (CtrA box) of *C. crescentus* CtrA, a global transcriptional regulator, was found at -300 bp from the translational start site of *ompA* gene. To determine whether CtrA binds to the promoter region of *ompA* gene, we performed EMSA. After the incubation of the biotinylated *ompA* promoter region (368 bp) with the purified MBP-rCtrA, the shifted band was detected (Fig. 5.6 A). The specificity of the binding was confirmed by adding 50-fold unlabeled DNA fragment or using MBP protein as negative controls.

**rCtrA activates the lacZ reporter fusion**

Because CtrA bound to DNA probe derived from the sequence upstream of *ompA* gene, we examined whether this expression of *ompA* could be transactivated by CtrA. The *lacZ* reporter fusion was constructed by inserting the *ompA* gene promoter fragment upstream of the translation start site of the promoterless *lacZ* gene in pACYC184. The *lacZ* reporter construct was transformed into *E. coli* BL21 (DE3) containing either pET33b(+) expressing rCtrA or pET33b(+) expressing recombinant *E. chaffeensis* NtrY (rNtrY) as a negative control. For the reporter constructs, IPTG induction resulted in a significant increase in β-galactosidase activity compared to samples lacking IPTG or compared to IPTG induction of the pET33b(+) expressing rNtrY (Fig. 5.6 B). The
expressions of rCtrA and rNtrY upon IPTG induction were confirmed by western blot analysis (Fig. 5.6 B).

5.5 Discussion

The results from this study provide a significant advancement in our understanding of the function of E. chaffeensis OmpA, a peptidoglycan-associated lipoprotein. Peptidoglycan-associated lipoproteins (PAL) are not covalently linked to the murein layer (Leduc et al., 1992), but interact with murein through the conserved α-helical motif formed by peptidoglycan binding motif (Koebnik, 1995). The 3-D structure shows that Haemophilus influenzae Pal interacts exclusively with the peptide portion. In particular, extensive H-bond and hydrophobic contacts to the m-Dap (meso-diaminopimelate) group, which is found in the cell walls of all Gram-negative bacteria, indicate that Pal specifically recognizes un-cross-linked Dap groups in the PG layer (Parsons et al., 2006). It was reported that PAL is necessary for resistance to detergents and antibiotics (Cascales et al., 2002) and for integrity of the cell envelope in E. coli (Rodriguez-Herva et al., 1996). Despite absence of peptidoglycan, in E. chaffeensis OmpA, the peptidoglycan binding motif sequence is conserved, which suggests that this motif of OmpA may also form an α-helical motif and interact with periplasmic proteins other than the protein portion of peptidoglycan to support the cell wall structure. In the present study, OmpA is highly expressed prior to bacterial release, further suggesting that OmpA may play an important role in the bacterial transient survival outside of the host cells.
Little is known about the adhesions of *E. chaffeensis* gp120, an outermembrane protein, is also highly expressed prior to the bacterial release, even shed into the morula cavity (Popov *et al.*, 2000). No evidents suggest that it plays a role in the bacterial outside survival, but a previous study suggested that it was an adhesin that might enhance the internalization of *E. chaffeensis* (Popov *et al.*, 2000). The present study showed anti-OmpA peptides antiserum inhibited the binding of *E. chaffeensis* to host cells and the bacterial infection in *vitro*, which suggested that OmpA was involved in the bacterial binding to the host cells and initiate the bacteria infection.

*Legionella* PAL is a strong antibody inducer in mice (Yoon *et al.*, 2002). Rabbit anti *H. influenzae* PAL antibodies passively protect infant rats from *H. influenzae* type b-induced meningitis (Munson and Granoff, 1985). In this study, we demonstrated that OmpA is bacterial surface exposed, which is an important characteristic for a vaccine antigen, indicating that the protein is accessible to potentially protective antibodies. OmpA was expressed by *E. chaffeensis* and antigenitic in infected dog and human, and anti-OmpA IgG acted as infection neutralizing antibody in *vitro*. Thus, OmpA may be included among potential HME vaccine candidates to protect human and animal from disease caused by the ehrlichial pathogens.

The expressions of certain bacterial proteins, especially those related to invasion and intracellular survival have been shown to be growth stage-dependent. OMP-1 and P28, which have porin activities, were up-regulated at early exponential phase and down-regulated during the stationary phase prior to bacterial release (Kumagai *et al.*, 2008). Genes encoding type IV secretion apparatus are differentially expressed (Cheng *et al.*, 2008). In the present study, we demonstrated that OmpA expression was also differential
and regulated by CtrA. Many other genes important for bacterial infection and survival are also predicted to be regulated by CtrA (Chapter 4). CtrA in a free-living aquatic bacterium, *C. crescentus*, is known to regulate more than 95 genes to coordinate the cell cycle development, but the expression of OmpA homolog (identity with *E. chaffeensis* OmpA is 22%) was not regulated by CtrA (Laub *et al.*, 2002). This suggests an evolutionary adaptation of CtrA system to regulate intracellular development of *E. chaffeensis*. Similar temporal expression patterns were observed with *surE* and *bolA*, which were predicted as downstream genes of CtrA in Chapter 4. Thus at least three proteins of *E. chaffeensis* predicted to be involved *E. chaffeensis* transient extracellular survival (Vieira *et al.*, 2004; Mura *et al.*, 2003; Santos *et al.*, 1999) appear to be regulated by CtrA (Chapter 4). These results indicate that CtrA regulates the expression of *ompA* to link the bacterial intracellular development to the bacterial survival and the next round of infection.
<table>
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<tr>
<th>Bacteria</th>
<th>GenBank No.</th>
<th>Identity</th>
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<td>YP_303196.1</td>
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<td>YP_154026.1</td>
<td>37%</td>
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<td><em>Anaplasma phagocytophilum HZ</em></td>
<td>YP_504946.1</td>
<td>33%</td>
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<td><em>Rickettsia rickettsii 'Sheila Smith'</em></td>
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<td><em>Rickettsia conorii str. Malish 7</em></td>
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<tr>
<td><em>Rickettsia canadensis str. McKiel</em></td>
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Table 5.1 OmpA amino acid sequence identity in the order *Rickettsiales*.

E value of $< 10^{-5}$ based on amino acid sequences was used as cut-off.
Fig. 5.1 Consensus sequences of the peptidoglycan binding motif of *E. chaffeensis* OmpA and *E. coli* Pal.

Consensus sequence of the OmpA (17 aa) and the Pal (16 aa) were aligned. The peptidoglycan binding motifs (NX$_2$LSX$_2$RGX$_2$VX$_3$L) are shown in bold and underlined. The positions of the motifs are shown lower or upper.
Fig. 5.2 Surface localization of \textit{E. chaffeensis} OmpA by double immunofluorescence labeling.

Host cell-free \textit{E. chaffeensis} was fixed in paraformaldehyde, (A) Bacteria were incubated with anti-\textit{E. chaffeensis} (red) and rabbit anti-OmpA peptide IgG (81-94 aa) (green). The panels on the right are superimposed images viewed with green and red filters. Ring-like \textit{E. chaffeensis} surface staining of OmpA was detected. As controls for immunofluorescence labeling, \textit{E. chaffeensis} were incubated with (B) rabbit pre-immune IgG, (C) dog pre-immune serum, (D) rabbit antiserum against a cytosolic protein (PleD of \textit{E. chaffeensis}) and secondary conjugated anti-rabbit IgG, or and secondary conjugated anti-dog IgG. The scale bar is 1 μm.
Fig. 5.2

A

Pre-immune dog serum

B

C

D

Anti-Ec  Anti-OmpA  Merge

Anti-Ec  Anti-OmpA  Merge

Anti-Ec  Pre-immune rabbit IgG  Merge

Anti-Ec  Anti-rPleD  Merge
Fig. 5.3 Synchronous growth of *E. chaffeensis* and the temporal expression of *ompA* gene. (A) Genomic DNA extracted from infected THP-1 cells at different time p.i. was subjected to quantitative PCR analysis. Data points reflect the numbers of bacteria relative to the amount determined at 0 h p.i. The values represent the means ± standard deviations from three specimens. (B) mRNA prepared from infected THP-1 cells at different time p.i. was subjected to quantitative PCR analysis. Transcript levels are relative to the amount of *ompA* transcript at 0 h p.i. The values represent the means ± standard deviations from three specimens. (C) Western blot analyses of samples from infected THP-1 cells at different time p.i. were performed using an anti-OmpA rabbit IgG to determine the expression of OmpA. All the samples are normalized by bacterial number. *E. chaffeensis* OmpA is indicated by arrow.
Fig. 5.3

A

Relative bacterial number

0h 24h 48h 72h

B

Relative ompA mRNA / bacterium

0h 24h 48h 72h

C

OmpA / bacterium

0h 24h 48h 72h

15 kD
Fig. 5.4 Antigenicity of recombinant *E. chaffeensis* OmpA protein.

After IPTG induction, the crude *E. coli* lysate was prepared by sonication. All samples were loaded onto 12% SDS-PAGE gel. Following separation, proteins were transferred to a PVDF membrane. (1) The membrane was incubated with mAb anti T7 tag at a 1:5000 dilution. (2) The membrane was incubated with the sera from HME human patient or experimental *E. chaffeensis* infected dog, at a 1:1000 dilution, followed by incubation of secondary antibody, horseradish peroxidase-conjugated goat anti human IgG and goat anti-dog IgG (KPL, Gaithersburg, MA), at a 1:1000 dilution. M, Prestained protein size standards. Each lane received 1 µg protein. The recombinant *E. chaffeensis* OmpA are indicated by arrows.
Fig. 5.5 Rabbit anti-OmpA IgG inhibits the binding and infection of *E. chaffeensis* to THP-1 cells.

(A) Inhibition of the binding of *E. chaffeensis* to THP-1 cells with rabbit anti-OmpA IgG. Numbers of bound or internalized *E. chaffeensis* were scored in 100 THP-1 cells in triplicate samples. (B) Inhibition of the infection of *E. chaffeensis* to THP-1 cells with rabbit anti-OmpA IgG. The percentage of infection is expressed as the number of bacteria per THP-1 cell under the indicated conditions divided by the number of bacteria per THP-1 cell incubated with medium multiplied by 100. * significantly different by one-way analysis of variance (ANOVA) (P < 0.01).
Fig. 5.5

A

Bacterial number / 100 cells

0 100 200 300 400

medium 20 100 500 20 100 500 ug/ml

Pre-immune IgG

Anti-OmpA peptides IgG

B

Relative bacterial number (%)

0 25 50 75 100 125

medium pre-immune anti-OmpA

*
Fig. 5.6 The expression of OmpA is regulated by CtrA.

(A) Binding of recombinant MBP-CtrA to the DNA probe derived from sequence upstream of *ompA* gene. For each panel: lane 1, DNA probe (0.1 pmol); lane 2, DNA probe incubated with MBP (25 ng); lane 3, DNA probe incubated with MBP-rCtrA (25 ng); lane 4, DNA probe incubated with MBP-rCtrA in the presence of a 50-fold excess of the corresponding unlabeled DNA probe. Shifted band is indicated by an arrowhead. Bands were visualized using the LightShift Chemiluminescent EMSA kit.

(B) CtrA activates the transcription of *ompA-lacZ* reporter fusions. β-galactosidase assays were used to measure the transcriptional activities of *lacZ* reporter constructs. The values reflect means ± standard deviations from three specimens. *, Significantly different (*P* < 0.01) compared to samples lacking IPTG or compared to IPTG induction of the pET33b(+) vector expressing an unrelated protein, rNtrY, by the Tukey Honestly Significant Differences test. Western blot analyses of samples from the β-galactosidase assays were performed using an anti-His-tag antibody to verify the expression of rCtrA and rNtrX. A representative of three independent experiments is shown in the lower panels. rCtrA and rNtrY are indicated by arrows.
Fig. 5.6

A

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<td><em>ompA</em> promoter region (368 bp)</td>
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B

β-galactosidase activity (Miller units)

- IPTG: - ___ - + ___ - ______ - + ___ - _
- rNtrY:   - ___ - + ___ - _
- rCtrA:   - ___ - + ___ - _

rNtrY: 40 kD
rCtrA: 31 kD


