Two-Component Regulatory Systems of *Anaplasma phagocytophilum* and Outer Membrane Protein P44 Expression Locus of *Anaplasma platys*

Dissertation

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Abstract

Gram-negative obligate intracellular bacterium, *Anaplasma phagocytophilum* is the etiologic agent of human granulocytic anaplasmosis (HGA), which is transmitted to humans by black-legged ticks. Two-component regulatory system (TCRS) composed of a histidine autokinase and a response regulator is a bacterial signal sensing and transduction system. TCRS allows bacteria to regulate cellular functions in response to environmental changes. After sequencing the *A. phagocytophilum* genome, our laboratory found six hypothetical proteins which have motifs of TCRS, and based on their amino acid sequence similarity named them as PleC, PleD, NtrY, NtrX, CckA, and CtrA. Our hypothesis is that these proteins make three pairs of TCRS (PleC-PleD, NtrY-NtrX and CckA-CtrA) and play important roles in regulating intracellular infection. Our genome sequence analysis also identified a candidate for His-containing phosphotransfer (Hpt) domain protein AhpT which serves as phospho-His intermediates between the hybrid kinase (CckA) and the response regulator (CtrA). My study demonstrated that *pleC, pleD, ntrY, ntrX, cckA, ahpT,* and *ctrA* were expressed during *A. phagocytophilum* intracellular infection in human promyelocytic HL-60 cells, and in HGA patient’s blood. Autokinase activity of rPleC, rNtrY, and rCckA and phosphotransfer from rPleC to rPleD, rNtrY to rNtrX, rCckA to rAhpT and rAhpT to rCtrA were detected. Diguanylate cyclase activity of rPleD was demonstrated by generating bacterial second messenger, cyclic-di-GMP (c-di-GMP) from GTP in vitro. UV cross-linking of the *A. phagocytophilum* lysate and rPleD with c-[32P]di-GMP detected an ~47-kDa endogenous protein and rPleD has c-di-GMP binding activity, suggesting them as c-di-GMP receptors. A novel hydrophobic c-di-GMP derivative, 2′-O-di(tert-butyldimethylsilyl)-c-di-GMP, inhibited *A.
*phagocytophilum* infection in HL-60 cells. These results suggest that PleD is a diguanylate cyclase and the c-di-GMP-receptor complex regulates *A. phagocytophilum* intracellular infection. NtrX and CtrA have DNA binding motif and 5 potential NtrX downstream target genes and 6 potential CtrA downstream target genes were detected by systematic evolution of ligands by exponential enrichment (SELEX) assay. Our results suggest that PleC-PleD, NtrY-NtrX and CckA-AhpT-CtrA are functional TCRS of *A. phagocytophilum* and play important roles in bacterial intracellular developmental cycle. *Anaplasma platys* is a Gram-negative obligate intracellular bacterium and the etiologic agent of infectious canine cyclic thrombocytopenia (ICCT), transmitted by brown dog ticks. Major outer membrane protein P44s are immunodominant 44-kDa major outer membrane proteins consist of a single central hypervariable region, an N-terminal conserved region and a c-terminal conserved region. Polymorphic *p44/msp2* expression loci are found in *A. phagocytophilum* and *Anaplasma marginale* genomes. Using DNA isolated from the blood specimens from naturally infected dogs and hemi-nested PCR, we cloned the *A. platys p44* expression locus and defined *A. platys tr1, omp-1X and p44* based on amino acid sequence similarity. Three more P44 hypervariable region sequences were obtained using P44 conserved N- and C-terminal regions designed primers. Our results provide the first information of *A. platys p44* polymorphic multigene family and their genomic expression locus.
Dedication

Dedicated to my family in Taiwan and my wife for support during the whole study
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Table of Contents

Abstract...........................................................................................................................................ii

Dedication........................................................................................................................................iv

Acknowledgments............................................................................................................................v

Vita..................................................................................................................................................vi

List of Tables..................................................................................................................................ix

List of Figures.................................................................................................................................x

Chapters

1. Introduction....................................................................................................................................1

2. *Anaplasma phagocytophilum* PleC Histidine Kinase and PleD Diguanylate Cyclase Two-component System and Role of Cyclic Di-GMP in Host Cell Infection
   2.1 Abstract.................................................................................................................................14
   2.2 Introduction.............................................................................................................................15
   2.3 Materials and methods..........................................................................................................18
   2.4 Results....................................................................................................................................24
   2.5 Discussion...............................................................................................................................31

3. CckA, HpT, and CtrA Phosphorelay and CtrA Targets in *Anaplasma phagocytophilum*
   3.1 Abstract.................................................................................................................................49
   3.2 Introduction.............................................................................................................................50
   3.3 Materials and methods..........................................................................................................53
   3.4 Results....................................................................................................................................57
   3.5 Discussion...............................................................................................................................64

4. *Anaplasma phagocytophilum* NtrY and NtrX Two-Component System, and Role of NtrX in Intracellular Infection
   4.1 Abstract.................................................................................................................................81
   4.2 Introduction.............................................................................................................................82
   4.3 Materials and methods..........................................................................................................84
4.4 Results...........................................................................................................88
4.5 Discussion.....................................................................................................93

5 Polymorphic Major Outer Membrane Protein p44 Expression Locus in *Anaplasma platys* from Naturally-infected Dogs
  5.1 Abstract....................................................................................................108
  5.2 Introduction.............................................................................................109
  5.3 Materials and methods..........................................................................112
  5.4 Results......................................................................................................113
  5.5 Discussion...............................................................................................117

Bibliography..........................................................................................................130
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Oligonucleotides used for cloning and expression of <em>Anaplasma phagocytophilum</em> sensor kinases and response regulators, and their mutants</td>
<td>35</td>
</tr>
<tr>
<td>2.2</td>
<td>Oligonucleotides used for <em>pleC-pleD</em> gene-specific real-time PCR in <em>Anaplasma phagocytophilum</em></td>
<td>36</td>
</tr>
<tr>
<td>3.1</td>
<td>Oligonucleotide primers used for cloning and expression CckAHK, CckARD, AhpT, CtrAD, CckAHKDH432A, CckARDH741A, AhpTH17A, CtrAD53A and CtrA downstream targets</td>
<td>68</td>
</tr>
<tr>
<td>3.2</td>
<td>Putative <em>Anaplasma phagocytophilum</em> CtrA regulon identified by SELEX</td>
<td>70</td>
</tr>
<tr>
<td>3.3</td>
<td>Hpt homologs found in sequenced genome of the family Anaplasmataceae</td>
<td>71</td>
</tr>
<tr>
<td>4.1</td>
<td>Oligonucleotides used for cloning and expression of <em>Anaplasma phagocytophilum</em> NtrYHKDH491A, NtrXR, NtrXRDD60A, NtrXDBD, and NtrX downstream targets</td>
<td>97</td>
</tr>
<tr>
<td>4.2</td>
<td>Putative <em>Anaplasma phagocytophilum</em> NtrX regulon identified by SELEX</td>
<td>98</td>
</tr>
<tr>
<td>5.1</td>
<td>Primers used for PCR amplification of <em>A. platys</em> P44ES cluster</td>
<td>122</td>
</tr>
<tr>
<td>5.2</td>
<td>Properties of <em>A. platys</em> p44ES cluster</td>
<td>123</td>
</tr>
</tbody>
</table>
**List of Figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Schematic representation of the domain structures of PleC and PleD in <em>Anaplasma phagocytophilum</em> and <em>Caulobacter crescentus</em></td>
</tr>
<tr>
<td>2.2</td>
<td><em>pleC</em> and <em>pleD</em> are transcribed by <em>Anaplasma phagocytophilum</em> in the blood of an HGA patient and in human myelocytic leukemia HL-60 cells</td>
</tr>
<tr>
<td>2.3</td>
<td>PleC and PleD are expressed by <em>Anaplasma phagocytophilum</em> in human myelocytic leukemia HL-60 cells</td>
</tr>
<tr>
<td>2.4</td>
<td>PleC and PleD expression in synchronously cultured <em>Anaplasma phagocytophilum</em></td>
</tr>
<tr>
<td>2.5</td>
<td><em>Anaplasma phagocytophilum</em> PleC has specific His-dependent autokinase activity, and phosphotransfer from <em>A. phagocytophilum</em> PleC to PleD is dependent on a specific Asp of PleD</td>
</tr>
<tr>
<td>2.6</td>
<td><em>Anaplasma phagocytophilum</em> rPleD has di-guanylate cyclase activity</td>
</tr>
<tr>
<td>2.7</td>
<td><em>Anaplasma phagocytophilum</em> produces a c-di-GMP binding protein</td>
</tr>
<tr>
<td>2.8</td>
<td>2’-O-TBDMS-c-di-GMP inhibits <em>Anaplasma phagocytophilum</em> infection in human myelocytic leukemia HL-60 cells</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic representation of the domain structures of CckA, AhpT, and CtrA in <em>Anaplasma phagocytophilum</em> and <em>Caulobacter crescentus</em>, and consensus sequences to identify Hpt domain protein</td>
</tr>
<tr>
<td>3.2</td>
<td>Expression of CckA, AhpT, and CtrA</td>
</tr>
<tr>
<td>3.3</td>
<td>CckA and CtrA are expressed by <em>Anaplasma phagocytophilum</em> in human myelocytic leukemia HL-60 cells</td>
</tr>
<tr>
<td>3.4</td>
<td>Figure 3.4. CckA autokinase activity and phosphotransfer relay CckA-HKD-CckA-RD-AhpT-CtrA</td>
</tr>
<tr>
<td>3.5</td>
<td>CtrA SELEX pull-down assay of putative CtrA downstream genes</td>
</tr>
<tr>
<td>4.1</td>
<td>Schematic representation of the domain structures of NtrX and NtrY in <em>Anaplasma phagocytophilum</em> and <em>Azorhizobium caulinodans</em></td>
</tr>
<tr>
<td>4.2</td>
<td><em>ntrY</em> and <em>ntrX</em> are transcribed by <em>Anaplasma phagocytophilum</em> in the blood of an HGA patient and in human myelocytic leukemia HL-60 cells</td>
</tr>
<tr>
<td>4.3</td>
<td>NtrY and NtrX are expressed by <em>Anaplasma phagocytophilum</em> in human myelocytic leukemia HL-60 cells</td>
</tr>
<tr>
<td>4.4</td>
<td><em>A. phagocytophilum</em> NtrY has specific His-dependent autokinase activity, and specific phosphotransfer from NtrY to NtrX is dependent on a specific Asp of</td>
</tr>
</tbody>
</table>
NtrX.................................................................................................................................104
4.5 NtrX SELEX and consensus sequence in the promoter regions.................................106
5.1 Strategy for designing *A. platys* p44ES cluster sequencing primers and *A. platys*
    P44ES locus ..................................................................................................................124
5.2 Synteny analysis of the *A. platys* p44ES cluster ...................................................125
5.3 Phylogenetic tree of Tr1 proteins of *A. platys, A. phagocytophilum, A. marginale* ....126
5.4 Phylogenetic tree of Omp-1X proteins of *A. platys, A. phagocytophilum, A. marginale, E. canis, E. chaffeensis, E. ewingii, E. ruminantium* .........................127
5.5 Phylogram of P44ES/Msp2 proteins of *A. platys, A. phagocytophilum, and A. marginale* .....................................................................................................................128
5.6 Membrane criterion profile for *A. platys* P44 .........................................................129
CHAPTER 1

INTRODUCTION

The family Anaplasmataceae

The family *Anaplasmataceae* belongs to the class *α*-proteobacteria, in the order *Rickettsiales* based upon 16S rRNA gene and *groESL* operon sequences, (Dumler, Barbet et al. 2001). The family *Anaplasmataceae* includes five genera, *Ehrlichia, Anaplasma, Neorickettsia, Aegyptianella*, and *Wolbachia*. Members of the family Anaplasmataceae are small gram-negative pleomorphic cocci and obligate intracellular bacteria, which replicate in membrane-bound vacuoles (parasitophorous vacuoles called morulae) in the cytoplasm of eukaryotic host cells, which are usually bone marrow or haematopoietic origin and invertebrate hosts (Rikihisa 1991).

The genus *Anaplasma*

The genus *Anaplasma* includes closely related species, *A. phagocytophilum* (Bakken, Dumler et al. 1994; Chen, Dumler et al. 1994), *A. marginale* (Theiler 1910), *A. centrale* (Theiler 1911), *A. bovis* (Donatien 1936), *A. ovis* (Lestoquard 1924), and *A. platys* (Harvey, Simpson et al. 1978; French and Harvey 1983). *A. phagocytophilum* includes organisms, previously called *Ehrlichia phagocytophilum, E. equi*, and the human
granulocytic ehrlichiosis agent (HGE agent) which share at least 99.1% nucleotide sequence similarity in 16S rRNA sequences and identical GroEL amino acid sequences (Dumler, Barbet et al. 2001).

**A. phagocytophilum**

*A. phagocytophilum* has been recently recognized in the U.S.A. as human pathogen causing emerging human granulocytic anaplasmosis (HGA, formerly human granulocytic ehrlichiosis) characterized by chills, headache, myalgia, and hematological abnormalities, including leukopenia and thrombocytopenia, and increased serum transaminase activities suggesting mild to moderate liver injury. Most patients are required hospitalization and respond to treatment with oxytetracycline (Dumler, Choi et al. 2005). The case-fatality rate is 0.7% and largely relates to complicating opportunistic infections, antecedent medical conditions, such as diabetes mellitus, elderly or immunocompromise (Bakken and Dumler 2000; Dumler, Madigan et al. 2007). Human infection occurs through the bite of infected ticks. HGA has become an emerging tick-borne zoonosis of global distribution, being reported in the United States (northeastern and mid-Atlantic, Upper Midwest, and Pacific Northwest states), Europe, and Asia (China, Siberian Russia, and Korea) (Dumler, Choi et al. 2005; Dumler, Madigan et al. 2007). These regions correspond to the distribution of *Ixodes* spp ticks (*I. scapularis* in the eastern United States (Telford, Lepore et al. 1995), *I. pacificus* in the western United States (Richter, Kimsey et al. 1996), *I. ricinus* in Europe (Ogden, Hailes et al. 1998), and *I. persulcatus* in Asia (Cao, Zhao et al. 2003; Kim, Kim et al. 2003). Tick infection occurs after sucking of the blood of infected mammals. The bacterium is transstadially, but not
transovarially transmitted in ticks. The mammalian natural reservoirs for *A. phagocytophilum* are wild rodents, e.g. white-footed mouse (*Peromyscus leucopus*) and dusky-footed wood rats (*Neotoma fuscipes*) in the eastern and western United States, respectively (Rikihisa 2003).

*A. phagocytophilum* has a small genome size of 1.47 Mb and lost most genes required for the biosynthesis of peptidoglycan and all genes required for the biosynthesis of LPS, thus can stealthily infect granulocytes equipped with powerful innate antimicrobial defenses (Lin and Rikihisa 2003; Hotopp, Lin et al. 2006). *A. phagocytophilum* acquired unique capability to take up host cell cholesterol and incorporate cholesterol into their membrane (Lin and Rikihisa 2003). Cholesterol has become indispensable for their survival and successful establishment of infection (Lin and Rikihisa 2003; Xiong, Wang et al. 2007; Xiong, Lin et al. 2009). A developmental cycle of *A. phagocytophilum* consists of two forms: small dense-cored cells (DC) which can bind HL-60 cells and has the ability to enter host cells, and large reticulate cells (RC) that are transformed from DC. RC again mature into DC, which are released upon host cell lysis (Woldehiwet and Scott 1982; Rikihisa 1991; Munderloh, Jauron et al. 1999). *A. phagocytophilum* employs APH_1387 and type IV substrates AnkA and Ats-1 to hijack and localize vesicular traffic creating pathogen-occupied vacuoles, be phosphorylated and bind to SHP-1 and translocated into host cell mitochondria inhibit apoptosis, respectively (Huang, Troese et al.; Niu, Kozjak-Pavlovic et al.; Lin, den Dulk-Ras et al. 2007).

Besides, *A. phagocytophilum* has three pairs of two-component regulatory systems (TCSs) to sense and respond to the environmental signals/changes during its life cycle such as internalization, replicative compartment establishment and maturation, interaction...
with host cell, and exocytosis/release or transmitted between different host cells.

Moreover, TCSs may participate in bacterial sensing and acquiring metabolites from host cells for survival and replication, since \textit{A. phagocytophilum} lacks most genes for intermediary metabolism, glycolysis, and amino acid biosynthesis (Hotopp, Lin et al. 2006).

\textbf{Two-component regulatory system}

The bacterial two-component regulatory system (TCS) also known as histidyl-aspartyl (His-Asp) phosphorelay system, is an ubiquitous signal sensing and transduction paradigm controlling response and adaptation to a variety of environmental conditions (Parkinson and Kofoid 1992). The TCSs are typically composed of a sensor histidine kinase (HK) and a cognate response regulator (RR). When the SK senses a particular environmental signal through the periplasmic sensor domain, dimerization and intermolecular autophosphorylation of the His residue in the cytoplasmic kinase domain occurs. The receiver domain of RR also has autokinase activity to use autophosphorylated cognate SK as the phosphoryl donor. This phosphorylation, in turn, regulates the output domain of the RR, which generally has DNA binding activity to control target gene transcription or diguanyl cyclase activity to generate bacterial second messenger cyclic (c) di-GMP. 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). Through sequence and domain structure analysis, and overall similarities in protein structures, we identified three
histidine kinases (homologs of NtrY, PleC, and CckA) and pairing response regulators (homologs of NtrX, PleD, and CtrA) in *A. phagocytophilum* genome (Cheng, Kumagai et al. 2006). All six genes from *A. phagocytophilum* are expressed in human leukocyte culture (Cheng, Kumagai et al. 2006). Pretreatment of host cell-free *A. phagocytophilum* with closantel (N-[5-chloro-4-[(R,S)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide), an inhibitor of histidine kinases (Stephenson, Yamaguchi et al. 2000), completely blocks the infection of host cells (Cheng, Kumagai et al. 2006). This result suggests that TCS plays an essential role in *A. phagocytophilum* infection of human leukocytes.

**PleC PleD**

Bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) is an ubiquitous second messenger that regulates cell surface-associated traits in bacteria such as switch between a free-living, planktonic and a sedentary, biofilm-related lifestyle. c-di-GMP is synthesized from GTP by the activity of diguanylate cyclases (DCGs) and is degraded by phosphodiesterases (PDEs). Genetic and biochemical studies identified the GGDEF domain as the catalytically active part of DGCs and the EAL and HD-GYP domain as two alternative and structurally unrelated domains with PDE activity. Several protein c-di-GMP downstream receptors have been identified such as PilZ domain containing proteins, proteins with degenerate GGDEF and EAL domains, and AAA+ ATPase domain of *Pseudomonas aeruginosa* FleQ (Schirmer and Jenal 2009). However, the known c-di-GMP protein receptors cannot explain the global cellular effects of c-di-GMP. To explain this, it has been proposed that highly conserved RNA motif called
GEMM (Genes for the Environment, for Membranes and for Motility) (Weinberg, Barrick et al. 2007) functions as riboswitch to control the downstream gene expression in response to c-di-GMP binding to the motif (Sudarsan, Lee et al. 2008). Furthermore, crystal structure of a c-di-GMP riboswitch aptamer bound to c-di-GMP has been reported (Smith, Lipchock et al. 2009). However, we did not find GEMM upstream of PleD of *A. phagocytophilum*. One of *A. phagocytophilum* response regulator PleD of TCS pairs has a GGDEF domain suggesting c-di-GMP signaling pathway exists and may also regulate the bacterial biofilm formation and pathogenesis. Therefore, we hypothesize that c-di-GMP signaling pathway in *A. phagocytophilum*, which is essential for infection of human leukocytes.

**CckA CtrA**

Bacterial differential developmental regulation has been found in free-living aquatic bacterium, *Caulobacter crescentus* which divides asymmetrically to produce two different progeny, a swarmer cell and a stalked cell, each with distinct morphological features and regulatory programs (Brown, Hardy et al. 2009; McAdams and Shapiro 2009). The cell cycles of both *Caulobacter* daughter cell types have a cyclical genetic circuit controlled by four master regulatory proteins DnaA, GcrA, CtrA and CcrM. These proteins are synthesized and cleared from the cell one after the other over the course of the cell cycle. The cycle of these four master regulatory proteins control activation of modular subsystems in the appropriate sequence and timing relative to each other. Among these four regulators, CtrA is a two-component response regulator conserved among α-proteobacteria playing an important role controlling cell cycle (Brassinga, Siam
et al. 2002). *Caulobacter* CtrA is highly regulated at transcription, phosphorylation, and degradation steps. The transcription of *ctrA* is controlled by CtrA feedback regulation. Phosphorylation of the CtrA occurs by phosphorelays initiated by the hybrid histidine kinase CckA, a histidine phosphotransferase (Hpt), called Chpt, and CtrA (Biondi, Reisinger et al. 2006). The phosphorylation state modulates CtrA DNA binding activity, but does not affect proteolysis (Ryan, Judd et al. 2002). CtrA degradation by the ClpXP protease depends on the colocalization of CtrA and ClpXP at the cell pole. When CpdR is phosphorylated by CckA, ClpXP is delocalized, and CtrA is not degraded (Iniesta, McGrath et al. 2006). The CtrA is brought to the activated polar ClpXP by the combined action of the RcdA localization factor and the PopA cyclic di-GMP effector protein (McGrath, Iniesta et al. 2006; Duerig, Abel et al. 2009). Among genes encoding DnaA, GcrA, CtrA and CcrM master regulatory proteins, only *dnaA* and *ctrA* are conserved in the *A. phagocytophilum* genome (Hotopp, Lin et al. 2006). Up to now, CtrA roles in *A. phagocytophilum* infection is unknown, although in *A. phagocytophilum*, *cckA* and *ctrA* are transcribed and histidine kinase activity is essential for infection (Cheng, Kumagai et al. 2006). Therefore, we hypothesize that in *A. phagocytophilum* a signaling pathway is composed of CckA, CtrA and an unknown histidine phosphotransferase and this pathway regulates *A. phagocytophilum* intracellular development cycle.

**NtrY NtrX**

Bacterial NtrC family transcriptional activators belong to AAA+ ATPases superfamily and interact with its functional partner, the RNA polymerase sigma factor 54 via
conserved GAFTGA portion (Wang, Lee et al. 1997; Zhang, Chaney et al. 2002).

Members of this family contain C-terminal helix-turn-helix (HTH) domains and function as transcriptional activators that bind DNA at promoter region and catalyze ATP-dependent structural transitions required for transcription initiation (Zhang, Chaney et al. 2002). The NtrC family proteins N-terminal include receiver domains connecting NtrC ATPase protein into the two-component signaling systems. NtrY-NtrX TCS are homologous to NtrB-NtrC TCS controlling expression of nitrogen-regulated gene \textit{nifA} that encodes a transcription factor in \textit{Azorhizobium caulinodans} (Pawlowski, Klosse et al. 1991). According to our laboratory genomic sequencing result, \textit{ntrY} and \textit{ntrX} genes are conserved in the \textit{A. phagocytophilum} genome (Hotopp, Lin et al. 2006). And up to now, the NtrY-NtrX role in \textit{A. phagocytophilum} has been poorly documented. Moreover, according to our laboratory previous study, \textit{A. phagocytophilum ntrY} and \textit{ntrX} are transcribed in human leukocytes (Cheng, Kumagai et al. 2006). Therefore, we hypothesize that NtrY-NtrX signaling pathway existence in \textit{A. phagocytophilum} and regulating cellular functions.

\textit{Anaplasma platys}

\textit{A. platys} (formerly, \textit{E. platys}) is an obligatory intracellular bacterium of platelets first described as a rickettsia-like agent in platelets of dogs from Florida and is the etiologic agent of infectious canine cyclic thrombocytopenia (ICCT) (Harvey, Simpson et al. 1978; French and Harvey 1983). Clinical signs of ICCT are fever, depression, and anorexia (French and Harvey 1983). Parasitemia and thrombocytopenia occur in cycles at approximately 10-to 14-day intervals (French and Harvey 1983). \textit{A. platys} DNA was
detected in dogs from Greece (Mylonakis, Koutinas et al. 2004), France (Inokuma, Fujii et al. 2002), Taiwan (Chang and Pan 1996), Spain (Sainz, Amusategui et al. 1999), China (Hua, Yuhai et al. 2000), Australia (Brown, Martin et al. 2001), Portugal (Cardoso, Tuna et al. 2008), Republic of Congo (Sanogo, Davoust et al. 2003), Japan (Unver, Rikihisa et al. 2003), and Thailand and Venezuela (Suksawat, Pitulle et al. 2001; Huang 2005). Despite global distribution of this infection, *A. platys* has never been culture isolated. Consequently, very little is known about this bacterium and the disease it causes because of cyclic and often low levels of organisms (French and Harvey 1983). In 1992 Anderson *et al.* (Anderson, Greene et al. 1992) reported the 16S rRNA gene sequence of *A. platys* from Lousiana. Subsequently *groEL* gene sequence of *A. platys* from Lousiana was determined (Yu, Zhang et al. 2001). Phylogenetic analysis of these sequences showed that this is a bacterium closely related to *A. phagocytophilum* and *A. marginale*, which led to reclassification of this bacterium into the genus *Anaplasma* (Dumler, Barbet et al. 2001). Later it was reported that although *A. platys* do not cross-react with serum antibodies from dogs infected with *E. canis* on IFA tests, moreover, the *A. platys* antigen cross-reacts with anti-*A. phagocytophilum* antibodies (Inokuma, Fujii et al. 2002). A polymerase chain reaction (PCR) test confirmed by DNA sequencing is considered to be the most reliable laboratory diagnostic test for *A. platys* infection. Currently, the bacterium is poorly known at any level. The transmission vector was proposed to be *Rhipicephalus sanguineus* (Brown dog tick), because *A. platys* DNA is detected mostly in this tick species (Inokuma, Raoult et al. 2000), but experimental transmission of *A. platys* by this tick was unsuccessful (Simpson, Gaunt et al. 1991).
Major outer membrane P44 family

In *A. phagocytophilum* and *A. marginale*, surface-exposed immunodominant 44-kDa major outer membrane proteins (P44s/Msp2s) are encoded by the *p44* (*msp2*) polymorphic multigene family (Barbet, Lundgren et al. 2000; Brayton, Palmer et al. 2002; Zhi, Ohashi et al. 2002). In *A. phagocytophilum*, P44 proteins consist of a single central hypervariable region of approximately 94 amino acid residues and an N-terminal and C-terminal conserved regions of approximately 186 and 146 amino acid residues, respectively (Lin, Zhi et al. 2002). A single polymorphic *p44/msp2* expression locus (*p44/msp2ES*) is found in the genome of *A. phagocytophilum* and *A. marginale*, respectively (Brayton, Kappmeyer et al. 2005; Hotopp, Lin et al. 2006). Both expression loci are found downstream of *tr1* genes encoding putative transcriptional factor and homologs of *E. chaffeensis omp-1* genes encoding polymorphic major outer membrane protein (Barbet, Lundgren et al. 2000; Barbet, Meeus et al. 2003; Lin, Rikihisa et al. 2003). Although the *A. phagocytophilum* genome has been greatly reduced (1.47 Mb), about 7% of this genome encodes *p44*s, a total of 113 *p44*s are present in *A. phagocytophilum* genome, including 22 full-length, 64 shorter (without start codons), 21 fragmented (contain only 5’ or 3’) and 6 truncated (containing only the hypervariable regions) *p44*s (Hotopp, Lin et al. 2006). And 65 out of these 113 *p44*s are expressed at the transcription level in either in cell culture, patients, experimentally infected horses, or ticks (Zhi, Ohashi et al. 1999; Lin, Zhi et al. 2002; Zhi, Ohashi et al. 2002; Lin, Rikihisa et al. 2003; Felek, Telford et al. 2004; Lin, Rikihisa et al. 2004; Wang, Rikihisa et al. 2004; Lin and Rikihisa 2005). Thus *p44*s are constitutively expressed under all conditions tested, suggesting an essential function for *A. phagocytophilum*. In *A. phagocytophilum*
p44/msp2ES, p44s and msp2 donor sequences elsewhere in the genome undergo nonreciprocal and nonsegmental recombination via RecF pathway to allow various p44/msp2ES expression under the same promoter (Lin, Zhang et al. 2006) however, in A. marginale p44/msp2ES has only 7-10 of donor alleles undergo segmental conversion followed by oligonucleotide recombination generating mosaic Msp2 (Brayton, Palmer et al. 2002). This mechanism is thought to facilitate P44/Msp2 antigenic variation for persistent infection and for adaptation to new environments such as transmission between tick and mammalian hosts (Brayton, Palmer et al. 2002; Zhi, Ohashi et al. 2002; Rikihisa 2003; Barbet, Lundgren et al. 2006; Lin, Zhang et al. 2006). Native P44 of A. phagocytophilum was shown to have porin activity this result might explain the P44 constitutively expression (Huang, Wang et al. 2007). There has been no study on A. phagocytophilum outer membrane proteins.

Objectives of my Ph.D. study are:

Objective 1. To study PleC and PleD functions in A. phagocytophilum

1-1. To investigate the expression of PleC and PleD pairs by A. phagocytophilum during cell culture and human patient acute phase infection.

1-2. To detect PleC autokinase activity and specific phosphotransfer between PleC and PleD.

1-3. To measure PleD DGC activity and identify c-di-GMP downstream targets and effects.
Objective 2. To study CckA and CtrA functions in *A. phagocytophilum*

2-1 To investigate the expression of CckA, AhpT, and CtrA by *A. phagocytophilum* intracellular infection in HL-60 cell culture.

2-2 To detect CckA autokinase activity and CckA-AhpT-CtrA specific phosphorelay

2-3 To identify CtrA downstream target genes by systematic evolution of ligands by exponential enrichment (SELEX) and their function by mutant complement.

Objective 3. To study NtrY and NtrX functions in *A. phagocytophilum*

3-1 To investigate the expression of NtrY and NtrX pairs by *A. phagocytophilum* during cell culture and human patient acute phase infection.

3-2 To determine NtrY autokinase activity and specific phosphotransfer between NtrY and NtrX.

3-3 To identify NtrX downstream target genes by SELEX and their function by mutant complement.

Objective 4. To clone *p44* expression locus of *A. platys*

4-1 To investigate the existence of *p44* expression locus in *A. platys*.

4-2 To study the P44 predicted location and hypervariable region.

4-3 To search specific antigenic region for diagnostic potential.

Our results showed that all three PleC-PleD, NtrY-NtrX, and CckA-AhpT-CtrA are functional TCSs and expressed by *A. phagocytophilum* in HL-60 cell culture. Besides, our results on potential downstream target genes provide new insights into the roles of
TCS in *A. phagocytophilum* obligatory intracellular infection. Our result also identified the entire 4-kb *A. platys* *p44ES* locus, containing *trl*, *omp-IX* and *p44* genes for the first time, providing the new understanding of the *p44* expression locus and the major surface antigen of *A. platys*.

Thereby, the new knowledge generated in my Ph.D. study is expected to expand the understanding of *A. phagocytophilum* TCS signal transduction pathways and major outer membrane protein P44 in *Anaplasma* species. The results are expected to facilitate the study to understand these pathogens to improve diagnosis and treatment, and to develop a potential vaccine for anaplasmosis.
2.1 Abstract

*Anaplasma phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis (HGA), has genes predicted to encode three sensor kinases, one of which is annotated PleC, and three response regulators, one of which is PleD. Prior to this study, the role of PleC and PleD in obligatory intracellular parasitism of *A. phagocytophilum*, and their biochemical activities are unknown. The present study illustrates the relevance of these factors by demonstrating that both pleC and pleD were expressed in an HGA patient. During *A. phagocytophilum* development in human promyelocytic HL-60 cells, PleC and PleD were synchronously upregulated at the exponential growth stage and downregulated prior to extracellular release. A recombinant PleC kinase domain
(rPleCHKD) has histidine kinase activity; no activity was observed when the conserved site of phosphorylation was replaced with alanine. A recombinant PleD (rPleD) has autokinase activity by using phosphorylated rPleCHKD as the phosphoryl donor, but not by two other recombinant histidine kinases. rPleCHKD could not serve as the phosphoryl donor for a mutant rPleD (a conserved aspartic acid, the site of phosphorylation, replaced by alanine) or two other A. phagocytophilum recombinant response regulators. rPleD had diguanylate cyclase activity to generate c-di-GMP from GTP in vitro. UV cross-linking of A. phagocytophilum lysate with rPleD and c-[32P]di-GMP detected an ~47-kDa endogenous protein, presumably c-di-GMP downstream receptor. A new hydrophobic c-di-GMP derivative, 2'-O-di(tert-butylidimethylsilyl)-c-di-GMP, inhibited A. phagocytophilum infection in HL-60 cells. Our results suggest that the two-component PleC-PleD system is a diguanylate cyclase and that a c-di-GMP-receptor complex regulates A. phagocytophilum intracellular infection.

2.2 INTRODUCTION

Anaplasma phagocytophilum is an obligatory intracellular bacterium and a member of the order Rickettsiales that infects granulocytes in various mammals (Dumler, Barbet et al. 2001). Infection of endothelial cells has been recently shown in vivo and in vitro (Munderloh, Lynch et al. 2004; Herron, Ericson et al. 2005). A. phagocytophilum causes human granulocytic anaplasmosis (HGA), an acute febrile disease that is potentially fatal, especially in elderly or immunocompromised individuals (Bakken, Dumler et al. 1994; Demma, Holman et al. 2005). Under an electron microscope, A. phagocytophilum is a pleomorphic bacterium of 0.2 to 2.0 µm in diameter, replicating in
the membrane-bound inclusion in the host cell cytoplasm. The developmental cycle of this bacterium consists of two forms: small dense cored cells (DC) with cell binding activity and the ability to enter host cells, and large reticulate cells (RC) that are differentiated from DC. RC again mature into DC, which are released upon host cell lysis (Woldehiwet and Scott 1982; Rikihisa 1991; Munderloh, Jauron et al. 1999; Ausmees, Mayer et al. 2001). However, little is known regarding the bacterial factors regulating \textit{A. phagocytophilum} intracellular growth and development.

The bacterial two-component system (TCS) is an ubiquitous signal transduction paradigm that controls response, adaptation, and resistance to a variety of environmental conditions (Parkinson and Kofoid 1992). TCSs are typically composed of a sensor kinase (SK) and a cognate response regulator (RR). In the cytoplasm, dimerization and intermolecular autophosphorylation of the His residue in the kinase domain occurs when the SK senses a particular environmental signal through the periplasmic sensor domain. The phosphoryl group is then transferred to an Asp residue in the phospho-receiver domain (hereafter called receiver domain) of a cognate RR (Parkinson and Kofoid 1992). This transfer, in turn, activates the output domain of the RR, which generally has DNA binding activity to regulate target gene transcription, or an enzymatic activity such as diguanlylate cyclase (DGC) associated with the GGDEF (Gly-Gly-Asp-Glu-Phe) domain containing the sequence motif GGDEF within the RR (Galperin, Nikolskaya et al. 2001). Genes predicted to encode three SKs and three RRs are found in \textit{A. phagocytophilum}, and an inhibitor of histidine kinases prevents \textit{A. phagocytophilum} infection of mammalian host cells (Cheng, Kumagai et al. 2006). Thus TCSs is considered essential for \textit{A.}
*phagocytophilum* infection. In a related bacterium, *Ehrlichia chaffeensis*, orthologs of these SKs and RRs were shown to constitute three functional pairs of TCSs (Kumagai, Cheng et al. 2006); however, a TCS has yet to be definitively identified for *A. phagocytophilum*.

One of the proven TCS pairs of *E. chaffeensis* (Kumagai, Cheng et al. 2006) and one of the predicted TCS pairs of *A. phagocytophilum* comprises the SK, PleC, and the RR, PleD, based on similarities of these proteins to the PleC and PleD produced by the aquatic free-living bacterium *Caulobacter crescentus* (Cheng, Kumagai et al. 2006). *C. crescentus* PleD has DGC activity to generate a bacterial second messenger, cyclic di-guanosine monophosphate (c-di-GMP) from GTP (Paul, Weiser et al. 2004). DGC activity has not been shown in any predicted PleD proteins containing a GGDEF motif in the order *Rickettsiales*, including *A. phagocytophilum* and *E. chaffeensis* (Cheng, Kumagai et al. 2006). Using mutation and reconstitution studies in *C. crescentus* and other bacteria, c-di-GMP was found to regulate bacterial cell surface–associated traits and community behavior such as cell-cell signaling, biofilm formation, motility, differentiation, and virulence (Romling, Gomelsky et al. 2005; Tamayo, Pratt et al. 2007).

Here, we demonstrate that during *A. phagocytophilum* development in human promyelocytic HL-60 cells, PleC and PleD were synchronously upregulated at the exponential growth stage and downregulated prior to extracellular release. *A. phagocytophilum* PleC has histidine autokinase activity and that phosphotransfer occurs from PleC to PleD, implying that PleC and PleD constitute a functional TCS. We also showed the evidence that *A. phagocytophilum* PleD has DGC activity.
2.3 MATERIALS AND METHODS

**Sequence analysis.** DNASTAR Protean program, PSORT analysis (http://psort.nibb.ac.jp), NCBI blast search (http://www.ncbi.nlm.nih.gov/BLAST), and Motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_query) were utilized to analyze deduced amino acid sequences. Protein motifs were determined by Motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_query) and ExPASy Scan Prosite of Redasoft Visual Cloning software (Redasoft, Toronto, Canada).

**Bacterial strains and culture.** *A. phagocytophilum* HZ was propagated in HL-60 cells, a human promyelocytic leukemia cell line (ATCC, Manassas, VA), in RPMI 1640 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine at 37°C in 5% CO₂/95% air. *Escherichia coli* strains, NovaBlue (Novagen, Madison, WI), DH5α (Invitrogen, Carlsbad, CA) and BL21(DE3) (Novagen), were cultured in Luria-Bertani broth (Sambrook, Fritsch et al. 1989) supplemented with either ampicillin (50 µg/ml) or kanamycin (50 µg/ml) as required.

**Reverse transcription PCR.** Total RNA was extracted from 5 × 10⁶ *A. phagocytophilum*–infected HL-60 cells (80% infected cells) with the RNeasy mini kit (Qiagen, Valencia, CA). Buffy coat specimens were prepared from the blood of HGA patient NY37 (Lin, Zhi et al. 2002), and total RNA was extracted with Trizol reagent (Invitrogen) and further purified with the RNeasy mini kit. RNA was reverse transcribed, and PCR was conducted as described (Lin, Zhi et al. 2002; Cheng, Kumagai et al. 2006).
To ensure the absence of DNA contamination in the RNA preparation, control assays were included that lacked reverse transcriptase.

**Construction of plasmids for expression of the histidine kinase domain (HKD) of SKs and full-length RRss.** Total DNA was extracted from *A. phagocytophilum*–infected HL-60 cells using a QIAamp DNA mini kit (Qiagen). The DNA fragments encoding HKDs of SKs and full-length of RRs were amplified by PCR with primers shown in Table 2.1 using *A. phagocytophilum* chromosomal DNA as a template. The PCR products were purified with the PCR purification kit (Qiagen). The amplified DNA fragments were digested with restriction enzymes and ligated into the restriction enzyme–digested vectors pET33b (Novagen) [for N-terminal His-tagged PleD (rPleDNHis), C-terminal His-tagged PleD (rPleDCHis), and rCckAHKD], pET11a (Novagen) (for rPleCHKD, rNtrX, and rCtrA), or pMALc2x (New England BioLabs, Ipswich, MA) (for rNtrYHKD). *E. coli* strain NovaBlue was transformed, plasmids were extracted, and the sequences of the cloned fragments were confirmed. Mutations that alter conserved His residues of rPleCHKD to Ala and conserved Asp residues of rPleDNHis to Ala were introduced using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers shown in Table 2.1. The mutations were confirmed by DNA sequencing.

**Expression and purification of recombinant proteins from *E. coli*.** Expression of recombinant proteins (rPleCHKD, rPleDNHis, rPleDCHis, rCckAHKD, rCtrA and rNtrX) was induced in *E. coli* BL21(DE3) and rNtrYHKD fused with maltose binding protein (MBP) was expressed in *E. coli* DH5α with 1 mM isopropyl-1-thio-β-D-
galactopyranoside. For antibody production all these proteins were purified from the *E. coli* insoluble fraction after solublization with 6 M urea using a HIS-Select Cartridge (Sigma-Aldrich, St. Louis, MO) or amylose resin (New England BioLabs). For biochemical assays, all these proteins except rCtrA were purified from the *E. coli* soluble fraction using a HIS-Select Cartridge or amylase resin and rCtrA was purified from the *E. coli* insoluble fraction followed by refolding as described (Kumagai, Cheng et al. 2006). Proteins were quantified with the BCA Protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

**Kinase and phosphotransfer assays.** Histidine autokinase activity of *A. phagocytophilum* rPleCHKD and phosphotransfer from rHKDs of SKs to recombinant RRs were assayed as described for *E. chaffeensis* (Kumagai, Cheng et al. 2006).

**Western blot analysis.** Proteins were separated by SDS-PAGE and transferred to a sheet of the nitrocellulose membrane as described (Kumagai, Cheng et al. 2006). The membrane was incubated with primary antibodies (1:500 dilution of antisera or 1:50 dilution of affinity-purified antibodies in Tris-buffered saline, 20 mM Tris-HCl pH 7.5, 0.15 M NaCl, with 1% skim milk) followed by incubation with a secondary antibody, horseradish peroxidase–conjugated goat anti-rabbit IgG (1:2,000) (KPL, Gaithersburg, MD). The membrane was then incubated with ECL Western blotting detection reagents (Pierce), and bands were visualized with the LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). Pre-immune rabbit sera were used as negative controls for primary antibodies. Rabbit anti-rPleCHKD and anti-rPleD were prepared by ProSci (Poway, CA) and Proteintech (Chicago, IL), respectively. Antibodies were pre-absorbed
with the soluble fraction of uninfected HL-60 cells or affinity purified using rPleC or rPleD (500 µg per gel), separated by SDS-PAGE, and transferred to a sheet of nitrocellulose membrane as described (Sambrook, Fritsch et al. 1989).

**Double-immunofluorescence labeling.** At 2 days post infection (p.i.), *A. phagocytophilum–infected* HL-60 cells (60% infected cells) were cytocentrifuged onto a glass slide and fixed for 15 s in Diff-Quik Fixative (Dade Behring Inc., Newark, DE) at room temperature. The fixed infected cells were incubated with rabbit anti-rPleCHKD serum at 1:100 dilution in phosphate-buffered saline (PBS: 136.5 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 27.5 mM KH2PO4, pH 7.4) or rabbit anti-rPleD antiserum at 1:20 dilution in PBS, and anti-P44 monoclonal antibody (mAb) 5C11 (IgG2b) at 1:100 dilution in PBS at 37°C for 1 h, followed by incubation with secondary antibodies (Alexa Fluor 488–conjugated goat anti-mouse IgG and Alexa Fluor 555–conjugated goat anti-rabbit IgG) (Molecular Probes, Eugene, OR) at a 1:100 dilution in PBS at 37°C for 1 h. As negative controls, *A. phagocytophilum–infected* HL-60 cells were incubated with pre-immune rabbit serum or mouse IgG2b isotype control (Wang, Kikuchi et al. 2006), as appropriate. Rabbit antisera (200 µl) were pre-absorbed on ice with 2 × 10^6 uninfected HL-60 cell sonicated lysate. Slides were analyzed using a Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments Inc., Melville, NY).

**Time course experiment.** *A. phagocytophilum–infected* HL-60 cells were harvested, sonicated on ice, passed through a 2.7-µm filter, and centrifuged at 18,000 × g for 10 min at 4°C to harvest host cell–free bacteria. Viability of bacteria was determined
by LIVE/DEAD BacLight kit (Invitrogen). To synchronize the infection, 1.46 × 10⁷ HL-60 cells were mixed with isolated host cell–free bacteria at 37°C for 3 h with constant shaking (40 rpm) on an orbital shaker (New Brunswick Scientific Inc., Edison, NJ), then washed three times with 10 ml RPMI 1640 medium to remove unbound bacteria, and resuspended in 72 ml RPMI 1640 medium with 5% fetal bovine serum. The infected cells at indicated time point cells were cytocentrifuged onto a glass slide and stained by Diff-Quik stain. Slides were analyzed in a Nikon alphaphot-2 light microscope (Nikon Instruments, Inc.). Cells were harvested at each time point by centrifuging at 18,000 × g at 4°C for 10 min, and stored at –80°C. To determine the amount of *A. phagocytophilum* in each sample, total DNA was extracted using the QIAamp DNA mini kit, and real-time PCR was performed using primers in Table 2.2 and the Brilliant SYBER Green QPCR core reagent kit (Stratagene) in real-time PCR equipment, MX3000P (Stratagene).

**DGC activity.** rPleDCHis was purified from *E. coli* soluble fraction as described for *E. chaffeensis* (Cheng, Kumagai et al. 2006). Protein (20 μg) was incubated with 20 mM acetyl phosphate for 30 min at room temperature and then with 50 nmol of GTP for 1-4 h at room temperature in reaction buffer (75 mM Tris-HCl, pH 8.0, 250 mM NaCl, 25 mM KCl, 10 mM MgCl₂, 1 mM DTT). After the sample was boiled at 95°C for 5 min, denatured proteins were pelleted by centrifugation and the resulting supernatant was filtered through a 0.22-μm filter and loaded onto a Synergi Fusion-RP C18 column (Phenomenex, Torrance, CA) attached to an ÄKTA purifier (GE Healthcare, Piscataway, NJ). Samples were eluted with a linear gradient of 3-20% methanol in 10 mM triethylammonium acetate buffer (pH 7.0), and eluted samples were detected at OD_{254}.  

22
Chemically synthesized c-di-GMP (Biolog, Bremen, Germany) was used as an HPLC standard.

**UV cross-linking with $^{32}\text{P}c$-di-GMP.** Recombinant *Pseudomonas aeruginosa* His$_6$-WspR, which has high DGC activity (Kulasakara, Lee et al. 2006), was purified from the *E. coli* soluble fraction using a HIS-Select Cartridge as described (Kumagai, Cheng et al. 2006). $\gamma$-$^{32}\text{P}$c-di-GTP was prepared by incubating 20 µg of rWspR, 100 µCi of $\gamma$-$^{32}\text{P}$-GTP (3,000 Ci /mmol, 10 mCi/ml) (PerkinElmer, Shelton, CT), and 80 pmol unlabeled GTP in 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT for 4 h at room temperature, then incubated with 10 u of calf intestine alkaline phosphatase (New England Biolabs) for 30 min at room temperature to hydrolyze unreacted GTP. c-di-GMP production and elimination of unreacted GTP in the reaction were confirmed by reverse-phase HPLC chromatography as described above after incubating 20 µg of rWspR and 100 pmol of unlabeled GTP under the same reaction conditions. To investigate *A. phagocytophilum* c-di-GMP binding proteins, the host cell–free *A. phagocytophilum* lysate (70 µg) and *A. phagocytophilum* rPleD (1 µg) were incubated with 0.1 µCi c$^{32}\text{P}$di-GMP with/without 2 µM of unlabeled c-di-GMP at room temperature for 10 min, UV cross-linked for 10 min, precipitated by 10% trichloroacetic acid, resuspended in SDS sample buffer, and subjected to SDS-PAGE. After electrophoresis, the gel was dried and exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA). The screen was scanned and bands quantified with a PhosphorImager 445 Si (Molecular Dynamics).
Effect of the c-di-GMP analog, 2'-O-TBDMS-c-di-GMP, on \textit{A. phagocytophilum} infection. 2'-O-TBDMS-c-di-GMP (C_{32}H_{58}N_{12}O_{14}P_{2}Si_{2}; MW = 952.99 Da) (>98% pure as determined by HPLC) is a c-di-GMP hydrophobic analog produced as an intermediate during chemical synthesis of c-di-GMP (Kawai, Nagata et al. 2003) and was dissolved in DMSO to make the stock solution. Host cell-free \textit{A. phagocytophilum} was incubated with 2'-O-TBDMS-c-di-GMP, at a final concentration of 0.05 mM in RPMI medium, or the same volume of DMSO (no more than 0.5% v/v) and incubated at 37°C for 2 h. After washing the treated bacteria twice with RPMI 1640 medium, the bacteria were added to uninfected HL-60 cells and continuously incubated in RPMI 1640 medium with 5% fetal bovine serum and 2 mM L-glutamine at 37°C. The cells were harvested, stained using Diff-Quik, and the percentage of \textit{A. phagocytophilum}–infected cells was scored in 100 HL-60 cells in triplicate culture wells. Images of Diff-Quik-stained cells were captured by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI) coupled to a Nikon microscope. Total DNA was extracted from an aliquot of samples and quantitative-PCR was performed using primer pairs in Table 2.2 and human GAPDH RT-F and human GAPDH RT-R to determine the relative number of organisms/HL-60 cells.

2.4 Results

\textbf{Sequence analysis of PleC and PleD.} Using hydropathy plots and the PSORT program, \textit{A. phagocytophilum} PleC (APH0944, 470 amino acid residues, after removal of
the 27-residue signal peptide, calculated molecular mass 50 kDa) was predicted to be an inner membrane protein with one transmembrane region and a histidine kinase domain (HKD) containing H, N, G1, G2 and G3 motifs (Parkinson and Kofoid 1992). The putative *A. phagocytophilum* PleC kinase domain (residues 206-470) has 41.5% amino acid sequence identity to the *C. crescentus* PleC kinase domain. The identity between *A. phagocytophilum* and *E. chaffeensis* PleC kinase domains is 67.9%, suggesting similar kinase activity. In contrast, the *A. phagocytophilum* PleC sensor domain (residues 1-205) has only 6.5% identity with the *C. crescentus* PleC sensor domain. While the stimulus for the *C. crescentus* PleC sensor domain is unknown, the higher identity (31.6%) between *A. phagocytophilum* and *E. chaffeensis* PleC sensor domains, probably reflects not only their close phylogenic relationship, but also their similar intracellular inclusion environment, which is distinct from the aquatic environment surrounding free-living *C. crescentus*.

*C. crescentus* PleD is an unorthodox RR consisting of a receiver domain (D1) with the phosphorylatable Asp, a receiver-like adaptor domain (D2), and the DGC domain(Ausmees, Mayer et al. 2001; Galperin, Nikolskaya et al. 2001). Alignment of *A. phagocytophilum* PleD (APH0551, 456 residues, calculated molecular mass 52 kDa) with *C. crescentus* PleD defined the three domains, D1, D2 and a GGDEF (Fig. 2.1). The D1 domain of *A. phagocytophilum* PleD contains the characteristic receiver residues (Stock 2003), including the predicted phosphorylation site, Asp53 (Fig. 2.1). The D2 domain contains Asp207, which corresponds to Asp53 of the D1 domain, but it does not contain the two other characteristic residues of the receiver domain of RRs (a hydroxyl-
containing residue and Lys). The C-terminal output domain of *A. phagocytophilum* PleD was found to contain four consensus regions characteristic of the GGDEF domain (Hecht and Newton 1995) associated to DGC activity (Ausmees, Mayer et al. 2001) (Fig. 2.1).

**Expression of PleC and PleD by *A. phagocytophilum***. We determined mRNA expression of *A. phagocytophilum pleC* and *pleD* in an HGA patient (NY37), who was previously confirmed to have HGA (Lin, Zhi et al. 2002), and in cultured cells by RT-PCR. Both *pleC* and *pleD* mRNAs were expressed by *A. phagocytophilum* in the blood of a patient NY37 and in HL-60 cells (Fig. 2.2). There was no amplicon detected in the absence of reverse transcriptase.

To assess PleC and PleD expression, localization, and function, DNA fragments encoding PleCHKD (Fig. 2.1, PleC, residues 206-470) and full-length PleD were cloned into *E. coli* expression vectors. Protein was expressed and purified to near homogeneity (Fig. 2.3A) from the insoluble inclusion bodies using nickel column chromatography. Rabbit antisera raised against rPleCHKD and rPleD specifically reacted with their respective recombinant proteins (Fig. 2.3A). The antisera also specifically recognized native proteins of *A. phagocytophilum* isolated from infected HL-60 cells (Fig. 2.3A). The size of each native protein correlated with the size based on the respective amino acid sequences. These antisera did not react with proteins derived from uninfected HL-60 cells (Fig. 2.3A). Pre-immune rabbit sera did not react with any proteins in uninfected HL-60 cells or *A. phagocytophilum* (data not shown).
Double immunofluorescence labeling of *A. phagocytophilum* in HL-60 cells with each of rabbit anti-rPleC and anti-rPleD and mouse mAb5C11 against *A. phagocytophilum* P44 confirmed that PleC and PleD were expressed by replicating *A. phagocytophilum* in microcolonies (morulae) in HL-60 cells (Fig. 2.3B). As negative controls for immunofluorescence labeling, *A. phagocytophilum*–infected HL-60 cells were incubated with the respective pre-immune rabbit serum and secondary conjugated anti-rabbit IgG or with the isotype control mouse IgG2b and secondary conjugated anti-mouse IgG. There was no detectable labeling with these sera, indicating that labeling with both mAb5C11 and rabbit anti-rPleC and anti-rPleD protein sera was specific (Fig. 2.3B).

**Synchronous culture of *A. phagocytophilum* and temporal expression of PleC and PleD.** Host cell–free *A. phagocytophilum* (liberated from heavily infected HL-60 cells by mild sonication) was filtered to yield bacteria of relatively homogenous size (0.2-0.5 μm) (Fig. 2.4A, Ap). Approximately 80% of the population was viable based on staining with LIVE/DEAD BacLight bacterial viability kit (Fig. 2.4A, L/D). A synchronous culture of *A. phagocytophilum* was prepared using this preparation as inoculum. At 0 h (0 h post washing to remove non-host-associated bacteria after 3 h incubation of HL-60 cells with host cell–free *A. phagocytophilum* at 37°C), only a few bacteria per host cell were localized by Diff-Quik staining. At 12 h p.i., almost every infected HL-60 cell contained 1 or 2 small morulae (1-2 μm in diameter); at 24 h p.i., almost every infected HL-60 cell had 1 or 2 intermediate-sized morulae (3-4 μm) loosely
packed with small bacteria. At 36 h p.i., almost every infected HL-60 cell had 1 or 2 intermediate-sized (3-4 μm) plus several small morulae (1-2 μm) densely packed with bacteria. At 48 h p.i., almost every infected HL-60 cell had several large morulae (3-5 μm) densely packed with bacteria. At 60 h p.i., almost every infected cell had several large morulae (3-10 μm) with clumped bacteria, and at 72 h p.i. almost every infected HL-60 cell began to lyse and remaining intracellular morulae were dispersed with some clumped bacteria (Fig. 2.4A). Quantitative PCR using a pair of primers specific for *A. phagocytophilum* pleD showed the lag phase of *A. phagocytophilum* growth for approximately 0-36 h and the exponential growth phase from 36-72 h p.i. (Fig. 2.4B).

After normalization using bacterial DNA, both PleC and PleD were synchronously detected starting at 36 h p.i. and peaked at 60 h p.i. in the mid-exponential growth stage (Fig. 2.4C).

**Autokinase activity of rPleCHKD and specific phosphotransfer from rPleCHKD to rPleD.** To characterize biological activities of properly folded *A. phagocytophilum* rPleCHKD and rPleD, recombinant proteins were purified from *E. coli* soluble extract. By culturing at lower temperature (20-30°C), rPleCHKD and rPleD became partially soluble and could be purified from the soluble *E. coli* sonicated extract by nickel affinity chromatography. rPleCHKD displayed autokinase activity (Fig. 2.5A) when [γ-32P]ATP was used as the phosphate donor. By aligning the *A. phagocytophilum* PleC sequence with other bacterial sensor kinases, we predicted that His244 of PleC
would be phosphorylated (Fig. 2.1). To confirm this prediction, we expressed and purified mutant rPleCHKD with His244 replaced by Ala through site-directed mutagenesis. Expression of the resulting mutant protein (rPleCHKDH244A) was induced and purified to near homogeneity using the same method as for the wild-type protein. No autokinase activity was detected for the mutant protein (Fig. 2.5A), indicating that the conserved His residue (His244 of PleC) is required for phosphorylation. rPleD alone did not have autokinase activity (Fig. 2.5A). When purified rPleD was mixed with autophosphorylated rPleCHKD, rPleD autokinase activity was detected by using phosphorylated rPleCHKD as the phosphoryl donor (Fig. 2.5A). When mutant recombinant PleD (rPleDD53A) was mixed with autophosphorylated rPleCHKD, no autokinase activity was detected (Fig. 2.5A), indicating that the conserved Asp53 is required for phosphorylation.

Of the three *A. phagocytophilum* RRs (rPleD and rNtrX purified from *E. coli* soluble fraction and rCtrA purified from *E. coli* insoluble fraction and refolded), only rPleD autokinase activity was detected by using phosphorylated rPleCHKD as phosphoryl donor (Fig. 2.5B). Of three autophosphorylated *A. phagocytophilum* rHKDs (rPleCHKD, rNtrYHKD, and rCckAHKD purified from *E. coli* soluble fraction), rPleD autokinase activity was detected only using phosphorylated rPleCHKD as phosphoryl donor (Fig. 2.5C). These results demonstrate specific phosphotransfer from PleC to PleD.
**DGC activity of rPleD.** To demonstrate DGC activity of *A. phagocytophilum* PleD, a C-terminal His-tagged recombinant PleD (rPleDCHis) was phosphorylated by acetylphosphate. Upon incubation of rPleDCHis with GTP, c-di-GMP was detected by HPLC using synthetic c-di-GMP as standard (Fig. 2.6).

**A. phagocytophilum[^32P]-c-di-GMP receptor.** DgrA, a c-di-GMP receptor that controls flagella motor function in *C. crescentus*, was recently detected using UV cross-linking with c-[^32P]di-GMP (Christen, Christen et al. 2007). The UV cross-linking assay with c-[^32P]di-GMP detected a ~47-kDa band of *A. phagocytophilum* native protein at the mid-exponential stage of growth (Fig. 2.7). Binding of c-[^32P]di-GMP was specific because a 1,000-fold excess of unlabeled c-di-GMP competitively blocked c-[^32P]di-GMP binding (Fig. 2.7). *A. phagocytophilum* PleD has an amino acid sequence similar to the binding site for allosteric product inhibition as described for *C. crescentus* PleD (Munderloh, Lynch et al. 2004; Paul, Weiser et al. 2004). Specific binding of c-[^32P]di-GMP was also demonstrated with *A. phagocytophilum* rPleDHNHis, suggesting allosteric inhibition of *A. phagocytophilum* PleD DGC by c-di-GMP (Fig. 2.7).

**Inhibitory effect of a hydrophobic derivative of c-di-GMP.** We used a pharmacological approach to investigate the function of c-di-GMP in *A. phagocytophilum*. *A. phagocytophilum* was preincubated with a newly developed hydrophobic derivative of c-di-GMP, 2'O-TBDMS-c-di-GMP, and added to uninfected
HL-60 cells. Pretreatment with at 0.05 mM (final concentration) 2'-O-TBDMS-c-di-GMP for 2 h significantly inhibited *A. phagocytophilum* infection in HL-60 cells compared to DMSO control as determined by real time PCR and scoring infected cells (Fig. 2.8). At 0 and 36 h, p.i., both 2'-O-TBDMS-c-di-GMP- and DMSO-treated bacteria were detected at similar levels on or in HL-60 cells by Diff-Quik staining (Fig. 2.8A). In contrast, at 84 h p.i., approximately 16 and 1% HL-60 cells were infected with *A. phagocytophilum* in DMSO and 2'-O-TBDMS-c-di-GMP-treated group, respectively (Fig. 2.8B). At 132 h p.i., approximately 20% and 1% HL-60 cells were infected with *A. phagocytophilum* in DMSO and 2'-O-TBDMS-c-di-GMP-treated group, respectively, and some heavily infected HL-60 cells in DMSO-treated group were lysed (Fig. 2.8).

2.5 Discussion

The present study demonstrates *A. phagocytophilum* PleCHKD autokinase activity and the direct and specific phosphotransfer from *A. phagocytophilum* PleC to *A. phagocytophilum* PleD. In *C. crescentus*, two SKs, PleC and DivJ, phosphorylate PleD *in vitro* (Paul, Weiser et al. 2004), and PleC kinase activity is required for PleD DGC activity *in vivo* (Paul, Jaeger et al. 2008). Amino acid identity between *C. crescentus*, PleCHKD and DivJHKD is 43.0%. We determined one of three *A. phagocytophilum* SKs is PleC, but not DivJ, since the HKD of *A. phagocytophilum* PleC has higher sequence identity to the HKD of *C. crescentus* PleC (37.4% identity) than to the HKD of *C. crescentus* DivJ (32.1% identity). These results are similar to those for *E. chaffeensis*
PleC and PleD (Kumagai, Cheng et al. 2006), and perhaps can be extended to \textit{pleC} and \textit{pleD} orthologs found in other members of the order Rickettsiales (Cheng, Kumagai et al. 2006). There is no functional homolog of DivJ in \textit{A. phagocytophilum}, since there was no observed cross-reaction with other putative \textit{A. phagocytophilum} TCSs, as no phosphotransfer was detected from \textit{A. phagocytophilum} rPleCHKD to other RR\textsc{s} (rNtrX and rCtrA) or from other HK\textsc{d}s (rNtrYHKD and rCckAHKD) to rPleD.

In the present study, the amount of PleD per bacterium drastically changed during \textit{A. phagocytophilum} growth. Although \textit{pleC} and \textit{pleD} do not constitute an operon, the synchronized pattern of PleC and PleD expression in cultured HL-60 cells (37°C) demonstrates that they are co-regulated in \textit{A. phagocytophilum} during intracellular developement. In \textit{C. crescentus}, the temporal expression of PleC and PleD levels during bacterial development has not been reported, however, temporal changes in PleD-GFP distribution has been reported: during stalk formation, PleD localizes to the cell pole where the stalk is developed, whereas in swarmer cells PleD is distributed diffusely throughout the cytoplasm (Paul, Weiser et al. 2004). Activated PleD at the stalk pole of \textit{C. crescentus} has been hypothesized to be responsible for the morphologic swarmer-to-stalk cell differentiation via the action of the messenger molecule c-di-GMP (Paul, Weiser et al. 2004). In \textit{A. phagocytophilum}, PleC and PleD expression peaked during the mid-exponential growth phase, and thus c-di-GMP might control the transition between reticulate cells and dense-cored cells. If the \textit{A. phagocytophilum} PleC-PleD system functions as a bacterial developmental cycle regulator, these proteins might be essential for obligatory intracellular parasitism (and thus survival) of \textit{A. phagocytophilum}. 
Mutation of either pleC or pleD abrogates normal cell differentiation; however, PleC and PleD are not essential for C. crescentus viability (Sommer and Newton 1989).

In the present study, we demonstrate that A. phagocytophilum rPleD has DGC activity. We found that the DGC activity of A. phagocytophilum rPleD was much lower than that of P. aeruginosa rWspR (data not shown). This result is consistent with the fact that no A. phagocytophilum protein has been predicted that contains an EAL(Glu-Ala-Leu) (www.sanger.ac.uk/Software/Pfam) (Simm, Morr et al. 2004) or HD-GYP domain (a subgroup of the HD superfamily of metal dependent phosphohydrolases that contain an additional GYP motif) (Ryan, Fouhy et al. 2006) associated with c-di-GMP-specific phosphodiesterase activity. While there may be some other types of PDE lurking about in the background waiting to be discovered, it is possible that A. phagocytophilum PleD DGC activity is low to avoid c-di-GMP overproduction. Additionally, A. phagocytophilum PleD contains a predicted interactive-site similar to the C. crescentus PleD interactive-site, which allows allosteric noncompetitive feedback inhibition by c-di-GMP (Christen, Christen et al. 2006). This feedback inhibition may maintain PleD DGC activity at a low level in A. phagocytophilum. In support of this hypothesis, the c-di-GMP cross-linking study showed c-di-GMP binding to A. phagocytophilum rPleD. A hydrophobic derivative of c-di-GMP, 2'-O-TBDMS-c-di-GMP, inhibited A. phagocytophilum infection in HL-60 cells. These data suggest that c-di-GMP levels need to be temporarily regulated during A. phagocytophilum development inside host cells.

Inhibitory mechanisms by 2'-O-TBDMS-c-di-GMP warrant further investigation, as this is the first c-di-GMP analog shown to have anti-microbial effects. This c-di-GMP
A derivative may potentially competitively block the *A. phagocytophilum* c-di-GMP receptors and uncouple c-di-GMP from the downstream events. Alternatively, when a large amount of the unregulated c-di-GMP derivative is taken up by *A. phagocytophilum*, it may dys-regulate global c-di-GMP signaling pathway.

Although c-di-GMP regulates bacterial cell surface–associated traits and community behavior such as cell-cell signaling, biofilm formation, motility, differentiation, and virulence (Romling, Gomelsky et al. 2005; Tamayo, Pratt et al. 2007), little is known about the downstream effector molecules. Recently, the c-di-GMP binding domain, PilZ, was identified in proteins from several bacteria, and PilZ-containing proteins are suggested to serve as adaptor molecules to downstream effectors (Amikam and Galperin 2006). A PilZ-containing protein, DgrA, was recently shown to indirectly control flagellar motor function in *C. crescentus* (Christen, Christen et al. 2007). Our c-di-GMP crosslinking study suggests that *A. phagocytophilum* expresses c-di-GMP binding proteins of ~47 kDa. Recently, it was reported that c-di-GMP may be sensed by riboswitch to regulate downstream genes (Sudarsan, Lee et al. 2008). These observations suggest c-di-GMP might play a versatile role in bacterial signal transduction pathway. Further characterization of c-di-GMP binding proteins and the mechanisms by which 2'-O-TBDMS-c-di-GMP inhibits *A. phagocytophilum* infection would provide new insights into the roles of c-di-GMP and TCSs in obligatory intracellular parasitism.
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<sup>a</sup> Restriction enzyme cleavage sites are underlined.

<sup>b</sup> NA: not applicable

Table 2.1 Oligonucleotides used for cloning and expression of *Anaplasma phagocytophilum* sensor kinases and response regulators, and their mutants.
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Table 2.2 Oligonucleotides used for gene-specific real-time PCR in *Anaplasma phagocytophilum*. 
Figure 2.1 Schematic representation of the domain structures of PleC and PleD in *Anaplasma phagocytophilum* and *Caulobacter crescentus*.

Numbers represent amino acids residues. The horizontal bars above PleC and PleD indicate regions cloned for the functional study. Percentages in parentheses between the two aligned proteins indicate sequence identity.
Figure 2.2. *pleC* and *pleD* are transcribed by *Anaplasma phagocytophilum* in the blood of an HGA patient and in human myelocytic leukemia HL-60 cells. M, molecular size marker; D, positive control (chromosomal DNA used as template) for the PCR reaction; + and – indicate the presence or absence of reverse transcriptase, respectively. Genes and base pair sizes of amplified products are indicated.
Figure 2.3. PleC and PleD are expressed by *Anaplasma phagocytophilum* in human myelocytic leukemia HL-60 cells.

**A.** Lanes 1 and 2, nickel affinity–purified rPleC; lanes 5 and 6, nickel affinity–purified rPleD; lanes 3 and 7, *A. phagocytophilum*-infected HL-60 cells; lanes 4 and 8, uninfected HL-60 cells. Lanes 1, 2, 5, and 6, 0.5 μg protein per lane; lanes 3, 4, 7, and 8, 20 μg protein per lane. Lanes 1 and 5, Coomassie brilliant blue stain; lanes 2-4, western blotting using anti-rPleC; lanes 6-8, western blotting using anti-rPleD. Note rPleCHKD (30 kDa) and *A. phagocytophilum* native PleC (50 kDa) recognition by anti-rPleC. rPleD (56 kDa) and *A. phagocytophilum* native PleD (52 kDa) recognition by anti-PleD.

**B.** The infected HL-60 cells at 2 days p.i. (60% infected cells) were double labeled for immunofluorescence. The following antisera were used: mAb anti-*A. phagocytophilum* P44 (anti-mouse IgG, green, P44), and anti-rPleC or anti-rPleD (anti-rabbit IgG, red, PleC or PleD). The panels on the right are superimposed images viewed with green and red filters (Merge) and a phase contrast image (Phase Contrast) to show the relative intracellular location of morulae. As controls for immunofluorescence labeling, *A. phagocytophilum*–infected HL-60 cells were incubated with pre-immune rabbit serum (Preimmune) and secondary conjugated anti-rabbit IgG, or with mouse isotype IgG2b control and secondary conjugated anti-mouse IgG. The scale bar is 5 μm.
Fig. 2.3
Figure 2.4. PleC and PleD expression in synchronously cultured *Anaplasma phagocytophilum*.

**A. Synchronously cultured *A. phagocytophilum***. Ap, Host cell–free *A. phagocytophilum* used as inoculum, Diff-Quik stain. L/D, LIVE/DEAD BacLight bacterial viability test of the host cell–free *A. phagocytophilum*; green: live bacteria, red: dead bacteria. 0-72, synchronously cultured *A. phagocytophilum* in HL-60 cells using host cell–free *A. phagocytophilum* harvested from 0 to 72 h p.i., Diff-Quik stain. The bacteria or morulae are indicated by arrowheads. The scale bar is 10 μm.

**B. Synchronous growth of *A. phagocytophilum* as determined by quantitative PCR.**
Total DNA was extracted at 0 to 72 h p.i. The amount of DNA was determined by quantitative PCR and is presented relative to the DNA content measured at 0 h p.i., which was defined as 1. The values are the mean ± standard deviations of triplicate samples.

**C. Temporal expression of PleC and PleD during *A. phagocytophilum* intracellular development.** PleC and PleD expression was determined by western blot analysis using antisera against *A. phagocytophilum* rPleCHKD and rPleD. Amounts of bacterial genome equivalents loaded per lane were normalized by real-time PCR using the single-copy genomic *pleD* as template. Note stronger band density of both PleC and PleD at 60 h p.i.
Fig. 2.4
**Figure 2.5.** *Anaplasma phagocytophilum* PleC has specific His-dependent autokinase activity, and phosphotransfer from *A. phagocytophilum* PleC to PleD is dependent on a specific Asp of PleD.

**A.** Autoradiogram showing the specific His-dependent autokinase activity of rPleCHKD. Ten micrograms each of the wild-type rPleCHKD (lane 1) and rPleCHKDH244A (lane 2) were incubated with \([\gamma-^{32}P]ATP\). Only the wild-type HKD was \(^{32}P\)-phosphorylated. Lanes 4 and 5, autoradiogram showing the specific Asp-dependent phosphotransfer of \(^{32}P\) from rPleCHKD to rPleDs. rPleCHKD (2 \(\mu\)g each) was incubated with \([\gamma-^{32}P]ATP\) followed by incubation with 20 \(\mu\)g of rPleD (lane 4) or rPleDD53A (lane 5). rPleD alone (lane 3) was incubated with \([\gamma-^{32}P]ATP\) as a negative control.

**B.** Autoradiogram showing phosphotransfer of \(^{32}P\) from rPleCHKD to three recombinant response regulators. rPleCHKD (10 \(\mu\)g each) was incubated with \([\gamma-^{32}P]ATP\) (lane 1, PleC alone) followed by incubation with 20 \(\mu\)g wild-type rPleD (lane 2), rNtrX (lane 3), or rCtrA (lane 4). Phosphotransfer was evident only for rPleD (lane 2).

**C.** Autoradiogram showing phosphotransfer of \(^{32}P\) from three sensor kinases to rPleD. rPleCHKD, rCckAHKD, and rNtrYHKD (10 \(\mu\)g each) were incubated with \([\gamma-^{32}P]ATP\) (lanes 1, 3 and 5, HKD alone), followed by incubation with rPleD (20 \(\mu\)g) (lanes 2, 4 and 6). Phosphotransfer occurred only from rPleC (lane 2). Numbers on the left indicate molecular mass in kDa.
Fig 2.5

A

B

C

- rPleD
- rPleC

- rNtrY
- rPleD
- rCckA
- rPleC
Figure 2.6. *Anaplasma phagocytophilum* rPleD has di-guanylate cyclase activity.

rPleDCHis (20 μg) was incubated with 50 nmol GTP for 1 h at room temperature, then the sample was boiled, centrifuged, filtered, loaded onto an HPLC reverse-phase RC-18 column, and eluted with a methanol gradient (dash line). The solid line is a representative elution profile, and retention times of GTP and chemically synthesized c-di-GMP standard are indicated.
Figure 2.7. *Anaplasma phagocytophilum* produces a c-di-GMP binding protein.

Autoradiogram of host cell-free *A. phagocytophilum* lysate (AP, 70 μg) and nickel affinity–purified rPleD (PleD, 1 μg) incubated with c-[\(^{32}\)P]di-GMP followed by UV cross-linking without (-) or with (+) 1,000-fold excess unlabeled c-di-GMP (2 μM). A ~47-kDa protein band was detected (arrow), and the c-[\(^{32}\)P]di-GMP binding was blocked by unlabeled c-di-GMP.
Figure 2.8. 2′-O-TBDMS-c-di-GMP inhibits *Anaplasma phagocytophilum* infection in human myelocytic leukemia HL-60 cells. *A. phagocytophilum* were pretreated with DMSO solvent control (DMSO) or 0.05 mM 2′-O-TBDMS-c-di-GMP, and after washing incubated with HL-60 cell in the absence of the compound.

A. Representative Diff-Quik-stained images of HL-60 cells infected with *A. phagocytophilum* pretreated with 0.05 mM 2′-O-TBDMS-c-di-GMP, or DMSO at 0, 36, 84 or 132 h p.i. Arrowheads, bacterial inclusion or bacteria. The scale bar is 10 μm.

B. Relative number of organisms/HL-60 cells at 84 and 132 h p.i. determined by real-time PCR in triplicate wells. Percentage of infected HL-60 cells at 84 and 132 h p.i. were counted in 100 HL-60 cells in triplicate wells.

* Significantly different from the DMSO solvent control by Student's *t*-test (*P* < 0.05, *n* = 3).
Fig. 2.8
CHAPTER 3

CCKA, AHPT, AND CTRA PHOSPHORELAY AND CTRA TARGETS IN

ANAPLASMA PHAGOCYTOPHILUM

3.1 Abstract

*Anaplasma phagocytophilum* is an obligatory intracellular bacterium of granulocytes, and causes human granulocytic anaplasmosis. Based on amino acid sequence homology, we previously predicted a two-component pair similar to *Caulobacter crescentus*: a hybrid sensor histidine kinase CckA, and response regulator CtrA in the *A. phagocytophilum* genome. In the present study, using *in silico* analysis, we identified a gene encoding an *A. phagocytophilum* histidine phosphotransferase (AhpT). RT-PCR, double immunofluorescence labeling, and western blot analysis showed that expressions of CckA, AhpT, and CtrA mRNA and CckA and CtrA proteins during *A. phagocytophilum* intracellular growth in human leukocytes. We in vitro reconstituted two phosphorelays initiated by *A. phagocytophilum* CckA, requiring AhpT, which lead to the phosphorylation of CtrA. To get insights into the entire network of transcription
regulation of the *A. phagocytophilum* genome by CtrA, we isolated a set of recombinant CtrA DNA binding domain (CtrADBD)-binding sequences using a modified method of genomic SELEX. From the DNA sequences of 14 independently isolated *A. phagocytophilum* DNA fragments by SELEX, the CtrA-binding sequences were identified in a total of six promoter regions on the *A. phagocytophilum* genome, including promoters of four known genes controlling nucleic acid biosynthesis, protein turnover, and iron-sulfur cluster assembly, and two hypothetical-unidentified genes. Of the four genes, only a single *ctrA* is shared with *Caulobacter* CtrA regulon. These results suggest that in *A. phagocytophilum* CckA-CtrA phosphorelay and CtrA autoregulation are similar to *Caulobacter*, however, cellular processes are regulated by CtrA through the control of distinct target genes.

3.2 Introduction

Human granulocytic anaplasmosis (HGA) caused by *Anaplasma phagocytophilum* is an emerging tick-borne zoonosis first defined in 1994. HGA is a moderate to severe systemic illness with approximately 40–60% of patients requiring hospitalization. The case-fatality rate for HGA is approximately 0.7% and largely relates to complicating opportunistic infections, although poor outcomes are also associated with antecedent medical condition, such as diabetes mellitus (Dumler, Madigan et al. 2007).

*A. phagocytophilum* belongs to the order Rickettsiales in the class α-proteobacteria (Dumler, Barbet et al. 2001). The life cycle of *A. phagocytophilum* involves a tick vector and mammalian hosts. *A. phagocytophilum* is an obligatory
intracellular bacterium that resides in human granulocytes (Goodman, Nelson et al. 1996). During the bacterial intracellular life cycle in human leukocytes, genes encoding and a putative transcription factor Tr1, and major surface protein P44s, are regulated by a DNA binding protein, ApxR (Wang, Kikuchi et al. 2007). A. phagocytophilum was shown to have biphasic intracellular growth stages (Woldehiwet and Scott 1982; Rikihisa 1991; Munderloh, Jauron et al. 1999), and a two-component pair: (PleC and PleD), a type IV secretion substrate (Ats-1), and type IV secretion machinery proteins (VirB6 and VirB9) were shown to be differentially expressed during intracellular growth in human leukocytes (Lai, Kumagai et al. 2009: Niu, 2006 #399: Niu, 2006 #400). However, regulation of A. phagocytophilum intracellular development is poorly understood.

A paradigm of bacterial developmental cycle regulation has been found in free-living aquatic bacterium, Caulobacter crescentus (Brown, Hardy et al. 2009). C. crescentus divides asymmetrically to produce two different progeny, a swarmer cell and a stalked cell, each with distinct morphological features and regulatory programs (McAdams and Shapiro 2009). The cell cycles of both Caulobacter daughter cell types have a cyclical genetic circuit controlled by four master regulatory proteins DnaA, GcrA, CtrA and CcrM. These proteins are synthesized and cleared from the cell one after the other over the course of the cell cycle. The cycle of these four master regulatory proteins control activation of modular subsystems in the appropriate sequence and timing relative to each other. Among genes encoding these four proteins, only dnaA and ctrA are conserved in the A. phagocytophilum genome (Hotopp, Lin et al. 2006).
CtrA is a two-component response regulator conserved among α-proteobacteria (Brassinga, Siam et al. 2002). Caulobacter CtrA is highly regulated at transcription, phosphorylation, and degradation steps. The transcription of ctrA is controlled by feedback regulation from two promoters controlled by CtrA. At the beginning of S phase, ctrA is transcribed from the ctrA P1 promoter, which contains a GAnTC site near the –35 region. As CtrA protein accumulates during S phase, it activates transcription from the ctrA P2 promoter and represses the P1 promoter (Domian, Reisenauer et al. 1999). P1 promoter is activated by CtrA in hemimethylated state and repressed by full methylation by CcrM (Reisenauer and Shapiro 2002). Phosphorylation of the CtrA occurs by two phosphorelays initiated by the hybrid histidine kinase CckA, each requires a histidine phosphotransferase (Hpt), called Chpt (Biondi, Reisinger et al. 2006). Another protein CpdR, a single domain response regulator, as it has CheY-like receiver domain, but lacks DNA binding domain, is also phosphorylated by CckA signal transduction pathway (Iniesta, McGrath et al. 2006). The phosphorylation state modulates CtrA DNA binding activity, but does not affect proteolysis (Ryan, Judd et al. 2002). CtrA degradation by the ClpXP protease depends on the colocalization of CtrA and ClpXP at the cell pole. CpdR, when in the unphosphorylated state, binds to ClpXP and localize at the cell pole. When CpdR is phosphorylated, ClpXP is delocalized, and CtrA is not degraded (Iniesta, McGrath et al. 2006). Therefore, CckA simultaneously activates CtrA and prevents its degradation by delocalizing the CpdR/ClpXP complex. The ClpXP is localized to the cell pole via unphosphorylated CpdR accumulation (Iniesta, McGrath et al. 2006). The CtrA is brought to the activated polar protease by the combined action of the RcdA localization factor and the PopA cyclic di-GMP effector protein (McGrath, Iniesta et al. 2006; Duerig,
Abel et al. 2009). Although ClpXP is found, CpdR, RcdA, or PopA homolog has not been found in the *A. phagocytophilum* genome.

Up to now, the CtrA role in *A. phagocytophilum* has been poorly documented. In *A. phagocytophilum*, histidine kinase activity is essential for infection, and a *C. crescentus* CtrA homolog and its potential cognate histidine kinase, CckA have been found in *A. phagocytophilum* (Cheng, Kumagai et al. 2006). To gain insight into the role of the CtrA central regulator in *A. phagocytophilum*, we characterized its function as a response regulator and searched for specific target genes. Our data indicate that this protein is a functional response regulator that is phosphorylated on a conserved Asp residue by two phosphorelays initiated by CckA. Since no useful genetic manipulative system and no naturally isolated mutant has been available for obligatory intracellular bacteria including *A. phagocytophilum*, making the genetic approach to examine functions of TCS impossible. Using method of genomic SELEX, we identified putative *A. phagocytophilum* CtrA target genes that are different from those described in *C. crescentus*, except for *ctrA*, supporting the CtrA network plasticity in α-proteobacteria. The predicted function of the product of these genes suggests that, in *A. phagocytophilum*, CtrA could control cellular events including nucleic acid biosynthesis, protein turnover, and iron-sulfur cluster assembly.
3.3 MATERIALS AND METHODS

**Sequence analysis.** DNAStar Protean program, PSORT analysis (http://psort.nibb.ac.jp), NCBI blast search (http://www.ncbi.nlm.nih.gov/BLAST), SMART tool (http://smart.embl.de/), and Motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_query) were utilized to analyze deduced amino acid sequences. Protein motifs were determined by Motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_query) and ExPASy Scan Prosite of Redasoft Visual Cloning software (Redasoft, Toronto, Canada).

**Bacterial strains and culture.** *A. phagocytophilum* HZ was propagated in HL-60 cells, a human promyelocytic leukemia cell line (ATCC, Manassas, VA), in RPMI 1640 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine at 37°C in 5% CO₂/95% air. *Escherichia coli* strains, NovaBlue (Novagen, Madison, WI), DH5α (Invitrogen, Carlsbad, CA) and BL21(DE3) (Novagen), were cultured in Luria-Bertani broth (Sambrook, Fritsch et al. 1989) supplemented with either ampicillin (50 µg/ml) or kanamycin (50 µg/ml) as required.

**Reverse transcription PCR.** Total RNA was extracted from $5 \times 10^6$ *A. phagocytophilum*-infected HL-60 cells (80% infected cells) with the RNeasy mini kit (Qiagen, Valencia, CA). RNA was reverse transcribed, and PCR was conducted as described (Lin, Zhi et al. 2002; Cheng, Kumagai et al. 2006). To ensure the absence of DNA contamination in the RNA preparation, control assays were included that lacked reverse transcriptase.
Construction of plasmids and expression of recombinant proteins, CckA histidine kinase domain (HKD), CckA receiver domain (RD), *Anaplasma* histidine phosphotransferase (AhpT), CtrA, and CtrA DNA binding domain (DBD). The construction of plasmids and expression of three *A. phagocytophilum* HKDs of SKs and three full-length RRs were previously described (Lai, Kumagai et al. 2009). Recombinant CckA receiver domain (rCckARD), AhpT, and CtrA DNA binding domain (rCtrA DBD) were cloned with the primers shown in Table 3.1. *E. coli* strain NovaBlue was transformed, plasmids were extracted and the sequences of the cloned fragments were confirmed. Mutations that alter Glu661 of rCckAHKD-RD to stop codon (CckAHKD), conserved His residues of rCckAHKD (rCckAHKD H432A) and rAhpT (rAhpT H17A) to Ala, conserved Asp residues of rCckARD (rCckARD D741A) and rCtrA (rCtrA D53A) to Ala were introduced using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers shown in Table 3.1. The mutations were confirmed by DNA sequencing. Expression of recombinant proteins (rPle CHKD, rPle D, rCckAHKD-RD, rCtrA, MBP-rNtrYHKD and rNtrX) were previously described (Lai, Kumagai et al. 2009). rCckAHKD, rCckARD, rCckAHKD H432A, rCckARD D741A, rAhpT, rAhpT H17A, rCtrA D53A and rCtrA DBD were induced in *E. coli* BL21(DE3) with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). For biochemical assays, all these proteins except rCtrA were purified from the *E. coli* soluble fraction using a HIS-Select Cartridge (Sigma-Aldrich, St. Louis, MO) or amylose resin (New England BioLabs, Ipswich, MA). rCtrA from the *E. coli* insoluble fraction was solubilized by 2 M urea and 1% sarkosyl and purified by a HIS –Select
Cartridge. Then excess urea and sarkosyl were removed by dialysis. Proteins were quantified with the BCA Protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

**Kinase and phosphotransfer assays.** Histidine autokinase activity of *A. phagocytophilum* rCckAHKD and phosphotransfer from rCckAHKD to rCckARD, from rCckARD to rHpt, and from rHpt to rCtrA were assayed as described for *A. phagocytophilum* PleC and PleD (Lai, Kumagai et al. 2009).

**Western blot analysis.** Western blot analysis was performed as previously described (Kumagai, Cheng et al. 2006). Pre-immune rabbit sera were used as negative controls for primary antibodies. Rabbit anti-rCckAHKD-RD and anti-rCtrA were prepared by ProSci (Poway, CA) and Proteintech (Chicago, IL), respectively. Antibodies were pre-absorbed with the soluble fraction of uninfected HL-60 cells or affinity purified using rCckAHKD-RD or rCtrA (500 µg per gel), separated by SDS-PAGE, and transferred to a sheet of nitrocellulose membrane as described (Sambrook, Fritsch et al. 1989).

**Double immunofluorescence labeling.** Double immunofluorescence labeling was performed as previously described (Lai, Kumagai et al. 2009). As negative controls, *A. phagocytophilum*–infected HL-60 cells were incubated with pre-immune rabbit serum or mouse IgG2b isotype control (Wang, Kikuchi et al. 2006) as appropriate. Rabbit antisera (200 µl) were pre-absorbed on ice with 2 × 10^6 uninfected HL-60 cell sonicated lysate. Slides were analyzed using a Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments Inc., Melville, NY).
Solid phase DNA binding and systematic evolution of ligands via exponential enrichment (SELEX). For *A. phagocytophilum* genomic DNA library construction DNA was isolated from purified *A. phagocytophilum* (Lai, Kumagai et al. 2009), digested by Tsp509I (New England Biolab), and purified by PCR purification kit (Qiagen). rCtrADBD was induced in *E. coli* BL21(DE3) with 1 mM IPTG at 20°C, and purified by 50 µl magnetic beads of MagneHis™ protein purification system (Promega, Madison, WI) equilibrated with binding buffer (50 mM sodium phosphate, 50 mM NaCl, 20 mM imidazole; pH 8.0). After three washes with 500 µl of binding buffer, to reduce *E. coli* DNA contamination, the beads were washed with 400 µl 1.25 M NaCl then equilibrated with interaction buffer (20 mM Tris-HCl, 10 mM MgCl₂, 100 mM KCl, 20 mM imidazole; pH 8.0). Subsequently, 50 µg of Tsp509I-digested *A. phagocytophilum* genomic DNA was added to the rCtrADBD bound magnetic beads, and the mixture was incubated for 1 h at 4°C with constant shaking. After the beads were washed five times with interaction buffer, the bound DNA was eluted with 1.25 M NaCl, and the eluted DNA was purified by the PCR purification kit, and ligated into the alkaline phosphatase (New England BioLabs)-treated EcoRI site of pUC19. These plasmids were used as templates to amplify bound DNA fragments by PCR using M13/pUC forward primer and M13/pUC reverse primer (Table 3.1) as primers. PCR products were purified by the PCR purification kit and incubated with rCtrADBD bound magnetic beads. This SELEX cycle was repeated 4 times and the final PCR products cloned by using TA cloning kit (Invitrogen).
3.4 Results

**Sequence analysis of CckA and CtrA.** Using PSORT program, *A. phagocytophilum* CckA (APH0582; 784 amino acid residues, after removal of the 30-residue signal peptide; calculated molecular mass, 88 kDa) was predicted to be an inner membrane protein with one transmembrane region, an HKD containing H, N, G1, F, and G2 motif and a receiver domain (Parkinson and Kofoid 1992) (Fig. 3.1). Two PAS domain were predicted in the sensor domain using SMART tool (Fig. 3.1). The putative *A. phagocytophilum* CckA kinase domain (residues 340-658) and CckA receiver domain (residues 692-814) have 46% and 37% amino acid sequence identity to the *C. crescentus* CckA kinase domain and receiver domain, respectively. *A. phagocytophilum* CtrA (APH1099; 276 amino acid residues; calculated molecular mass, 31 kDa) was predicted to be a cytoplasmic protein with one hydrophobic region and a receiver domain and a DNA binding domain (Fig. 3.1). The putative *A. phagocytophilum* CtrA receiver domain (residues 1-119) and DNA binding domain (residues 120-276) have 47% and 58% amino acid sequence identity to the *C. crescentus* CtrA receiver domain and DNA binding domain, respectively.

**Search for Hpt in A. phagocytophilum.** Similar to *Caulobacter* CckA, *A. phagocytophilum* CckA is a hybrid kinase, which can directly phosphorylate a response regulator or initiate a phosphorelay in which a phosphoryl group is passed intramolecularly to the kinase's own receiver domain, then to a histidine phosphotransferase (Hpt), and finally to a response regulator. Hpts are difficult to identify
by sequence homology as they require conservation of only a small number of crucial residues, and none were predicted in the sequenced members of the order Rickettsiales. However, previously a weak consensus sequence deduced from several Hpt proteins have been reported (Ishige, Nagasawa et al. 1994). We searched Hpt candidate proteins in \textit{A. phagocytophilum} genome using the consensus (D-X-X-X-h-X-X-X-h-H-+-h-+-G-X-A-X-X-X-G-h; X is any amino acid, h is a hydrophobic amino acid, + is a positively charged residue), however, no candidate proteins were detected. We then used several less stringent consensus sequences to search \textit{A. phagocytophilum} Hpt proteins, and among them, the consensus sequence (h-X-X-X-h-H-X-h-X-S/G-S/A/G) yielded the hit of 16 ORFs. Five out of the 16 were annotated as hypothetical proteins. Besides the short consensus sequence, Hpt proteins share characteristic structural features: i) Hpt proteins are comprised of <250 amino acids, ii) X-ray crystallography of the Hpt domain of \textit{E. coli} ArcB revealed six $\alpha$-helixes with the conserved phosphorylatable His in one of the $\alpha$-helixes (Kato, Mizuno et al. 1997). A similar structure was determined for \textit{Saccharomyces cerevisiae} Hpt protein Ypd1p (Song, Lee et al. 1999; Xu and West 1999). Two \textit{A. phagocytophilum} ORFs (APH0203 and APH0827) matched the criteria, however, APH0827 is hydrophobic and predicted as an inner membrane protein with the potential phosphorylatable His residue likely within lipid bilayer, thus we concluded APH0203 is a putative Hpt and annotated as \textit{Anaplasma} histidine phosphotransferase (AhpT). BLAST search was conducted using APH0203 as a query and Hpt homologus were found in all sequenced members of the family \textit{Anaplasmataceae} (Table 3.2). \textit{Ehrlichia chaffeensis} homolog ECH0343 (E value: 5e-34) was annotated as \textit{Ehrlichia} histidine phosphotransferase (EhpT) which has a conserved His with some homologous amino
acids surrounding the His residue and the His residue lies in an \( \alpha \)-helix. No \( E. \) chaffeensis ortholog was detected for APH0827. The putative Hpt proteins of \( A. \) phagocytophilum and \( E. \) chaffeensis share some, but not all sequence features with \( C. \) crescentus ChpT that has been demonstrated as a histidine phosphotransferase (Biondi, Reisinger et al. 2006) (Fig. 3.1).

**Expression of CckA, AhpT, and CtrA by \( A. \) phagocytophilum in human leukocytes.** We determined the mRNA expression of \( A. \) phagocytophilum CckA, AhpT, and CtrA in cultured cells by RT-PCR. \( cckA, \) \( ahpT, \) and \( ctrA \) mRNA were transcribed by \( A. \) phagocytophilum in HL-60 cells (Fig. 3.2A). No amplicon was detected in the absence of reverse transcriptase.

To assess CckA and CtrA expression, localization, and function, DNA fragments encoding CckAHKD-RD and full-length CtrA were cloned into \( E. \) coli expression vectors. The proteins were expressed and purified to near homogeneity (Fig. 3.2B and 3.2C) from the insoluble inclusion bodies using nickel column chromatography. Rabbit antisera raised against rCckAHKD-RD and rCtrA specifically reacted with their respective recombinant proteins (Fig. 3.2B and 3.2C). The antisera also specifically recognized native proteins of \( A. \) phagocytophilum isolated from infected HL-60 cells (Fig. 3.2B and 3.2C). These antisera did not react with proteins derived from uninfected HL-60 cells (Fig. 3.2B and 3.2C). Preimmune rabbit sera did not react with any proteins in uninfected HL-60 cells or \( A. \) phagocytophilum (data not shown).
Double-immunofluorescence labeling of *A. phagocytophilum* in HL-60 cells with rabbit anti-rCckAHKD-RD and anti-rCtrA and mouse MAb 5C11 against *A. phagocytophilum* P44 confirmed that CckA and CtrA were expressed by replicating *A. phagocytophilum* in microcolonies (morulae) in HL-60 cells (Fig. 3.3). As negative controls for immunofluorescence labeling, *A. phagocytophilum*-infected HL-60 cells were incubated with the respective preimmune rabbit serum and secondary conjugated anti-rabbit IgG or with the isotype control mouse IgG2b and secondary conjugated anti-mouse IgG. There was no detectable labeling with these sera, indicating that labeling with both MAb 5C11 and rabbit anti-rCckA and anti-rCtrA protein sera was specific (Fig. 3.3).

**Autokinase activity of rCckAHKD and specific phosphotransfers from**

**rCckAHKD to rCckARD, from rCckARD to rAhpT, and from rAhpT to rCtrA.** To characterize the biological activities of properly folded *A. phagocytophilum* rCckAHKD, rCckARD, rCckAHKD-RD and rAhpT, recombinant proteins were purified from *E. coli* soluble fraction. By culturing bacteria at lower temperature (20 to 30°C), rCckAHKD, rCckARD, rCckAHKD-RD and rAhpT became soluble and could be purified from the *E. coli* soluble fraction by nickel affinity chromatography. rCtrA was refolded by 2 M urea and 1% Sarkosyl and purified from the *E. coli* insoluble fraction. rCckAHKD displayed autokinase activity when [γ-^32^P]ATP was used as the phosphate donor (Fig. 3.4A). By aligning the *A. phagocytophilum* CckAHKD, CckARD, AhpT and CtrA sequences with other bacterial SKs and RRs, we predicted that His432 of CckA, Asp741 of CckA, His17
of AhpT and Asp53 of CtrA would be phosphorylated (Fig. 3.1A). To confirm this prediction, we cloned mutant rCckAHKD with His432, mutant rCckARD with Asp 741, mutant rAhpT His17 and mutant rCtrA with Asp53 replaced by Ala through site-directed mutagenesis. Expression of the resulting mutant proteins (rCckAHKDH432A, rCckARDD741A, rAhpTH17A and rCtrAD53A) was induced, and it was purified to near homogeneity using the same method as for the wild-type protein. rCckA displayed autokinase activity (Fig. 3.4A) when [γ-32P]ATP was used as the phosphate donor. No autokinase activity was detected for the mutant rCckAHKD (Fig. 3.4A), indicating that the conserved His residue (His432 of CckA) is required for phosphorylation. When purified rCckARD was mixed with autophosphorylated rCckAHKD, rCckARD autokinase activity was detected by using phosphorylated rCckAHKD as the phosphoryl donor (Fig. 3.4B). When mutant recombinant CckARD (rCckARDD741A) was mixed with autophosphorylated rCckAHKD, no autokinase activity was detected (Fig. 3.4B), indicating that the conserved Asp741 is required for phosphorylation. rCckARD alone did not have autokinase activity (Fig. 3.4B). When purified rAhpT was mixed with autophosphorylated rCckARD or rCckAHKD-RD, rAhpT histidine phosphotransferase activity was detected by using phosphorylated rCckARD or CckAHKD-RD as the phosphoryl donor (Fig. 3.4D). When mutant recombinant AhpT (rAhpTH17A) was mixed with autophosphorylated rCckARD or rCckAHKD-RD, no histidine phosphotransferase activity was detected (Fig. 3.4C and 3.4D), indicating that the conserved His17 is required for phosphorylation. rAhpT alone did not have autokinase activity (Fig. 3.4C and 3.4D). When purified rCtrA was mixed with autophosphorylated rAhpT, rCtrA phosphorylation was detected by using phosphorylated rAhpT as the
phosphoryl donor (Fig. 3.4E). When mutant recombinant CtrA (rCtrADD53A) was mixed with autophosphorylated rAhpT, no CtrA autokinase activity was not detected (Fig. 3.4E), indicating that the conserved CtrAAasp53 is required for phosphorylation. rCtrA alone did not have autokinase activity (Fig.3.4E).

**SELEX.** To study the downstream target genes of *A. phagocytophilum* CtrA, a recombinant CtrA DNA binding domain (rCtrADBD) was purified from *E. coli* by magnetic beads to homogeneity and nearly single band in GelCode™ blue protein stain (Fig. 3.5A), and no detectable band in 1.25 M NaCl and interaction wash fractions, indicating that rCtrADBD was not eluted during purification and wash condition. After incubation of Tsp509I-digested *A. phagocytophilum* genomic DNA library with magnetic beads-bound rCtrADBD, 14 independently DNA fragments were isolated with Tsp509I enzyme site AATT on both ends. Total 6 of 14 fragments were in promoter regions. Two of them were found upstream of hypothetical proteins (APH0595 and APH0484), and four of them (*purC, clpX, hscB* and *ctrA*) have known function. The consensus CtrA binding motif sequences (CtrA box) TTAAN7TTAAC (9-mer), and TTAACCAT (8-mer) have been identified in CtrA promoter region without and with two miss matches, respectively (Fig. 3.5B). The Caulobacter CtrA-binding consensus sequence TTAAN7TTAA with up to 3 base miss matches were found in all other five promoter regions. PurC, phosphoribosylaminoimidazole-succinocarboxamide synthase [EC:6.3.2.6] is involved in purine nucleotide metabolism (Zhang, Morar et al. 2008). ClpX is an ATP-dependent protease ATP-binding subunit belonging heat-shock protein
100 family (Grimaud, Kessel et al. 1998). ClpX subunits form a six membered ring stabilized by binding of ATP, and function as ATP-dependent molecular chaperone and are the regulatory subunit of the ClpXP protease (Kim, Wu et al. 2003). HscB (heat shock cognate) in bacteria encodes a 20-kDa J-type co-chaperone, designated Hsc20, that functions with the hsp70-class molecular chaperone HscA/Hsc66 (Kawula and Lelivelt 1994; Seaton and Vickery 1994; Vickery, Silberg et al. 1997). In vitro studies have shown that HscB/Hsc20 regulates interactions between the Fe-S cluster assembly protein IscU and the HscA/Hsc66 chaperone (Hoff, Silberg et al. 2000; Silberg, Hoff et al. 2001; Hoff, Ta et al. 2002; Vickery and Cupp-Vickery 2007).

3.5 Discussion

The CckA-CtrA system was well studied in C. crescentus to play a key role in controlling cell cycle-related events (McAdams and Shapiro 2009). CtrA homolog, CzcR is described in the Rickettsia prowazekii. Caulobacter CtrA and Rickettsia CzcR have similar binding patterns in replication origins of both bacteria (Brassinga, Siam et al. 2002). Although CckA and CtrA pair has been described in A. phagocytophilum (Cheng, Kumagai et al. 2006), their biochemical activity and functions have not been known. The present study is the first report of Hpt specific biochemical activities and phosphorelay of CckA-Ahpt-CtrA in the order Rickettsiales. Caulobacter CckA has a transmembrane region and a PAS-like motif in the sensor domain which is important for the stable accumulation of CckA at the new bacterial pole (Angelastro, Sliusarenko et al.). PAS motifs in prokaryotes often are found in the sensor domain of histidine kinases, where
they are involved in sensory functions or protein-protein interactions that regulate the activity of signal transduction system (Taylor and Zhulin 1999). One transmembrane region and two PAS domains were detected within in *A. phagocytophilum* CckA sensor domain, suggest it plays a sensor role on the membrane and might interact with other protein via PAS domain. There is also a single strongly hydrophobic region (aa 72-88) in both *A. phagocytophilum* and *E. chaffeensis* CtrA receiver domain, and we believe that this is the reason why rCtrA is insoluble and in *E. chaffeensis* one half of native CtrA is in the membrane fraction (Kumagai, Cheng et al. 2006).

In the present study, to overcome insolubility of CtrA, we used a mild solubilization method using 2 M urea and 1% Sarkosyl as described by (Tao, Liu et al.; Singh and Panda 2005) followed by His tag purification and demonstrated that *A. phagocytophilum* CtrA could be phosphorylated by *A. phagocytophilum* CckA via a AhpT domain protein in vitro. Our results suggest *A. phagocytophilum* Hpt protein AhpT is specifically involved in the phosphorelay consisting of CckA-AhpT-CtrA as in *C. crescentus* (Biondi, Reisinger et al. 2006).

The three component CckA-AhpT-CtrA configuration may integrate multiple signals into CtrA. In *A. tumefaciens* hybrid kinase VirA-VirG system, it has been suggested that while xenognostic sugar and low pH induces the phosphotransfer from VirA-HKD to VirA-RD, phenolic compounds induce the phosphotransfer from VirA-HKD to VirG (Mukhopadhyay, Gao et al. 2004). The Hpt protein which may be involved in this phosphorelay has not been identified. In the proposed model for *E. coli* ArcB (comprised of SD, HKD, RD, and Hpt domain)-ArcA system, phosphotransfer from
ArcB-Hpt to ArcA is mainly responsible for the adaptation to anaerobic conditions, whereas phosphotransfer from ArcB-HKD to ArcA appears to operate under fully aerobic conditions in response to a certain metabolic state (Matsushika and Mizuno 1998). Although the present study did not find the direct phosphotransfer from CckAHKD to CtrA in *A. phagocytophilum*, in *E. chaffeensis* direct phosphotransfer from CckAHKD to CtrA has been demonstrated (Kumagai, Cheng et al. 2006).

Although natural stimuli to activate *A. phagocytophilum* CckA have not been identified, we have found *cutA* gene encoding a periplasmic divalent metal cation tolerance protein (Tanaka, Tsumoto et al. 2004) is only 48 and 103 bp downstream of *cckA* of both *A. phagocytophilum* and *E. chaffeensis*, respectively. The short intergenic space between *cckA* and *cutA* suggests they may be an operon, and have related function. Perhaps CckA is sensing divalent metal cations.

In the present study, we demonstrated that *A. phagocytophilum* rCtrADBD has DNA binding activity using SELEX method. We found that *purC*, *clpX*, *hscB* and *ctrA* could be downstream target regulated by CtrA. In *Caulobacter* CtrA regulates ClpP (Skerker and Laub 2004). These results suggest CtrA regulates cell-cycle related events such as nucleotide metabolism, protein turnover, and iron-sulfur cluster assembly. Although none of these genes except *ctrA* were shared with *Caulobacter* CtrA, *Caulobacter* and *A. phagocytophilum* CtrA target different genes during their evolution to adapt to distinct survival and growth niche. One of these SELEX fragments (*purC*) is located at 500 bp upstream of the translation start site of *purC*. In *E. coli* and *Bacillus subtilis* the expression of genes involved with synthesis of inosine monophosphate is
regulated by a repressor protein encoded by \textit{purR} which binds to DNA operator (Gots, Benson et al. 1977; Saxild, Brunstedt et al. 2001). In \textit{B. subtilis} a guanine riboswitch provides a regulatory role to the 12-gene \textit{purEKBCSQLFMNHD} operon for de novo purine biosynthesis (Mandal, Boese et al. 2003). Riboswitch was not detected within 600 bp upstream of \textit{A. phagocytophilum purC} using RiboSW (Chang, Huang et al. 2009) and unlike \textit{E. coli} or \textit{B. subtilis} regulatory \textit{purR} is detected and \textit{purA}, \textit{purB}, \textit{purC}, \textit{purD}, \textit{purE}, \textit{purF}, \textit{purH}, \textit{purK}, \textit{purM} and \textit{purN} are scattered in the \textit{A. phagocytophilum} genome, and how purine transport and purine nucleotide biosynthesis are regulated in \textit{A. phagocytophilum} is unknown. Therefore, we kept \textit{purC} in the candidate list for the CtrA regulon.

CckA mediated CtrA cell cycle regulatory mechanism is evolutionarily conserved among \(\alpha\)-proteobacteria. In \textit{C. crescentus}, CtrA turnover is regulated by CdpR, RcdA and PopA cyclic di-GMP effector protein via ClpXP protease complex (Duerig, Abel et al. 2009). No \textit{A. phagocytophilum} protein has significant similarity to CtrA regulatory proteins CdpR, RcdA, or PopA. It is possible that in \textit{A. phagocytophilum} ClpX is directly regulated by CtrA to auto-regulate CtrA by protease degradation. Lastly, our SELEX assay also identified 2 hypothetical proteins could be CtrA downstream target genes, further characterization of these unknown proteins would provide new insights into the roles of CckA-AhpT-CtrA signal transduction pathway in obligatory intracellular parasitism.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Direction</th>
<th>Primer sequence$^a$</th>
<th>Target DNA size (bp)</th>
<th>Enzyme</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>CckAHKD</td>
<td>Forward</td>
<td>CTACCAAGAATATACGCAACTTAAGGTAAGAGTAGGAATTCGC</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCGATTTCTACTTCTTGACCTTAAGTTTGCGTATATTCCTTGTAAG</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CckARD</td>
<td>Forward</td>
<td>AACCATGGCGAGCACTGTGTTTGCTCGTAG</td>
<td>389</td>
<td>Ncol</td>
<td>pET-33b(+)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AACTCGAGTTTCTGGCTTTGCACGCTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AhpT</td>
<td>Forward</td>
<td>AACCATGGGCATGACCCAGAGCTGCTAGGC</td>
<td>636</td>
<td>Ncol</td>
<td>pET-33b(+)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AACTCGAGGGCTTCTTCCGACATAATCCGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CtrADB</td>
<td>Forward</td>
<td>AACCATGGCCACTTAAGAAAGATACGGTATGAGTAGAGC</td>
<td>392</td>
<td>Ncol</td>
<td>pET-33b(+)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AACTCGAGGGCTTCTTCCGACATAATCCGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CckAHKDH432A</td>
<td>Forward</td>
<td>CAATTAACCCGCTGCTGATAGCCAGCGATTTTATAATATATCTGACC</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTCACTATATTAAATACGGCTGCTATCCACCGGTATTTG</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CckARDD741A</td>
<td>Forward</td>
<td>CACATAGATGTTATTATCACGCGCGGGTGTAATGCCAGGTACTAGT</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>Reverse</td>
<td>restriction enzyme cleavage sites</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------</td>
<td>--------------------------------</td>
<td>-----------------------------------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>AhpTH17A</td>
<td>GCTTTTGACGCAAGCTGCGCTGATGTTGGGGT</td>
<td>ACCCAGACACCCGCAACATCGCCAGCAGCTCGCTGCAAAGC</td>
<td>[underlined]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CtrAD53A</td>
<td>GATTATGACGGTTTATACTGGCTATACCTTCCTGGAAGATT</td>
<td>AATCTTCCAGGAAAGTGTATAAGCCAGTATAAACAACGCTATAATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13/pUC</td>
<td>GTAAAACGACGCGCCAGT</td>
<td>CAGGAACAGCCTATGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Restriction enzyme cleavage sites are underlined.

* NA: not applicable

Table 3.1 Oligonucleotide primers used for cloning and expression CckAHKD, CckARD, AhpT, CtrADB, CckAHKDH432A, CckARDD741A, AhpTH17A, CtrAD53A and CtrA downstream targets.
<table>
<thead>
<tr>
<th>Bacteria strain</th>
<th>GenBank No. (locus)</th>
<th>% identity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anaplasma phagocytophilum</em> HZ</td>
<td>YP_504825 (APH_0203)</td>
<td>100</td>
</tr>
<tr>
<td><em>Anaplasma marginale</em> St. Maries</td>
<td>YP_154130 (AM982)</td>
<td>51.9</td>
</tr>
<tr>
<td><em>Anaplasma centrale</em> Israel</td>
<td>YP_003328318 (ACIS_00360)</td>
<td>50.5</td>
</tr>
<tr>
<td><em>Ehrlichia chaffeensis</em> Arkansas</td>
<td>YP_507163 (ECH_0343)</td>
<td>34.0</td>
</tr>
<tr>
<td><em>Ehrlichia ruminantium</em> Welgevonden</td>
<td>YP_180521 (Erum6570)</td>
<td>32.0</td>
</tr>
<tr>
<td><em>Ehrlichia canis</em> Jake</td>
<td>YP_303294 (Ecaj_0664)</td>
<td>33.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent amino acid sequence identities of the entire open reading frames are relative to the *A. phagocytophilum* gene by the alignment result generated with CLUSTAL W method.

Table 3.2 Hpt homologs found in sequenced genome of the family Anaplasmataceae.
<table>
<thead>
<tr>
<th>Locus ID</th>
<th>Gene name</th>
<th>Protein function</th>
<th>From ATG¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>APH_0119</td>
<td><em>purC</em></td>
<td>Phosphoribosylaminomidazole-succinocarboxamide synthase</td>
<td>-546 to -616</td>
</tr>
<tr>
<td>APH_0969</td>
<td><em>clpX</em></td>
<td>ATP-dependent Clp protease, ATP-binding subunit ClpX</td>
<td>-256 to -413</td>
</tr>
<tr>
<td>APH_0595</td>
<td>NA</td>
<td>Hypothetical protein</td>
<td>-217 to -397</td>
</tr>
<tr>
<td>APH_0484</td>
<td>NA</td>
<td>Hypothetical protein</td>
<td>-36 to -217</td>
</tr>
<tr>
<td>APH_0677</td>
<td><em>hscB</em></td>
<td>Co-chaperone protein hscB</td>
<td>-291 to -389</td>
</tr>
<tr>
<td>APH_1099</td>
<td><em>ctrA</em></td>
<td>DNA-binding response regulator CtrA</td>
<td>-462 to 123</td>
</tr>
</tbody>
</table>

¹. Pulled-down DNA fragments

Table 3.3 Putative *A. phagocytophilum* CtrA regulon identified by SELEX
Figure 3.1. Schematic representation of the domain structures of CckA, AhpT, and CtrA in *Anaplasma phagocytophilum* and *Caulobacter crescentus*, and consensus sequences to identify Hpt domain protein.

A. The numbers on each protein are amino acids residues. The conserved histidine (H) in HKs and the conserved aspartate (D) in RRs, GenBank accession numbers, and predicted molecular masses are shown. The percentages in the parentheses between the two aligned proteins indicate amino acid identity.

B. Alignment of amino acid sequences surrounding the conserved His residues in Hpt proteins of *A. phagocytophilum* and *E. chaffeensis*. Consensus 1 is from (Ishige, Nagasawa et al. 1994) and consensus 2 is the sequence used for Hpt protein search in *A. phagocytophilum* genome. Cc ChpT, *C. crescentus* ChpT; h, hydrophobic residue; +, positively charged residue; x, any amino acid; *, conserved His residue to be phosphorylated. The numbers on the left sides are amino acid numbers of each protein. The regions presented here lie in α-helix.
Fig 3.1
Figure 3.2. Expression of CckA, AhpT, and CtrA

A. M, molecular size marker; D, positive control (chromosomal DNA used as template) for the PCR reaction; + and – indicate the presence or absence of reverse transcriptase, respectively. Genes and base pair sizes of amplified products (the parentheses) are indicated.

B. Lanes 1 and 2, nickel affinity–purified rCckA, lane 3 *A. phagocytophilum*–infected HL-60 cells, and lane 4 uninfected HL-60 cells. Lane 1, GelCode™ blue protein stain and lanes 2-4, western blotting using anti-rCckAHKD-RD. Note rCckAHKD (47 kDa) and *A. phagocytophilum* native CckA (88 kDa) recognition by anti-rCckAHKD-RD.

C. Lanes 1 and 2, nickel affinity–purified rCtrA, lane 3 *A. phagocytophilum*–infected HL-60 cells, and lane 4 uninfected HL-60 cells. Lane 1, GelCode™ blue protein stain and lanes 2-4, western blotting using anti-rCtrA. Note rCtrA (32 kDa) and *A. phagocytophilum* native CtrA (31 kDa) recognition by anti-rCtrA.
Fig 3.2

A. **cckA (672 bp)**

M  D  +  -

B. 1  2  3  4

100-  50-

A. **ahpt (456 bp)**

M  D  +  -

B. 1  2  3  4

C. 1  2  3  4

37-  25-

A. **ctrA (385 bp)**

M  D  +  -

B. 1  2  3  4

C. 1  2  3  4

CckA  rCckA

CtrA
Figure 3.3. CckA and CtrA are expressed by *Anaplasma phagocytophilum* in human myelocytic leukemia HL-60 cells. The infected HL-60 cells at 2 days p.i. (60% infected cells) were double labeled for immunofluorescence. The following antisera were used: mAb anti-*A. phagocytophilum* P44 (anti-mouse IgG, green, P44), and anti-rCckAHKD-RD or anti-rCtrA (anti-rabbit IgG, red, CckA or CtrA). The panels on the right are superimposed images viewed with green and red filters (Merge) to show the colocalization. The scale bar is 5 µm.
Figure 3.4. CckA autokinase activity and phosphotransfer relay CckA-HKD-CckA-RD-AhpT-CtrA

A. Autoradiogram showing the specific His-dependent autokinase activity of rCckAHKD. Ten micrograms each of the wild-type rCckAHKD (lane 1) and rCckAHKDH432A (lane 2) were incubated with \( [\gamma^{32}\text{P}]\text{ATP} \). Only the wild-type HKD was \( ^{32}\text{P} \)-phosphorylated.

B. The specific Asp-dependent phosphotransfer of \( ^{32}\text{P} \) from rCckAHKD to rCckARD. rCckAHKD was incubated with \( [\gamma^{-32}\text{P}]\text{ATP} \) (lane 1) followed by incubation with rCckARD (lane 2) or rCckARDD53A (lane 3). rCckARD alone (lane 4) was incubated with \( [\gamma^{-32}\text{P}]\text{ATP} \) as a negative control.

C. The specific His-dependent phosphotransfer of \( ^{32}\text{P} \) from rCckARD to rAhpT. rCckAHKD was incubated with \( [\gamma^{-32}\text{P}]\text{ATP} \) followed by incubation with rCckARD then followed by incubation with rAhpT (lane 1) or rAhpTH17A (lane 2). rAhpT alone (lane 3) was incubated with \( [\gamma^{-32}\text{P}]\text{ATP} \) as a negative control.

D. The specific His-dependent phosphotransfer of \( ^{32}\text{P} \) from rCckAHKD-RD to rAhpT. rCckAHKD-RD was incubated with \( [\gamma^{-32}\text{P}]\text{ATP} \) (lane 2) followed by incubation with rAhpT (lane 3) or rAhpTH17A (lane 4). rAhpT alone (lane 1) was incubated with \( [\gamma^{-32}\text{P}]\text{ATP} \) as a negative control.

E. The specific Asp-dependent phosphotransfer of \( ^{32}\text{P} \) from rAhpT to rCtrA. rCckAHKD-RD was incubated with \( [\gamma^{-32}\text{P}]\text{ATP} \) followed by incubation with rAhpT
(lane 1) then followed by incubation with rCtrA (lane 2) or rCtrAD53A (lane 3). rCtrA alone (lane 4) was incubated with \([\gamma^{32}P]ATP\) as a negative control.

Fig 3.4
Figure 3.5 CtrA SELEX pull-down assay of putative CtrA downstream genes.

A. DNA fragment containing *A. phagocytophilum* CtrA DNA binding domain was cloned into pET33b vector, expressed, and purified using magnetic beads of MagneHis™ protein purification system. Purified protein was subjected to 12% SDS-PAGE analysis followed by GelCode™ blue protein stain. rCtrADBD (lane 1). M, Prestained protein size standards.

B. Mapping putative/consensus CtrA binding site in the ctrA promoter. The CtrA recognition motif TTAAN7TTAAC is boxed. The CtrA recognition motif TTAACCAT with 2 miss match is underlined. The presumed translational start codon is marked by bent arrow.
Fig. 3.5

A

M 1 2

20

15

CtrA DBD

B

-500 TTGCAATCCTGCTGGGTTTTGCAATTAGGATTCAATTGGCATATTGATTCGTAAATTTAGGAGTAT

-420 AGCAAAATGTTCTTCATATAGTTTTGCAAGATTAAAGCTCTACCTACCTATGACGATGCAATGGAAT

-340 GGGAGCTGTATATAGCGTTACGGGAAGGGGTTTTTGTTAGATTAATATTCGTTATGAACTGTCAAC

-260 CTGTGCAATGTTTCTGTTACCCCTGTGCTGGCTGCTGCTGCTGCTGAGTTATGTTCTTAATGACCGACTTTTGCT

-180 AGGGGATGACCGTGGGGGACTCTCTAGTGTAATAGCTAAGAAATAATATGATATACATTTTCGCTAGACACCTGCCTAA

-100 TAATGTACGTATGCACTTTCTCTCTCAGGAGGTGATGGAAGAAACTTTTGGATCGTGTGGTGGGCTGCGGTTTGT

CtrA

-20 TGAGTTTTTGGGAGGGATTACCTGCTGTTAAATGAGATGTTGCGCCTGGGCTAAAGGAGTCTGAGGGTCTTTTG
CHAPTER 4

ANAPLASMA PHAGOCYTOPHILUM NTRY AND NTRX TWO-COMPONENT SYSTEM, AND ROLE OF NTRX IN INTRACELLULAR INFECTION

4.1 Abstract

*Anaplasma phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis (HGA), replicates in human granulocytes. In *A. phagocytophilum* we predicted a pair of putative two-component systems (TCS) designated as NtrY-NtrX based on amino acid sequence homology. Although DNA sequences encoding the NtrY-NtrX pair is found in many proteobacteria, their expression, regulation, and functions have not known well. The present study showed that both *ntrY* and *ntrX* were transcribed in the blood of an HGA patient. A recombinant NtrY kinase domain (MBP-rNtrYHKD) had specific histidine autokinase activity; no activity was observed when the conserved histidine was replaced with alanine. A recombinant NtrX receiver domain (rNtrXRD) was phosphorylated using phosphorylated MBP-rNtrYHKD as the phosphoryl donor, but not using other recombinant histidine kinases: CckA or PleC as the donor. MBP-rNtrYHKD could not transfer the phosphoryl group to a mutant rNtrXRD (with a conserved aspartic acid replaced by alanine) or two other *A. phagocytophilum*
recombinant response regulators. From the DNA sequences of 21 independently isolated
*A. phagocytophilum* DNA fragments by SELEX using NtrX, the NtrX-binding sequences
were identified in a total of five promoter regions on the *A. phagocytophilum* genome,
including promoters of an oxidoreductase FAD-binding protein, and a bicistronic operon
containing a hypothetical protein and PstB transporter, three additional hypothetical
unidentified genes. Palindromic consensus sequences were detected upstream of all these
genes. These results suggest NtrY-NtrX conserved two-component signal transduction
system, but evolved to regulate new genes with loss of nitrogen fixation in *A.
*phagocytophilum*.

4.2 Introduction

Human granulocytic anaplasmosis (HGA) caused by *Anaplasma phagocytophilum* is an emerging tick-borne zoonosis first identified in 1990. HGA is a
moderate to severe systemic illness with approximately 40–60% of patients requiring
hospitalization and treatment with oxytetracycline (Dumler, Choi et al. 2005). The case-
fatality rate for HGA is approximately 0.7% and largely relates to complicating
opportunistic infections, although poor outcomes are also associated with antecedent
medical condition, such as diabetes mellitus, elderly or immunocompromise (Dumler,
Madigan et al. 2007).

The bacterial two-component regulatory system (TCS) is an ubiquitous signal
sensing and transduction paradigm controlling response and adaptation to a variety of
environmental conditions (Parkinson and Kofoid 1992). The TCSs are typically composed of a histidine kinase (HK) and a cognate response regulator (RR). When the SK senses a particular environmental signal through the periplasmic sensor domain, dimerization and intermolecular autophosphorylation of the His residue in the cytoplasmic kinase domain occurs. The receiver domain of RR also has autokinase activity to use autophosphorylated cognate SK as the phosphoryl donor. This phosphorylation, in turn, regulates the output domain of the RR, which generally has DNA binding activity to control target gene transcription. *A. phagocytophilum* was found sensitive to HK inhibitor, closantel, \( N\)-(5-chloro-4-[(R,S)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl)-2-hydroxy-3,5-diiodobenzamide) in vitro, indicating that HK function is essential for *A. phagocytophilum* infection (Cheng, Kumagai et al. 2006).

Our analysis of the *A. phagocytophilum* HZ genome sequence (Hotopp, Lin et al. 2006) predicted total three TCS pairs, one of which were named NtrY-NtrX (Cheng, Kumagai et al. 2006) based on similarities of these proteins to the NtrY and NtrX, respectively produced by the *Azorhizobium caulinodans*, a nitrogen-fixing bacterium (Pawlowski, Klosse et al. 1991). The NtrX protein has been identified as a transcriptional activator of genes involved in the metabolic control of nitrogen sources. In *A. caulinodans* both NtrY/NtrX and NtrB/NtrC TCS pairs control expression of NifA, a transcription factor that regulates nitrogen fixation genes. Furthermore, *Azospirillum brasilense nifR3ntrBC* deletion mutant (HDK1) was complemented for nitrate-dependent growth by the *A. brasilense ntrYX* genes carried by plasmid pL46(Ishida, Assumpcao et al. 2002). A 52 bp NtrXD52E binding promoter sequence was reported in the upstream of
which encodes pigment binding proteins in a photosynthetic, nitrogen-fixing bacterium, *Rhodobacter capsulatus* (Gregor, Zeller et al. 2007). However, NtrY-NtrX expression, regulation, and functions in a non-nitrogen-fixing obligatory intracellular pathogen, *A. phagocytophilum* are unknown.

Here we demonstrate NtrY and NtrX transcripts in the blood of a HGA patient, and NtrY and NtrX protein expression during *A. phagocytophilum* development in human promyelocytic leukemia cell line HL-60. We also showed *A. phagocytophilum* NtrY histidine autokinase activity and specific NtrX receiver domain phosphorylation using phosphorylated NtrY as the phosphoryl donor, implying that *A. phagocytophilum* NtrY and NtrX constitute a functional TCS. Since no useful genetic system is available for obligatory intracellular bacteria including *A. phagocytophilum* or no naturally isolated mutant has been available, we used Systematic Evolution of Ligands by Exponential Enrichment (SELEX) system (Shimada, Fujita et al. 2005) to identify downstream genes regulated by NtrX in *A. phagocytophilum*.

### 4.3 Materials and methods

**Sequence analysis.** DNASTAR Protean program, PSORT analysis (http://psort.nibb.ac.jp), NCBI blast search (http://www.ncbi.nlm.nih.gov/BLAST), SMART tool (http://smart.embl.de/), and Motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_query) were utilized to analyze deduced amino acid sequences. Protein motifs
were analyzed by Motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_query) and ExPASy Scan Prosite of Redasoft Visual Cloning software (Redasoft, Toronto, Canada).

**Bacterial strains and culture.** *A. phagocytophilum* HZ was propagated in HL-60 cells, a human promyelocytic leukemia cell line (ATCC, Manassas, VA), in RPMI 1640 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine at 37°C in 5% CO₂/95% air. *Escherichia coli* strains, NovaBlue (Novagen, Madison, WI), DH5α (Invitrogen, Carlsbad, CA) and BL21(DE3) (Novagen), were cultured in Luria-Bertani broth (Sambrook, Fritsch et al. 1989) supplemented with either ampicillin (50 µg/ml) or kanamycin (50 µg/ml) as required.

**Reverse transcription PCR.** Total RNA was extracted from 5 × 10⁶ *A. phagocytophilum*–infected HL-60 cells (80% infected cells) with the RNeasy mini kit (Qiagen, Valencia, CA). Buffy coat specimens were prepared from the blood of HGA patient NY37 (Lin, Zhi et al. 2002), and total RNA was extracted with Trizol reagent (Invitrogen) and further purified with the RNeasy mini kit. RNA was reverse transcribed, and PCR was conducted as described (Lin, Zhi et al. 2002; Cheng, Kumagai et al. 2006). To ensure the absence of DNA contamination in the RNA preparation, control assays were included that lacked reverse transcriptase.

**Construction of plasmids and expression of recombinant proteins.** The construction of plasmids and expression of three *A. phagocytophilum* HKDs of SKs and three full-length RRs were previously described (Lai, Kumagai et al. 2009). Mutations that alter conserved His residues of MBP-rNtrYHKD to Ala (MBP-rNtrYHKDH491A),
Arg135 of rNtrX to stop codon (NtrXRD), and conserved Asp residues of rNtrXRD to Ala (rNtrXRDD60A) were introduced using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers shown in Table 4.1. The mutations were confirmed by DNA sequencing. Recombinant NtrX DNA binding domain (rNtrXDBD) was cloned with the primers shown in Table 4.1.

Expression of recombinant proteins (rPleCHKD, rPleD, rCckAHKD, rCtrA, MBP-rNtrYHKD and rNtrX) were previously described (Lai, Kumagai et al. 2009). rNtrXRD, rNtrXRDD60A and rNtrXDBD were induced in \textit{E. coli} BL21(DE3) and MBP-rNtrYHKDH491A was expressed in \textit{E. coli} DH5α with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). For biochemical assays, all these proteins except rCtrA were purified from the \textit{E. coli} soluble fraction using a HIS-Select Cartridge (Sigma-Aldrich, St.Louis, MO) or amylose resin (New England BioLabs, Ipswich, MA) and rCtrA was purified from the \textit{E. coli} insoluble fraction followed by refolding as described (Kumagai, Cheng et al. 2006). Proteins were quantified with the BCA Protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

**Kinase and phosphotransfer assays.** Histidine autokinase activity of \textit{A. phagocytophilum} rNtrYHKD and specific phosphotransfer from rHKDs of SKs to recombinant RRss were assayed as previously described (Kumagai, Cheng et al. 2006; Lai, Kumagai et al. 2009).

**Western blot analysis.** Western blot analysis was performed as previously described (Kumagai, Cheng et al. 2006). Pre-immune rabbit sera were used as negative
controls for primary antibodies. Rabbit anti-rNtrYHKD and anti-rNtrX were prepared by Proteintech (Chicago, IL) and ProSci (Poway, CA), respectively. Antibodies were pre-absorbed with the soluble fraction of uninfected HL-60 cells or affinity purified using rNtrYHKD or rNtrX (500 µg per gel) separated by SDS-PAGE, and transferred to a sheet of nitrocellulose membrane as described (Sambrook, Fritsch et al. 1989).

**Double-immunofluorescence labeling.** Double-immunofluorescence labeling was performed as previously described (Kumagai, Cheng et al. 2006). As negative controls, *A. phagocytophilum*–infected HL-60 cells were incubated with pre-immune rabbit serum or mouse IgG2b isotype control (Wang, Kikuchi et al. 2006), as appropriate. Rabbit antisera (200 µl) were pre-absorbed on ice with $2 \times 10^6$ uninfected HL-60 cell sonicated lysate. Slides were analyzed using a Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments Inc., Melville, NY).

**Solid phase DNA binding assay/SELEX.** *A. phagocytophilum* genomic DNA library was extracted from host cell-free purified *A. phagocytophilum* prepared as described (Lai, Kumagai et al. 2009), digested by Tsp509I (New England Biolab), and purified by PCR purification kit (Qiagen). rNtrXDBD was induced in *E. coli* BL21(DE3) with 1 mM IPTG at 20°C, and purified by 50 µl portion of Millipore PureProteome Nickel Magnetic Beads (Millipore, Temecula, CA) equilibrated with binding buffer (50 mM sodium phosphate, 50 mM NaCl, 20 mM imidazole; pH 8.0). After three washes with 500 µl of binding buffer, to reduce *E. coli* DNA fragments contamination, the beads were washed with 400 µl 1.25 M NaCl then equilibrated with interaction buffer (20 mM Tris-HCl, 10 mM MgCl2, 100 mM KCl, 20 mM imidazole; pH 8.0). Subsequently, 50 µg
of Tsp509I-digested *A. phagocytophilum* genomic DNA was added to the magnetic beads, and the mixture was incubated for 1 h at 4°C with constant shaking. After the beads were washed five times with 500 µl of interaction buffer, the bound DNA was eluted with 400 µl 1.25 M NaCl, and the eluate was purified by the PCR purification kit, then ligated into the alkaline phosphatase (New England BioLabs)-treated EcoRI site of pUC19. These plasmids were used as templates to amplify bound DNA fragments by PCR amplification using M13/pUC forward primer and M13/pUC reverse primer (Table 4.1). PCR products were purified by the PCR purification kit and incubated with rNtrXDBD bound magnetic beads. This SELEX cycle was repeated 1-4 times, and the final PCR products cloned by using TA cloning kit (Invitrogen).

4.4 Results

**Sequence analysis of NtrX and NtrY.** *A. phagocytophilum* NtrX (GenBank No. YP_504819) displayed significant homology (43.0% identity over the entire length, 47% for the putative receiver domain and 41% for the effector domain, respectively to *A. caulino
dans* NtrX (Pawlowski, Klosse et al. 1991) (Fig. 4.1A). The N-terminal region of NtrX contains a response regulatory domain and a conserved aspartate residue is also present in this domain, suggesting that *A. phagocytophilum* NtrX is activated by phosphorylation by the cognate histidine kinase NtrY (Kumagai, Cheng et al. 2006). NtrX and NtrC in other bacteria are activated by sigma54 RNA polymerase (σ54) which depends on activators that contain ATPase domains of the AAA+ class. The central
region of *A. phagocytophilum* NtrX contains an AAA+ ATPase domain: a NtrC family-specific Walker A (P-loop motif GXXGXGK[DE]) of AAA+ ATPase-specific helix-2 insert clade and a Walker B motif (DEXX), which are responsible for ATP binding and ATP hydrolysis, respectively; Sensor I motif bearing a conserved polar residue mediating interactions that are critical for ATP hydrolysis (Iyer, Leipe et al. 2004), and sensor II motif with a conserved arginine (Iyer, Leipe et al. 2004; Zhang, 2002 #353: Lee, 2003 #350). Sensor II appears to be critical in binding the ATP molecule to facilitate its hydrolysis and undergoes conformational changes depending on the presence of ATP or ADP (Iyer, Leipe et al. 2004). *A. phagocytophilum* NtrX arginine finger region which is also essential for ATP hydrolysis is conserved, however, one arginine is substituted with a cystine (C303) (Zhang, Chaney et al. 2002). The other arginine (R309) is conserved. σ54 interaction domain GAFTGA found in *A. brasilense* NtrX or NtrC (Zhang, Chaney et al. 2002) is absent in *A. phagocytophilum* NtrX (Fig. 4.1B). The helix-turn-helix (HTH) DNA binding domain of FIS family is located in the NtrX C-terminal region (Yuan, Finkel et al. 1991)(Fig.4.1A).

*A. phagocytophilum* NtrY (GenBank No.YP_504761) displayed the highest homology to *A. caulinoceans* NtrY (30% identity over the entire length, 23% for the putative sensor domain and 46% for the putative histidine kinase domain, respectively) including a sensor domain with an uncleavable signal sequence predicted by PSORT and 3 or 5 transmembrane regions were predicted by TMHMM2 in SMART tool (http://smart.embl.de/) or PSORT, respectively, a HAMP domain for dimerization interface, and a histidine kinase domain (Fig. 4.1A).
Expression of NtrY and NtrX by *A. phagocytophilum* in HGA patient blood

and cell culture. We determined the mRNA expression of *A. phagocytophilum* *ntrY* and *ntrX* in an acute stage patient (NY37) who was previously confirmed to have HGA (Lin, Zhi et al. 2002) and in cultured cells by reverse transcription PCR. Both *ntrY* and *ntrX* mRNA was expressed by *A. phagocytophilum* in the blood of patient NY37 and in HL-60 cells (Fig. 4.2). No amplicon was detected in the absence of reverse transcriptase.

To assess NtrY and NtrX protein expression, localization, and function, DNA fragments encoding NtrYHKD (Fig. 4.1 NtrY, residues 324 to 700), full-length NtrX, NtrXRD and NtrXDBD were cloned into *E. coli* expression vectors. The proteins were expressed and purified to near homogeneity from the insoluble inclusion bodies using nickel affinity chromatography (Figs.4.3A and 4.3B). Rabbit antisera raised against rNtrYHKD and rNtrX specifically reacted with their respective recombinant proteins (Figs. 4.3A and 4.3B). The antisera also specifically recognized native proteins of *A. phagocytophilum* isolated from infected HL-60 cells (Figs. 4.3A and 4.3B). The size of each native protein was correlated with the size based on the respective amino acid sequences. These antisera did not react with proteins derived from uninfected HL-60 cells (Figs. 4.3A and 4.3B). Preimmune rabbit sera did not react with any proteins in uninfected HL-60 cells or *A. phagocytophilum* (data not shown).

Double-immunofluorescence labeling of *A. phagocytophilum* in HL-60 cells with rabbit anti-rNtrY and anti-rNtrX and mouse MAb 5C11 against *A. phagocytophilum* P44 confirmed that NtrY and NtrX were expressed by replicating *A. phagocytophilum* in microcolonies (morulae) in HL-60 cells (Fig. 4.3C). As negative controls for
immunofluorescence labeling, *A. phagocytophilum*-infected HL-60 cells were incubated with the respective preimmune rabbit serum and secondary conjugated anti-rabbit IgG or with the isotype control mouse IgG2b and secondary conjugated anti-mouse IgG. There was no detectable labeling with these sera, indicating that labeling with both MAb 5C11 and rabbit anti-rNtrY and anti-rNtrX protein sera was specific (Fig. 4.3C).

**Antokinase activity of rNtrYHKD and specific phosphotransfer from rNtrYHKD to rNtrX.** To characterize the biological activities of properly folded *A. phagocytophilum* rNtrYHKD and rNtrXRD, recombinant proteins were purified from *E. coli* soluble extract. By culturing bacteria at lower temperature (20 to 30°C), rNtrYHKD and rNtrXRD became soluble and could be purified from the soluble *E. coli* sonicated extract by amylose resin or nickel affinity chromatography. rNtrYHKD displayed autokinase activity (Fig. 4.4A) when \( [\gamma-32P]ATP \) was used as the phosphate donor. By aligning the *A. phagocytophilum* NtrY and NtrX sequences with other bacterial SKs and RRss, we predicted that His491 of NtrY and Asp60 of NtrX would be phosphorylated (Fig. 4.1A). To confirm this prediction, we expressed and purified both mutant rNtrYHKD with His491 and mutant rNtrXRD with Asp60 replaced by Ala through site-directed mutagenesis. Expression of the resulting mutant proteins (rNtrYHKD\(H491A\) and rNtrXRD\(D60A\)) was induced, and it was purified to near homogeneity using the same method as for the wild-type protein. No autokinase activity was detected for the mutant rNtrYHKD (Fig. 4.4A), indicating that the conserved His residue (His491 of NtrY) is required for phosphorylation. When purified rNtrXRD was mixed with autophosphorylated rNtrYHKD, rNtrX autokinase activity was detected by using
phosphorylated rNtrYHKD as the phosphoryl donor (Fig. 4.4A). When mutant recombinant NtrXRD (rNtrXRDD60A) was mixed with autophosphorylated rNtrYHKD, no autokinase activity was detected (Fig. 4.4A), indicating that the conserved Asp60 is required for phosphorylation. rNtrXRD alone did not have autokinase activity (Fig. 4.4A).

Of the three *A. phagocytophilum* RRs (rNtrXRD and rPleD purified from the *E. coli* soluble fraction and rCtRA purified from the *E. coli* insoluble fraction and refolded), only rNtrXRD autokinase activity was detected by using phosphorylated rNtrYHKD as a phosphoryl donor (Fig. 4.4B). Of three autophosphorylated *A. phagocytophilum* rHKDs (rNtrYHKD, rPleCHKD, and rCckAHKD, purified from the *E. coli* soluble fraction) rNtrXRD autokinase activity was detected only using phosphorylated rNtrYHKD as a phosphoryl donor (Fig. 4.4C). These results demonstrate specific phosphotransfer from NtrY to NtrX.

**SELEX.** To study the downstream target genes of *A. phagocytophilum* NtrX, a recombinant NtrX DNA binding domain (rNtrXDBD) was purified from *E. coli* by magnetic beads to homogeneity and nearly single band in GelCode™ blue protein stain, and no detectable band in interaction wash fractions, indicating that rNtrXDBD was not eluted during purification and wash condition (Fig. 4.5A). After incubation of Tsp509I treated *A. phagocytophilum* DNA library with magnetic beads purified rNtrXDBD five *A. phagocytophilum* promoter regions were detected via TA cloning (Table 4.2). There are several GTTT rich sequence amount these five promoter regions of these five genes suggesting GTTT may be the consensus sequence of NtrX binding.
NtrX target promoters and their downstream target genes. Five NtrX downstream target promoters were identified by SELEX. Three of them (APH0234, APH0354, and APH1318) were hypothetical proteins, APH0325 is an oxidoreductase FAD-binding protein, and APH0312 is also a hypothetical protein. However, phosphate ABC transporter, ATP binding protein, \textit{pstB} (APH0313) is 6 bp downstream of APH0312, thus APH0312 and APH0313 are likely a bicistronic operon regulated by NtrX.

4.5 Discussion

The NtrY-NtrX system was identified in \textit{A. caulinodans} (Pawlowski, Klosse et al. 1991) and in \textit{A. brasilense} (Ishida, Assumpcao et al. 2002) as a homolog of NtrB-NtrC system that regulates the expression of nitrogen regulated (Ntr) genes; however, to date the biochemical evidence of phosphotransfer from NtrY to NtrX was demonstrated only in \textit{E. chaffeensis} (Kumagai, Cheng et al. 2006). The present study is the first report of specific biochemical activities of NtrY to NtrX in \textit{A. phagocytophilum}. NtrY is predicted to be a transmembrane protein in \textit{A. caulinodans} (Pawłowski, Klosse et al. 1991) and \textit{A. brasilense} (Ishida, Assumpcao et al. 2002). Three hydrophobic regions in the sensor domain were detected in \textit{A. phagocytophilum} NtrY, suggesting that it plays a membrane associated sensor role. The present study demonstrates \textit{A. phagocytophilum} NtrYHKD autokinase activity and the direct and specific phosphotransfer from \textit{A. phagocytophilum} NtrYHKD to NtrXRD. We had tried the full length \textit{A. phagocytophilum} rNtrX for
specific phosphotransfer, but unlike *E. chaffeensis* rNtrX, we could not detect full length rNtrX phosphorylation by cognate rNtrY in vitro (Kumagai, Cheng et al. 2006). Besides, signal from autophosphorylated MBP-rNtrYHKD also decreased during incubation with rNtrX in *A. phagocytophilum* (data not shown). Assumpcao *et al.* (Assumpcao, de Souza et al. 2007) demonstrated that *A. brasilense* NtrX AAA+ domain has ATPase activity, suggesting that *A. phagocytophilum* NtrX AAA+ domain could interfere the rNtrYHKD-rNtrX phosphotransfer in vitro by ATP depletion via the ATPase activity. The σ54 interaction domain GAFTGA is absent in *A. phagocytophilum* and *E. chaffeensis* NtrX, similar to *A. caulinodans* NtrX. Although *A. caulinodans* has σ54, *A. phagocytophilum* and *E. chaffeensis* lost σ54 (Hotopp, Lin et al. 2006), suggesting that loss of NtrX σ54 interaction domain may have preceded the loss of functional partner σ54 during reductive evolution of these obligatory intracellular bacteria. An AAA+ family response regulator, *Pseudomonas aeruginosa* FleQ binds and is regulated by the bacterial secondary messenger c-di-GMP (Hickman and Harwood 2008). Although we did not detect cross-talk from NtrY to PleD or CtrA, or from PleC or CckA to NtrX by biochemical phosphotransfer assay (Lai, Kumagai et al. 2009), we cannot deny the possibility that *A. phagocytophilum* NtrX is indirectly regulated by PleC-PleD via c-di-GMP (Schirmer and Jenal 2009). In *E. chaffeensis*, c-di-GMP did not bind to rNtrX in vitro (Kumagai, Y and Y. Rikihisa, unpublished data).

Functions of the NtrY-NtrX pair have been implied through bacterial mutation studies: In *A. caulinodans* both *ntrC* and *ntrX* mutants display retarded growth on nitrate, a reduction of *nifA* expression under nitrogen fixation conditions and a severely disturbed
symbiotic phenotype (Pawlowski, Klosse et al. 1991). However, free-living nitrogen fixation rates between \textit{ntrC} and \textit{ntrX} mutants differ and their growth patterns in medium containing different amino acids as N sources are distinct (Pawlowski, Klosse et al. 1991). The \textit{A. brasilense ntrYX} genes complement \textit{A. brasilense nifR3ntrBC} deletion mutant for nitrate-dependent growth (Ishida, Assumpcao et al. 2002). However, NtrX downstream target genes found in \textit{Rhodobacter capsulatus}, such as \textit{nifA}, \textit{puf}, or \textit{puc} (Gregor, Zeller et al. 2007) have not been identified in the \textit{A. phagocytophilum} genome. Moreover, \textit{A. phagocytophilum} NtrX downstream genes and consensus sequences implied by using SELEX were distinct and no homologs were detected in other nitrogen-fixing bacteria. Taken together, with the loss of ability to fix nitrogen, NtrY/NtrX evolved to regulate different sets of their target genes.

In the present study, we demonstrated that \textit{A. phagocytophilum} rNtrXDBD has DNA binding activity using SELEX method. We found that \textit{pstB} and \textit{thiO} could be downstream target genes regulated by NtrX. PstB is a component of the phosphate specific transport (PST) system mediating Pi uptake, which contains a transmembrane channel of two proteins PstC and PstA and a peripherally membrane-bound PstB (Levitz, Friedberg et al. 1985). All three \textit{pst} genes are present in \textit{A. phagocytophilum} (\textit{pstB}: APH0313, \textit{pstA}: APH0370, and \textit{pstC}: APH1060), although they do not constitute operon unlike in most other bacteria. ThiO is oxidoreductase, FAD-binding protein involved in thiamine biosynthesis (Settembre, Dorrestein et al. 2003). Besides, there are three hypothetical proteins which could be NtrX downstream target genes. Further
characterization of functions of these proteins could provide new insights into the roles of NtrY-NtrX signal transduction pathway in obligatory intracellular parasitism.
<table>
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<sup>a</sup> Restriction enzyme cleavage sites are underlined.

<sup>b</sup> NA: not applicable

Table 4.1. Oligonucleotides used for cloning and expression of *Anaplasma phagocytophilum* NtrYHKDH491A, NtrXRD, NtrXRDD60A, NtrXDBD, and NtrX downstream targets.
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Table 4.2. Putative *Anaplasma phagocytophilum* NtrX regulon identified by SELEX.
**Figure 4.1. Schematic representation of the domain structures of NtrX and NtrY in* Anaplasma phagocytophilum* and* Azorhizobium caulinodans.**

**A.** The numbers on each protein are amino acids residues. The conserved histidine (H) in HKs and the conserved aspartate (D) in RRs, GenBank accession numbers, and predicted molecular masses are shown. The bars above HKs and RRs are regions cloned for biochemical analysis. The percentages in the parentheses between the two aligned proteins indicate amino acid identity.

**B.** Structure-based sequence alignment of* A. ph* NtrX (*A. phagocytophilum* NtrX),* E. ch* NtrX (*E. chaffeensis* NtrX),* A. ca* NtrX (*A. caulinodans* NtrX),* A. br* NtrX (*A. brasilense* NtrX),* E. co* NtrC (*Escherichia coli* NtrC), and* K. pn* NtrC (*Klebsiella pneumonia* NtrC). Boxes contain conserved regions found within the AAA+ families including the Walker A, Walker B, Sensor I, Sensor II, Arginine finger, and σ54 interaction motifs.
Figure 4.2. ntrY and ntrX are transcribed by *Anaplasma phagocytophilum* in the blood of an HGA patient and in human myelocytic leukemia HL-60 cells. Total RNA was prepared from *A. phagocytophilum*-infected HL-60 cells and RT-PCR was performed using specific primers. D, positive control (chromosomal DNA was used as a template); + and – indicate the presence and absence of reverse transcriptase. Genes and base pair sizes of amplified products (the parentheses) are indicated.
Figure 4.3. NtrY and NtrX are expressed by *Anaplasma phagocytophilum* in human myelocytic leukemia HL-60 cells.

A. Lanes 1 and 2, Amylose resin affinity–purified MBP-rNtrYHKD; lane 3 *A. phagocytophilum*-infected HL-60 cells; lane 4 uninfected HL-60 cells. Lane 1, GelCode™ blue protein stain and lanes 2-4, western blotting using anti-MBP-rNtrYHKD. Note MBP-rNtrYHKD (84 kDa) and *A. phagocytophilum* native NtrY (77 kDa) recognition by anti-MBP-rNtrYHKD.

B. Lanes 1 and 2, nickel affinity–purified rNtrX, lane 3 *A. phagocytophilum*–infected HL-60 cells, and lane 4 uninfected HL-60 cells. Lane 1, GelCode™ blue protein stain and lanes 2-4, western blotting using anti-rNtrX. Note rNtrX (54 kDa) and *A. phagocytophilum* native NtrX (51 kDa) recognition by anti-rNtrX.

C. The infected HL-60 cells at 2 days p.i. (60% infected cells) were double labeled for immunofluorescence. The following antisera were used: mAb anti-*A. phagocytophilum* P44 (anti-mouse IgG, green, P44), and anti-MBP-rNtrYHKD or anti-rNtrX (anti-rabbit IgG, red, NtrY or NtrX). The panels on the right are superimposed images viewed with green and red filters (Merge) and a phase contrast image (Phase Contrast) to show the colocalization. The scale bar is 5 µm.
Fig 4.3

A  1  2  3  4  
100-  
50-  
rNtrY-MBP  
Native NtrY  

B  1  2  3  4  
50-  
rNtrX  
Native NtrX  

C

<table>
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Figure 4.4. *A. phagocytophilum* NtrY has specific His-dependent autokinase activity, and specific phosphotransfer from NtrY to NtrX is dependent on a specific Asp of NtrX.

A. Autoradiogram showing the specific His-dependent autokinase activity of MBP-rNtrYHKD. Ten micrograms each of the wild-type MBP-rNtrYHKD (lane 1) and MBP-rNtrYHKDH491A (lane 2) were incubated with $[\gamma^{32}\text{P}]\text{ATP}$. Only the wild-type HKD was $^{32}\text{P}$-phosphorylated. Lanes 4 and 5, autoradiogram showing the specific Asp-dependent phosphotransfer of $^{32}\text{P}$ from MBP-rNtrYHKD to rNtrXRD, MBP-rNtrYHKD was incubated with $[\gamma^{32}\text{P}]\text{ATP}$ followed by incubation with 20 µg of rNtrXRD (lane 4) or rNtrXRDD60A (lane 5). rNtrXRD alone (lane 3) was incubated with $[\gamma^{32}\text{P}]\text{ATP}$ as a negative control.

B. Autoradiogram showing phosphotransfer of $^{32}\text{P}$ from MBP-rNtrYHKD to three recombinant response regulators. MBP-rNtrYHKD (10 µg each) was incubated with $[\gamma^{32}\text{P}]\text{ATP}$ (lane 1, MBP-rNtrYHKD alone) followed by incubation with 20 µg wild-type rNtrXRD (lane 2), rPleD (lane 3), or rCtrA (lane 4). Phosphotransfer was evident only for rNtrXRD (lane 2).

C. Autoradiogram showing phosphotransfer of $^{32}\text{P}$ from three sensor kinases to rNtrXRD. MBP-rNtrXHKD, rPleCHKD, and rCckAHKD (10 µg each) were incubated with $[\gamma^{32}\text{P}]\text{ATP}$ (lanes 1, 3 and 5, HKD alone), followed by incubation with rNtrXRD (20 µg) (lanes 2, 4 and 6). Phosphotransfer occurred only from MBP-rNtrYHKD (lane 2). Numbers on the left indicate molecular mass in kDa.
Figure 4.5. NtrX SELEX and consensus sequence in the promoter regions.

A. DNA fragment containing *A. phagocytophilum* NtrX DNA binding domain was cloned into pET33b vector, expressed, and purified using magnetic beads of Millipore PureProteome™ protein purification system. Purified protein was subjected to 12% SDS-PAGE analysis followed by GelCode™ blue protein stain. rNtrXDBD was purified to nearly single band (lane 1) and no detectable band in interaction wash fraction (lane 2). Numbers on the left indicate molecular mass in kDa.

B. Mapping putative/consensus NtrX binding sites in the promoter regions of SELEX fragments. The TA cloned SELEX fragments were underlined. The palindromic motifs GAAA/TTTC inside these fragments are boxed. The presumed translational start codons are marked by bent arrows.
Fig 4.5

A

B

ATTAGCGATATAACTTACTGAGCGCTGATGCTGGGAAAAGATCTCTGATTATCTTTCAGTTATCAGCTAAGTT
GTATATGAAAAATTTATTTTTTTGTCGATGGAAATTTCCCTGTGGGAAAAGATTTATATATATGTGAGCTATG
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AAAAGACCAAGATTTGCGAAAAAGCAATGAAAGAAGCTCGTACGGTATTATTATTACGATGTGGAAAGAAAGATCA
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ACTCAGGCGCAAGATATTATCAAGTTATGTTATAGTTATGGTTATATTTAAAGTTACAGAAAGCAGAAGACGTTA
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---

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AAAAATCTTGTATGTCTTGGAATTTTTTCTCTTATAAGTATGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT

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ATGGGAGCTAT

APHO312

APHO354

APHO0234

APHO0325

APH1318
CHAPTER 5

POLYMORPHIC MAJOR OUTER MEMBRANE PROTEIN P44 EXPRESSION
LOCUS IN ANAPLASMA PLATYS

5.1 Abstrate

*Anaplasma platys* infects platelets and is known to cause canine infectious cyclic thrombocytopenia. Despite global distribution of this infection, *A. platys* has never been culture isolated. Consequently, very little is known about this bacterium and the disease it causes. In this study, we cloned the genetic locus similar to the polymorphic major outer membrane protein *p44/msp2* expression loci (*p44ES/msp2ES*) of *Anaplasma marginale* and *Anaplasma phagocytophilum*, using DNA isolated from the blood specimens from three dogs naturally infected with *A. platys*. The *A. platys p44ES* is situated downstream of a putative transcriptional regulator, *tr1* and an *Anaplasma phagocytophilum omp-1X* homolog, named *omp-1X* in this study, in a 4-kb genomic region. Polymorphic *p44ES* sequences are characterized by conserved 5’ and 3’ regions and the central hypervariable region. Predicted molecular masses of the four mature *A. platys* P44s range from 43.3 to 43.5 kDa. Comparative amino acid sequence analyses among *tr1s*, *omp-1X* homologs,
and p44/msp2 from A. platys, with those from A. phagocytophilum and A. marginale, showed 86.4 and 73.1 % (tr1), 45.9~46.3 and 39.8% (omp-1s), and 55.0~56.9% and 41.5~42.1% (p44/msp2) identity, respectively. Phylogenetic analysis of omp-1X and p44/msp2 from A. platys are more closely related to those from A. phagocytophilum than from A. marginale. These results suggest that this locus starts from tr1 followed by tandem genes encoding β-barrel outer membrane proteins had evolved from the common ancestor of Anaplasma and Ehrlichia spp, and this genomic locus drives antigenic variation of A. platys P44. The study provides insights into the pathogenic properties of A. platys and molecular data to develop species-specific diagnostic tests.

5.2 Introduction

Anaplasma platys (formerly Ehrlichia platys) is first described as a rickettsia-like agent in platelets of dogs from Florida with infectious canine cyclic thrombocytopenia (ICCT) in 1978 (Harvey, Simpson et al. 1978). Authors pointed out morphological and biological similarity of this bacterium to Ehrlichia canis in infected dogs and Anaplasma marginale in infected cattle, two members of the family Anaplasmataceae, well-known at that time. Clinical signs of ICCT are fever, depression, and anorexia (French and Harvey 1983). Parasitemia and thrombocytopenia occur in cycles at approximately 10-to 14-day intervals (French and Harvey 1983). Seropositive dogs have been found in Florida, Pennsylvania, Texas, Louisiana, Illinois, California, Arkansas, Mississippi, Idaho, and North Carolina, and high rates of A. platys and E. canis dual positive dogs were noted
(French and Harvey 1983). Based on indirect fluorescence antibody test using the platelet-rich plasma from a dog experimentally infected with ICCT, minimal serologic cross-reaction is thought to occur between *A. platys* and *E. canis*, and the authors proposed the new name “*Ehrlichia platys*” for this bacterium (French and Harvey 1983). In 1992 Anderson *et al.* (Anderson, Greene et al. 1992) reported the 16S rRNA gene sequence of *A. platys* from Lousiana. Subsequently *groEL* gene sequence of *A. platys* from Lousiana was determined (Yu, Zhang et al. 2001). Phylogenetic analysis of these sequences showed that this is a distinct bacterium closely related to *Anaplasma phagocytophilum* and *A. marginale*, which led to reclassification of this bacterium into the genus *Anaplasma* (Dumler, Barbet et al. 2001). Later it was reported that although *A. platys* do not cross-react with serum antibodies from dogs infected with *E. canis* on IFA tests, the *A. platys* antigen cross-reacts with anti-*A. phagocytophilum* antibodies (Inokuma, Fujii et al. 2002). *A. platys* DNA was detected in dogs from Greece (Mylonakis, Koutinas et al. 2004), France (Inokuma, Fujii et al. 2002), Taiwan (Chang and Pan 1996), Spain (Sainz, Amusategui et al. 1999), China (Hua, Yuhai et al. 2000), Australia (Brown, Martin et al. 2001), Portugal (Cardoso, Tuna et al. 2008), Republic of Congo (Sanogo, Davoust et al. 2003), Japan (Unver, Rikihisa et al. 2003), and Thailand and Venezuela (Suksawat, Pitulle et al. 2001; Huang 2005). *A. platys* has been detected from brown dog tick *Rhipicephalus sanguineus* in Okinawa, Japan (Inokuma, Raoult et al. 2000), Spain (Sparagano, de Vos et al. 2003), and Republic of Congo (Sanogo, Davoust et al. 2003). However, whether *R. sanguineus* is a biological vector of *A. platys* has not been experimentally demonstrated (Simpson, Gaunt et al. 1991). *A. platys* has never been culture isolated. Consequently this bacterium is poorly
known at molecular, cellular, or immunologic level, and no antigen has been identified for this bacterium as of today.

In *A. phagocytophilum* and *A. marginale*, surface-exposed immunodominant 44-kDa major outer membrane proteins (P44s/Msp2s) are encoded by the *p44 (msp2)* polymorphic multigene family (Barbet, Lundgren et al. 2000; Brayton, Palmer et al. 2002; Zhi, Ohashi et al. 2002). In *A. phagocytophilum*, P44 proteins consist of a single central hypervariable region of approximately 94 amino acid residues and an N-terminal and C-terminal conserved regions of approximately 186 and 146 amino acid residues, respectively (Lin, Zhi et al. 2002). A single polymorphic *p44/msp2* expression locus (*p44/msp2ES*) is found in the genome of *A. phagocytophilum* and *A. marginale*, respectively (Brayton, Kappmeyer et al. 2005; Hotopp, Lin et al. 2006). Both expression loci are found downstream of *tr1* genes encoding putative transcriptional factor and homologs of *Ehrlichia chaffeensis omp-1* genes encoding polymorphic major outer membrane protein (Barbet, Lundgren et al. 2000; Barbet, Meeus et al. 2003; Lin, Rikihisa et al. 2003). At *p44/msp2ES*, *p44s* and *msp2* donor sequences elsewhere in the genome undergo recombination via RecF pathway to allow various *p44/msp2ES* expression under the same promoter (Brayton, Palmer et al. 2002; Lin, Zhang et al. 2006). This mechanism is thought to facilitate P44/Msp2 antigenic variation persistent infection and for adaptation to new environments such as transmission between tick and mammalian hosts (Brayton, Palmer et al. 2002; Zhi, Ohashi et al. 2002; Rikihisa 2003; Barbet, Lundgren et al. 2006; Lin, Zhang et al. 2006). Native P44 of *A. phagocytophilum* was shown to have porin activity (Huang, Wang et al. 2007). In the present study, we first
examined whether a similar p44 expression locus exist in A. platys. Second, we compared the expression loci among three Anaplasma species. Third, we analyzed the structure, in particular, hypervariable region of A. platys P44s. This study provides the first information of A. platys p44 (msp2) polymorphic multigene family and their genomic expression locus.

5.3 Materials and methods

Cloning A. platys expression locus. DNA specimens from three dogs naturally infected with A. platys at Lara, Venezuela in 2007 were used as the template for the amplification and sequencing process. A. platys infection of the dog was confirmed by PCR and sequencing of the 16S rRNA of A. platys as well as by observation of bacterial inclusions (morulae) in platelets in the blood smear. By aligning A. phagocytophilum and A. marginale p44/msp2 expression loci, several degenerate primers were designed on conserved regions (Fig. 5.1A, Table5.1). Using the first primer pair F1 and R1, and the second primer pair F1 and R2, the (hemi-) nested touchdown PCR amplified A. platys trl and omp-1X sequences. In order to avoid truncated p44 pseudogenes in A. platys genome, primer F3 5’ upstream of the predicted p44 open reading frame (ORF) was designed. p44ES sequences were amplified by nested touchdown PCR with primer pairs F2 and R3, and F3 and R4. Amplification was performed as previously described (Zhang, Xiong et al. 2008). The amplified DNA fragments were cloned using TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced with M13 Forward or M13 Reverse sequencing primers.
All sequencing data were assembled using SeqMan program of DNASTAR software (DNASTAR Inc., Madison, WI). The deduced amino acid sequences of *A. marginale*, *A. phagocytophilum*, and *A. platys* *tr1*, *Omp-IX* and *p44ES* were aligned using the MegAlign program of DNASTAR software by Clustal W method.

**Protein structure analysis.** The P44 secondary structure was predicted by hydrophobicity and the hydrophobic moment profile method as previously described (Jeanteur, Lakey et al. 1991; Huang, Wang et al. 2007). Antigenic Index and surface probability were examined by Protean program of DNASTAR.

**Nucleotide sequence accession number.** The *A. platys* *tr1-omp-IX-p44ES* locus from two dogs were assembled and deposited at GenBank under accession No. GQ868750 and GU357491, respectively. Additional *p44ES* and *p44* sequences are given GenBank accession No. GU357492, GU357493, GU357494, GU357495, GU357496 and GU357497.

### 5.4 Results

**A. platys trl-omp1-p44ES cluster sequencing and assembly.** Three degenerate primers and one primer at highly conserved region of *tr1* upstream region were designed based on conserved regions of the aligned *tr1-omp1X-omp1N-p44* cluster of *A. phagocytophilum* and YP_154239 (hypothetical protein AM1138) (transcriptional regulator)- YP_154240 (outer membrane protein 1) (outer membrane protein 4)-YP_154241 (*msp2* operon associated gene 3) (outer membrane protein 3)-YP_154243
(msp2 operon associated gene 2)-YP_154244 (msp2 operon associated gene 1)-YP_154245 (msp2) cluster of A. marginale (Fig. 5.1A and Table 5.1). The first touchdown PCR (Roux and Hecker 1997) was designed to amplify the entire fragment using primers F1 and R1. The PCR products (undetectable) from the two dog DNA specimens were then used as templates for hemi-nested touchdown PCRs using primers F1 and R2 (Fig. 5.1A and Table 5.1). As a result, one single band about 2,100 bp was amplified (fragment A). The PCR products were TA cloned and sequenced. The sequence results showed this fragment contained A. platys tr1 and omp-1X. The fragment A downstream region was amplified by nested touchdown PCR using the PCR products obtained with primers F1 and R1 as templates, with primer F2 designed based on the fragment A sequence and primer R3 designed based on p44 (msp2) sequences conserved between A. phagocytophilum and A. marginale. As a result, a single band about 1,100 bp was amplified (fragment B). The PCR products were TA cloned and sequenced. The sequence results showed that this fragment contained a partial A. platys p44 sequence. To amplify A. platys full-length p44, primer F3 was designed based on fragment B sequence and primer R4 was designed based on the conserved region of valS found downstream of p44 (msp2) in both A. phagocytophilum and A. marginale. Another touchdown PCR was done using primers F1 and R4. The PCR products were then used as templates for hemi-nested touchdown PCRs using primers F3 and R4. As a result, one single band about 1,700 bp was amplified (fragment C). The PCR products were TA cloned and sequenced. The sequence results showed this fragment contains full length A. platys p44 sequences. The final assembled sequences 3,957 bp from dogs 1 and 2, respectively contained the entire A. platys p44ES locus, and the G+C content was 47.46~47.51 %. Identities of the
A. platys Tr1, Omp-1X and P44 amino acid sequences deduced from base sequences relative to corresponding sequences of A. marginale and A. phagocytophilum were organized in Table 5.2.

**A. platys Tr1 structure.** Two almost identical (98.4%) A. platys tr1 sequences were obtained from two dogs. The predicted molecular mass of A. platys Tr1 is 21.0~21.1 kDa with isoelectric point of 5.50 (Table 5.2). The amino acid sequences of A. platys Tr1 were deduced from ORFs. Subcellular localization of Tr1 was analyzed by PSORTb version 2.0.4 ([http://www.psort.org/psortb](http://www.psort.org/psortb)) as cytoplasm (Gardy, Laird et al. 2005). A putative N-terminal helix-turn-helix DNA binding domain suggests that Tr1 is a transcription regulator. The amino acid sequences identity between A. platys Tr1 and A. phagocytophilum Tr1 (YP_505749) or A. marginale AM1138 (YP_154239) were 85.3~84.2% and 73.5~74.6%, respectively.

**A. platys OMP-1X structure.** Two almost identical (99.1%) A. platys omp-1x sequences were obtained from two dogs. The predicted molecular mass of A. platys OMP-1Xs are 31.9 kDa with isoelectric point of 7.27 (Table 5.2). A putative signal peptide with cleavage site between position 23 and 24 was predicted in OMP-1X by SignalP 3.0 server trained on Gram-negative bacteria ([http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)) (Bendtsen, Nielsen et al. 2004). OMP-1X was predicted to be an outer membrane protein by PRED-TMBB. PRED-TMBB is a Web server capable of predicting transmembrane strands and the topology of β-barrels in OMPs of gram-negative bacteria based on a hidden Markov model (Bagos, Liakopoulos et al. 2004). The PRED-TMBB results showed A. platys OMP-1Xs have transmembrane
segments and predicted as an outer membrane protein. The amino acid sequence identities between *A. platys* OMP-1X and *A. phagocytophilum* OMP-1X (YP_505750) or *A. marginale* outer membrane protein 1 (YP_154240) were 45.9–46.3% and 39.8%, respectively. When *A. platys* OMP-1X was compared with phylogenetically closest OMP-1 homologs in each species: *A. phagocytophilum* OMP-1X (YP_505750), *A. marginale* OMP1 (YP_154240), *E. canis* P30-19 (AAK28700), *E. ruminantium* Map1-related protein (YP_180721), *E. ewingii* OMP-1-1 (ABO36240) and *E. chaffeensis* OMP-1M (YP_507903) by ClustalW method, and the alignment report showed there is an unique region in *A. platys* OMP-1X amino acid sequence which may have potential for use in differential diagnosis.

**A. platys** P44ES **structure.** One, two, and one mature P44ES (total four sequences) were obtained, respectively from each of 3 dog DNA specimens (GenBank No. GQ868750, GU357491, GU357492 and GU357493). A putative signal peptide with cleavage site between position 21 and 22 was predicted for P44ES by SignalP 3.0 server trained on Gram-negative bacteria. Predicted molecular masses of the four mature *A. platys* P44s range from 41.2 kDa to 41.4 kDa with isoelectric points of 5.30 to 5.72 (Table 5.2). Alignment of four *A. platys* P44 proteins revealed a single central hypervariable region of approximately 50 amino acid residues and N-terminal and C-terminal conserved regions of approximately 200 and 160 amino acid residues, respectively. *A. platys* P44s were predicted to be an outer membrane protein by P-Sort analysis. The amino acid sequences of four *A. platys* P44s were analyzed by posterior decoding method of PRED-TMBB. The PRED-TMBB results showed *A. platys* P44s
have transmembrane segments comprised of antiparallel β-strands and predicted as an outer membrane protein. And hydrophobicity and the hydrophobic moment profile method predicted 16 β-strands. Three more different partial p44 sequences were identified from dog 2 and 3 DNA specimen. Although more sequence data need to be compared, the hypervariable region seemed to be less variable than those of A. phagocytophilum, and conserved amino acids C, C, W, A found within A. phagocytophilum P44 hypervariable region (Lin, Zhi et al. 2002), were also detected within A. platys P44 hypervariable region. As A. phagocytophilum full-length P44s (Huang, Wang et al. 2007), the C-terminal residue of A. platys P44 has also phenylalanine. The amino acid sequences identity between A. platys P44ES and A. phagocytophilum P44-18ES (YP_505752) or A. marginale msp2 (YP_154245) were 55.0–56.9% and 41.5–42.1%, respectively.

5.5 Discussion

In the present study, for the first time, the entire 4-kb A. platys p44ES locus, containing tr1, omp-1X and p44 genes, was sequenced, providing the new insight into the p44 expression locus and the major surface antigen of A. platys. From each infected dog different p44ES sequences were detected, suggesting mixed P44 allele population of A. platys is present in the dog blood in a given time point similar to A. phagocytophilum p44 expression in humans and horses (Lin, Rikihisa et al. 2003; Wang, Rikihisa et al. 2004) or A. marginale msp2 in the blood of cattle (Eid, French et al. 1996; French, McElwain
et al. 1998)) (Palmer, Bankhead et al. 2009). In addition three more different hypervariable regions were detected in partial \textit{A. platys} \textit{p44} gene sequences, suggesting \textit{p44}-expression locus of \textit{A. platys} is also the site of active recombination, and multiple \textit{p44} donor (reserve) sequences also exist in the \textit{A. platys} genome. Using primer pairs designed in the present study, it would be possible to obtain more complete \textit{A. platys} \textit{p44} repertoire genes would be possible to provide P44 antigen diversity among \textit{A. platys} strains in various geographic regions in the future.

Our synteny analysis suggests that the \textit{p44/msp2} clusters existed in a common ancestor of the present-day three \textit{Anaplasma} species. Furthermore, the locus appears to have been diverged primarily by duplicating \textit{omp-1}-like sequences in between \textit{tr1} and \textit{p44/msp2s}: \textit{A. marginale}, \textit{A. phagocytophilum}, and \textit{A. platys} have 4, 2, 1 \textit{omp-1}-like sequences, respectively. Three species of \textit{Anaplasma} infect different host cells, namely neutrophils, erythrocytes, and platelets. The comparative study of between \textit{A. phagocytophilum}, \textit{A. marginale}, and \textit{A. platys} \textit{P44/Msp2s}, and \textit{OMP-1} homologs could provide a new window of opportunity to investigate different \textit{Anaplasma} host cell tropism. It is also interesting to see whether the \textit{p44/msp2}-expression locus and host cell type/ligands had coevolved.

\textit{Tr1} is highly expressed by \textit{A. phagocytophilum} in tick cells than in human leukemic HL-60 cells suggesting it may regulate genes for the tick stage of infection cycle (Barbet, Agnes et al. 2005; Wang, Cheng et al. 2007; Nelson, Herron et al. 2008).
In *A. phagocytophilum* tr1, two omp-ls and *p44E* are co-expressed (Wang, Cheng et al. 2007). In the cattle blood, except for the third msp2-associated genes were co-expressed (Lohr, Brayton et al. 2002). Since Omp-1s are major surface antigens of *Ehrlichia* species (Ohashi, Zhi et al. 1998; Unver, Rikihisa et al. 1999; Yu, McBride et al. 2000; Singu, Liu et al. 2005; Ganta, Cheng et al. 2007), it may also have a role in *A. platys* infection cycle.

In *A. phagocytophilum* and *A. marginale* P44/Msp2 transcripts were distinct between mammals and ticks advocates multiple physiological change adaptation between different host environments (Rurangirwa, Stiller et al. 1999; Zhi, Ohashi et al. 2002; Nelson, Herron et al. 2008). Furthermore *A. phagocytophilum* *p44* gene conversion in mammalian hosts suggests its role in antigenic variation (Barbet, Meeus et al. 2003; Felek, Telford et al. 2004; Wang, Rikihisa et al. 2004; Lin and Rikihisa 2005). And in cattle *A. marginale* MSP2s allow antigenic variation for persist infection (Barbet, Lundgren et al. 2000; Brayton, Palmer et al. 2002; Palmer, Bankhead et al. 2009). Above all, P44 may have an important role in *A. platys* persist/cyclic infection cycle, however whether *A. platys* P44/Msp2 undergoes nonsegmental conversion such as *A. phagocytophilum* generating identical P44s from large number of donor alleles in different human patient and culture samples or segmental conversion such as *A. marginale* generating mosaic Msp2s from small number of donor alleles followed by oligonucleotide recombination is still unclear (Lin, Rikihisa et al. 2003; Palmer, Bankhead et al. 2009).
The P44 of *A. phagocytophilum* is the major surface antigen useful for serologic diagnosis of human granulocytic anaplasmosis (Dumler, Asanovich et al. 1995; Zhi, Ohashi et al. 1998; Hsieh, DiPietrantonio et al. 1999; JW, Wu et al. 1999; Aguero-Rosenfeld, Kalantarpour et al. 2000), and has a role in the interaction between *A. phagocytophilum* and host cells (Kim and Rikihisa 1998; Park, Choi et al. 2003; Wang, Cheng et al. 2007). P44s also has a porin activity for passive diffusion of hydrophilic solutes (Huang, Wang et al. 2007). *A. platys* has P44 is predicted to have similar β-barrel structure as *A. phagocytophilum* P44, and therefore, expected to be porin.

Based on the present study recombinant or peptide–based OMP-1X and P44 antigen can be prepared to be tested for applicability for *A. platys* serodiagnosis. P44 can be also a specific and sensitive target for PCR diagnosis for human granulocytic anaplasmosis (Massung and Slater 2003), thus can be tested for *A. platys* infection in the future.

Since the only available source of *A. platys* DNA was a small amount of DNA purified from infected dog blood specimen, we employed touchdown PCR. Incorrect base calls resulting from amplification were minimized by using high-fidelity Taq polymerase. So far, only a few *A. platys* genes, including the 16S rRNA gene, *groEL* and *gltA* have been reported (Anderson, Greene et al. 1992; Yu, Zhang et al. 2001; Inokuma, Fujii et al. 2002). Applying a similar approach to that used here, it would be possible to obtain DNA sequences of other genomic regions to further our study of this uncultivable organism. However, caution should be paid in designing primers, since between *A.
*phagocytophilum* and *A. marginale* there are many genomic regions without synteny or having inversions such as valS.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5'-ATTATGTATGATTTATCCTAAGTTATCTGAG-3'</td>
<td><em>tr1/orf3</em> upstream highly conserved region</td>
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<tr>
<td>F2</td>
<td>5'-GGGATATCGCGTTGATAGGG-3'</td>
<td><em>A. platys</em> <em>omp1-x</em> downstream genomic walking</td>
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<tr>
<td>F3</td>
<td>5'-GGTTTGTGTGCTGGTGATGGAGG-3'</td>
<td>Genomic walking from <em>A. platys p44</em> upstream</td>
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<tr>
<td>R1</td>
<td>5’-GCAAACCTAACCACCAAYTCMCCACC-3’</td>
<td><em>p44ES/Msp2</em> C-terminal highly conserved region</td>
</tr>
<tr>
<td>R2</td>
<td>5’-TATACTAAAAAAAGAATTAAGTCAAGAG-3’</td>
<td>Conserved intergenic region between <em>omp1-x/Omp4</em> and <em>omp1-n/Omp3</em></td>
</tr>
<tr>
<td>R3</td>
<td>5’-ATGGTAGAASCCCCAGCAA-3’</td>
<td><em>p44ES/Msp2</em> N-terminal conserved region</td>
</tr>
<tr>
<td>R4</td>
<td>5’-CACGTNTTTAGTTACTGCCA-3’</td>
<td><em>p44ES/Msp2ES</em> downstream <em>valS</em> gene</td>
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<td><em>A. platys</em> P44 hypervariable region cloning</td>
</tr>
<tr>
<td>HVR</td>
<td>5’-TACTTAGGTCTTCCGCTTTCGC-3’</td>
<td><em>A. platys</em> P44 hypervariable region cloning</td>
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**Table 5.1.** Primers used for PCR amplification of *A. platys* P44ES cluster
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<th>aa number</th>
<th>Predicted signal peptide AA number</th>
<th>Molecular Mass (Da)</th>
<th>Isoelectric point</th>
<th>Subcellular localization</th>
<th>Signal peptide</th>
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<tr>
<td>Hypothetical transcriptional regulator (Tr1)</td>
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<td>7.27</td>
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<td>21</td>
<td>41329.4</td>
<td>5.53</td>
<td>Outer membrane</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 5.2. Properties of *A. platys* p44ES cluster
**Figure 5.1.** Strategy for *A. platys* p44ES cluster sequencing and *A. platys* P44ES locus. **A.** *A. phagocytophilum* p44ES and *A. marginale* msp2ES were aligned to design primer F1 (targeting *trl/orf3* upstream highly conserved region) and degenerate primers R1 (targeting p44ES/msp2 C-terminal highly conserved region), R2 (targeting conserved intergenic region between *omp-1X/omp4* and *omp-1N/omp3*), R3 (targeting p44ES/msp2 N-terminal conserved region) and R4 (based on p44ES/msp2ES downstream conserved valS gene). Primers F2 and F3 were designed based on the sequence results. **B.** The final sequence (4,009 bp) was assembled with the SeqMan program in the DNASTAR software. Genes are represented as boxes with arrows indicating their orientation.
Figure 5.2. Synteny analysis of the *A. platys* p44ES cluster relative to *A. phagocytophilum* and *A. marginale* using the Artemis comparison tool. Each red box corresponds to a good match, and blue box corresponds to a good inverse match. Score Cutoffs: 10
Figure 5.3. Phylogenetic tree of Tr1 proteins of *A. platys*, *A. phagocytophilum*, *A. marginale*. The tree was constructed using DNASTAR MegAlign ClustalW method.
Figure 5.4. Phylogenetic tree of Omp-1X proteins of *A. platys*, *A. phagocytophilum*, *A. marginale*, *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. ruminantium*. The tree was constructed using DNASTAR MegAlign ClustalW method.
Figure 5.5. Phylogram of P44ES/Msp2 proteins of *A. platys*, *A. phagocytophilum*, and *A. marginale*. A total of 23 P44/Msp2s were segregated into 3 clusters. The tree was constructed using the Neighbor-Joining (NEIGHBOR program from PHYLIP) method based on the alignment generated with CLUSTAL W; 1,000 bootstrap replications were performed. The nodes supported by bootstrap values greater than 60% are labeled.
Figure 5.6. Membrane criterion profile for *A. platys* P44. The solid blue line shows normal β-strands. The dotted red line shows twisted β-strands. The Y axis is hydrophobic moment plot to predict hydrophobic or β-strands within the porin sequence. The x axis shows the amino acid number, starting from the N terminus of mature P44. Numbers in red at the top of each peak are predicted β-strands, from the N to C terminus.
Bibliography


