Published in final edited form as:

Mol Microbiol. 2011 December ; 82(5): 1217–1234. doi:10.1111/j.1365-2958.2011.07885.x.

Insights into the CtrA Regulon in Development of Stress Resistance in Obligatory Intracellular Pathogen *Ehrlichia chaffeensis*

Zhihui Cheng¹, Koshiro Miura^{1,†}, Vsevolod L. Popov², Yumi Kumagai^{1,§}, and Yasuko Rikihisa^{1,*}

¹Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio 43210

²Department of Pathology, Center for Biodefense and Emerging Infectious Diseases, Institute for Human Infections and Immunity, The University of Texas Medical Branch Galveston, TX 77555

Summary

Ehrlichia chaffeensis is an obligate intracellular bacterium that causes human monocytic ehrlichiosis. Ehrlichiae have a biphasic developmental cycle consisting of dense-cored cells (DCs) and reticulate cells (RCs). Isolated DCs are more stress resistant and infectious than RCs. Here, we report that a response regulator, CtrA was upregulated in human monocytes at the late growth stage when DCs develop. *E. chaffeensis* CtrA bound to the promoters of late-stage transcribed genes: *ctrA*, *ompA* (peptidoglycan-associated lipoprotein), *bolA* (stress-induced morphogen), and *surE* (stationary phase survival protein), which contain CtrA-binding motifs, and transactivated *ompA*, *surE*, and *bolA* promoter-*lacZ* fusions in *Escherichia coli*. OmpA was predominantly expressed in DCs. *E. chaffeensis* binding to and subsequent infection of monocytes were inhibited by anti-OmpA IgG. *E. chaffeensis* BolA bound to the promoters of genes encoding outer surface proteins TRP120 and ECH_1038, which were expressed in DCs, and transactivated *trp120* and *ECH_1038* promoter-*lacZ* fusions. *E. chaffeensis* *bolA* complemented a stress-sensitive *E. coli* *bolA* mutant. *E. coli* expressing *E. chaffeensis* *surE* exhibited increased resistance to osmotic stress. Our results suggest that *E. chaffeensis* CtrA plays a role in coordinating development of the stress resistance for passage from the present to the next host cells through its regulon.

Introduction

Human monocytic ehrlichiosis (HME) is caused by infection with *Ehrlichia chaffeensis*, which belongs to the family *Anaplasmataceae*, the order *Rickettsiales* in the class α -proteobacteria (Dumler *et al.*, 2001). HME is an acute flu-like illness often characterized by fever, headache, myalgia, anorexia and chills, and frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevated levels of serum hepatic aminotransferases, for which approximately 40-60% of patients require hospitalization (Paddock and Childs, 2003). The estimated case fatality rate for HME is approximately 3% (Paddock and Childs, 2003). HME became a reportable disease in 1999, and about 1,000 cases were reported to the CDC in 2010, representing an approximately tenfold increase in HME incidence over a 10-year period (CDC, 2010).

*To whom correspondence should be addressed: Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, Ohio 43210-1093. Phone: (614) 292-5661. Fax: (614) 292-6473. rikihisa.1@osu.edu.

†Current address: Department of Molecular Genetics, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-0192, Japan.

§Current address: Research Institute for Microbial Diseases, Osaka University, Yamada-oka 3-1, Suita, Osaka 5650871, Japan.

E. chaffeensis lacks endotoxin or exotoxin, but its ability to adapt to the host environment is an important virulence factor (Rikihisa, 2010). *E. chaffeensis* is an obligatory intracellular bacterium that replicates as aggregates called morulae in membrane-bound inclusions inside human blood monocytes (Maeda *et al.*, 1987; Dawson *et al.*, 1991). By electron microscopy, *E. chaffeensis* is a polymorphic bacterium (0.2-2.0 μm in diameter), and can be categorized as small dense-cored cells (DCs) or large reticulate cells (RCs) (Popov *et al.*, 1995). In cell culture, a biphasic developmental cycle has been reported: initially small DCs bind to and internalize into host cells, and then develop into large RCs. After replication in expanding inclusions, the RCs transform back into DCs (Zhang *et al.*, 2007; Cheng *et al.*, 2008). Subsequently, bacteria exit the host cells either by exocytosis, by rupturing the cell membrane, or by being transported to adjacent cells through filopodia to establish a widespread infection (Thomas *et al.*, 2010). By light microscopy, it is not possible to distinguish individual RCs and DCs in morulae (Rikihisa, 2010). However, when freshly isolated from host cells, smaller bacteria (<0.5 μm) are more densely stained with basic dye than larger bacteria (>0.5 μm), therefore, we previously defined them as DCs and RCs, respectively: DCs are more resistant to strong sonication and more infectious than RCs (Zhang *et al.*, 2007; Cheng *et al.*, 2008).

Many questions regarding the intracellular replication and development of *Ehrlichia* spp. remain to be addressed, including how these processes are induced, regulated, and coordinated. Although the *E. chaffeensis* developmental cycle resembles those of *Chlamydia* and *Coxiella* spp., eukaryotic histone H1 homologs that are required for chromosomal condensation during the DC-like stages of the *Chlamydia* and *Coxiella* spp. (Hackstadt *et al.*, 1991; Heinzen and Hackstadt, 1996) are not found in *E. chaffeensis* or in any other members of the family *Anaplasmataceae* (Dunning Hotopp *et al.*, 2006). The differentiation from the RC to the DC occurs at the late stage of intracellular growth, suggesting this differentiation is induced in response to host cell nutrient depletion and high bacterial densities. However, genes encoding the nutritional stress response proteins RelA and SpoT (Braeken *et al.*, 2006; Dalebroux *et al.*, 2010), and proteins required for the biosynthesis of quorum-sensing pheromones (Antunes *et al.*, 2010) have not been found in *Ehrlichia* genomes (Dunning Hotopp *et al.*, 2006). *Ehrlichia* spp. encode only two sigma factors: a constitutive σ^{70} factor and a single alternative σ factor, σ^{32} (Dunning Hotopp *et al.*, 2006). The paucity of alternative sigma factors suggests that intracellular development of these bacteria relies on transcription factors to regulate constitutive σ^{70} -type promoters.

Two-component regulatory systems (TCSs), which are composed of a histidine sensor kinase and a cognate response regulator, are signal sensing and transduction mechanisms widely distributed among bacteria (Wuichet *et al.*, 2010). In *E. chaffeensis*, three pairs of TCSs are present (Cheng *et al.*, 2006; Kumagai *et al.*, 2006). The histidine kinases CckA, NtrY, and PleC can specifically phosphorylate the cognate response regulators CtrA, NtrX, and PleD, respectively (Kumagai *et al.*, 2006). NtrX and CtrA have a DNA-binding motif and are considered as transcriptional regulators (Cheng *et al.*, 2006; Kumagai *et al.*, 2006). NtrX is found among proteobacteria, but absent in some members of the family *Anaplasmataceae* (Cheng *et al.*, 2006). CtrA is conserved among α -proteobacteria including all sequenced members of the family *Anaplasmataceae* (Hallez *et al.*, 2004; Cheng *et al.*, 2006), and extensive studies of CtrA of the free-living bacterium *Caulobacter crescentus* have been undertaken (Quon *et al.*, 1996; Domian *et al.*, 1997; Laub *et al.*, 2002; Ryan *et al.*, 2002; Bastedo and Marczyński, 2009; Spencer *et al.*, 2009). In *C. crescentus*, CtrA is essential and upregulated in non-replicating swarmer cells, and acts as a molecular switch that coordinates cell-cycle progression and morphogenesis by directly regulating at least 95 genes (Quon *et al.*, 1996; Domian *et al.*, 1997; Laub *et al.*, 2002). The *ctrA* homolog of *Rickettsia prowazekii*, *czcR*, is able to complement a temperature-sensitive *ctrA* mutation of *C. crescentus*, suggesting that CtrA activities are conserved between *R. prowazekii* and *C.*

crescentus (Brassinga *et al.*, 2002). However, the role of CtrA in members of the order *Rickettsiales* has not been documented so far. Although targeted gene knockout has been reported in *R. prowazekii* (Driskell *et al.*, 2009), CtrA knockout or knockdown has not been reported in members of the order *Rickettsiales*. In this paper, to gain insights into the role of CtrA in *E. chaffeensis* intracellular infection, we determined the CtrA regulon and characterized CtrA-regulated gene functions by temporal gene and protein expression analysis, electrophoretic mobility shift assays (EMSAs), *lacZ* reporter assays in *Escherichia coli*, and complementation tests using *E. coli* mutants.

Results

DCs are more resistant to oxidative stress than RCs

Reactive oxygen is an important host anti-microbial mechanism (Babior, 1999). *E. chaffeensis* lacks [Cu, Zn]-SOD, Mn-SOD, catalases, peroxidases, GSH peroxidase/reductase, and the OxyR/SoxRS regulon (Dunning Hotopp *et al.*, 2006). *E. chaffeensis* is sensitive to H₂O₂, and treatment with paraquat (1,1'-dimethyl-4,4-bipyridinium dichloride), which generates both intracellular and extracellular O₂⁻ (Hassan and Fridovich, 1979; Hassett *et al.*, 1987; Lin and Rikihisa, 2007). Since *E. chaffeensis* DCs are more resistant to sonication than RCs (Cheng *et al.*, 2008), we examined whether DCs are more resistant to oxidative stress than RCs. The viability of bacteria freshly isolated from host cells was approximately 85% as determined by the BacLight bacterial viability test and could infect host cells (data not shown). After incubation with 5 μM paraquat at 37°C for 15 min in SPK (sucrose potassium phosphate) buffer, the viability of RCs was 46%, which was significantly lower than that of DCs (82%) (Fig. 1). After treatment with 20 μM paraquat, the viabilities of RCs and DCs were 1% and 16%, respectively (Fig. 1). As a negative control, after incubation with RPMI alone, no significant difference could be detected (Fig. 1). These results indicate that DCs are more resistant to oxidative stress than RCs.

E. chaffeensis CtrA is upregulated in the late stage of intracellular growth

Under our synchronous infection condition, based on determination of the amount of bacterial 16S rRNA normalized against the amount of host cell *gapdh* mRNA, intracellular bacterial growth began with an approximately 24-h lag phase, followed by exponential replication that ended around 72 h p.i. (Fig. 2A). After normalization using bacterial 16S rRNA, it was found that *ctrA* mRNA was upregulated and particularly CtrA protein was upregulated by greater than 20-fold at 72 h compared to 0 h p.i. (Fig. 2B), suggesting that the level of CtrA protein is regulated during *E. chaffeensis* intracellular growth and development. Since the differentiation from RCs to DCs gradually occurs around 72 h p.i. (Zhang *et al.*, 2007), we investigated CtrA amounts in individual bacteria. Host cell-free bacteria were immunostained with anti-CtrA serum. While both RCs and DCs expressed CtrA, DCs had tendency of being labeled more than RCs (Fig. 2C). The labeling specificity was confirmed by the result that no signal could be detected using pre-immune rabbit or dog serum (Cheng *et al.*, 2006) or the respective secondary antibody alone (Fig. S1). Post-embedding immunogold electron microscopy of *E. chaffeensis*-infected THP-1 cells with the anti-CtrA serum (Fig. 2D) confirmed the immunofluorescence labeling observation. CtrA labeling was seen both on the bacterial membrane and cytoplasm, in agreement with our previous report on isolated bacterial fractions based on Western blot analysis (Kumagai *et al.*, 2006).

Bioinformatic analysis of potential CtrA regulon in *E. chaffeensis*

E. chaffeensis CtrA has 51-56% amino acid sequence identity to CtrA orthologs of *C. crescentus*, *Brucella abortus*, *Rhodobacter capsulatus*, *Rhodospirillum centenum*, and *Sinorhizobium meliloti* (Fig. 3). The C-terminal DNA-binding domain of *E. chaffeensis*

CtrA has 60-64% identity to those of *C. crescentus*, *B. abortus*, *R. capsulatus*, *R. centenum*, and *S. meliloti* (Fig. 3). The putative DNA sequence recognition α helix ($\alpha 3$) for the OmpR-like winged helix-turn-helix DNA-binding motif (Martinez-Hackert and Stock, 1997; Lang and Beatty, 2000; Bird and Mackrell, 2011) is conserved in CtrA orthologs of α -proteobacteria, including *E. chaffeensis* (Fig. 3). This suggests that *E. chaffeensis* CtrA recognizes DNA sequences similar to those determined in *C. crescentus* (Quon *et al.*, 1996; Laub *et al.*, 2002). In addition, we found that CtrA from members of the family *Anaplasmataceae* has a C-terminal extension (20-40 amino acids) (Fig. 3 and S2), and amino acid sequences of the extension are conserved among *Ehrlichia* spp., but not within the family *Anaplasmataceae* (Fig. S2).

To identify potential *E. chaffeensis* CtrA regulon, two *C. crescentus* consensus CtrA-binding motifs (9-mer, TTAAN₇TTAAC; 8-mer, TTAACCAT) (Quon *et al.*, 1996; Laub *et al.*, 2002) were used in homology searches. Analysis of the *E. chaffeensis* genome sequence identified 28 genes with the 9-mer and 21 genes with the 8-mer binding motif in the upstream regions between -50 to -400 bp of the putative translational start site (ATG) (Table S1). Genes with 1-bp mismatch binding motif are too many to list and will be provided upon request. One 9-mer and one 8-mer consensus CtrA-binding motif were found at -281 and -305 bp, respectively, in the promoter region of *E. chaffeensis ctrA* relative to the putative translational start site.

***E. chaffeensis* CtrA binds to the promoter regions of *ctrA*, *ompA*, *bolA*, and *surE*, and transactivates the *lacZ* fusions**

Based on stress resistance studies in *E. coli* (Li *et al.*, 1994; Santos *et al.*, 1999; Cascales *et al.*, 2002) and our bioinformatic analysis, we selected three genes: *ompA* (one 9-mer binding motif, and one 9-mer containing 1-bp mismatch), *bolA* (one 9-mer), and *surE* (one 9-mer containing 1-bp mismatch, and one 8-mer containing 1-bp mismatch) for functional analysis of *E. chaffeensis* CtrA regulon. By quantitative RT-PCR, we examined the expression patterns of *ompA*, *bolA*, and *surE* in synchronously cultured *E. chaffeensis* in THP-1 cells. After normalization using bacterial 16S rRNA, the expression of all three genes was upregulated at the late stage of intracellular growth (Fig. 4).

To investigate our bioinformatic prediction, EMSA was performed using recombinant glutathione-S-transferase-*E. chaffeensis* CtrA (GST-CtrA). Upon incubation of GST-CtrA with the biotinylated probes derived from the promoter regions of *ctrA*, *ompA*, *bolA*, and *surE*, two shifted bands were detected except for the *bolA* promoter probe, with which one shifted band was detected (Fig. 5A). The binding specificity was confirmed by the result that no band shift occurred when 50-fold of the corresponding unlabeled competitor was added (Fig. 5A). As a negative control, a DNA probe derived from the *glnA* promoter region, which does not contain a CtrA-binding motif, did not shift upon incubation with GST-CtrA (Fig. 5A). To determine whether *E. chaffeensis* CtrA indeed binds to *C. crescentus* consensus CtrA-binding motifs, GST-CtrA was incubated with oligo DNA probes (24 bp) derived from the *E. chaffeensis ctrA* promoter region, containing the 9-mer (-300 to -277 bp, ATTATTAATAAAGTATTAACCTTGT, the consensus sequence is underlined), the 8-mer (-320 to -297 bp, TACGTGTGTTAACCATTCTTTATTA, the consensus sequence is underlined), or a scrambled 9-mer binding motif (ATTAAATTTAAAGTAAATTCCTTGT, the scrambled consensus sequence is underlined). Upon incubation with GST-CtrA, shifted bands were detected only for the probes containing the 9-mer and the 8-mer binding motif (Fig. 5B). To measure the K_d value of the CtrA binding, we varied the concentration of GST-CtrA. The K_d value of GST-CtrA binding to the *bolA* promoter was calculated to be 28.9 ± 8.7 nM (Fig. 5C).

To confirm our CtrA EMSA results, we incubated Alu I-digested bacterial DNA with GST-CtrA. The PCR using the pulled down DNA as a template, detected the promoter regions of *ompA*, *bolA*, and *surE* (Fig. 5D). None of these sequences were detected in the DNA pulled down with recombinant GST-*E. chaffeensis* BolA (GST-BolA) (Fig. 5D).

We next examined whether CtrA transactivates *ompA*, *bolA*, and *surE*. The *ompA*, *bolA*, and *surE-lacZ* fusions were transformed into *E. coli* BL21 (DE3) strain expressing recombinant His-*E. chaffeensis* CtrA (His-CtrA), or recombinant His-*E. chaffeensis* NtrY histidine kinase domain (His-NtrYHKD), an unrelated protein as a negative control (Cheng *et al.*, 2006). For each of the three fusions, His-CtrA induction by isopropyl-thio- β -D-galactoside (IPTG) resulted in a significant increase in β -galactosidase activity compared to His-NtrYHKD induction (Fig. 5E), demonstrating transactivation of the three different genes by CtrA. The expression of His-CtrA and His-NtrYHKD was confirmed by Western blot analysis (Fig. 5E). The *lacZ* fusion containing the *glnA* promoter was not transactivated despite the expression of His-CtrA (Fig. 5E).

OmpA expression is upregulated at the late stage of intracellular growth

Outer membrane protein A (OmpA), also called peptidoglycan-associated lipoprotein (PAL), is conserved among Gram-negative bacteria and plays a major role in stabilizing the outer membrane (Cascales *et al.*, 2002; Paul-Satyaseela *et al.*, 2006). To study the role of OmpA in *E. chaffeensis* infection, we examined OmpA protein expression by *E. chaffeensis* during bacterial intracellular development. After normalization using bacterial 16S rRNA, Western blot analysis showed that OmpA protein was highly upregulated at 72 h compared to 0 h p.i. (Fig. 6A). Rabbit anti-OmpA IgG preferentially labeled individual bacteria smaller than 0.5 μ m (>80%), and only approximately 20% of bacteria larger than 0.5 μ m were labeled, suggesting that OmpA is expressed mostly by DC-form bacteria (Fig. 6B). Post-embedding immunogold electron microscopy of *E. chaffeensis*-infected THP-1 cells with the anti-OmpA IgG (Fig. 6C) confirmed the immunofluorescence labeling observation.

***E. chaffeensis*-infected animals develop antibodies against OmpA, and anti-OmpA IgG reduces bacterial binding and infection**

Because OmpA is exposed on the bacterial surface (Kumagai *et al.*, 2010), we examined whether infected animals develop antibodies against OmpA. Sera from *E. chaffeensis*-infected dogs recognized purified recombinant His-*E. chaffeensis* OmpA (His-OmpA), whereas normal dog serum did not (Fig. 7A). This result suggests that OmpA and antibody against OmpA are relevant for *E. chaffeensis* infection *in vivo*. Because OmpA is highly expressed by DCs, which are the infectious form of *E. chaffeensis* (Zhang *et al.*, 2007), we next examined whether antibody monospecific to OmpA is able to inhibit *E. chaffeensis* infection *in vitro*. Rabbit anti-OmpA IgG inhibited the binding of *E. chaffeensis* to THP-1 cells in a dose-dependent manner (Fig. 7B). Furthermore, rabbit anti-OmpA IgG blocked *E. chaffeensis* infection of THP-1 cells (Fig. 7C). Pre-immune rabbit IgG and culture medium had no effects on bacterial binding and infection (Fig. 7B and 7C).

***E. chaffeensis* bolA complements an *E. coli* bolA mutant**

BolA homologs are widely found among proteobacteria and eukaryotes (Huynen *et al.*, 2005). Induction of *bolA* expression in *E. coli* by various stresses, such as detergents, hyperosmosis and sudden carbon starvation, leads to a round cellular morphology and alterations in the properties of the outer membrane for resistance to the stresses (Santos *et al.*, 1999; Freire *et al.*, 2006). After the detergent stress, the growth rate and the proportion of round bacteria of an *E. coli* *bolA* mutant were significantly less than those of the wild type strain, and *E. chaffeensis* *bolA* complemented the *E. coli* *bolA* mutant (Fig. 8). The expression of *E.*

chaffeensis *bolA* in *E. coli* was confirmed by RT-PCR using specific primers (data not shown).

***E. chaffeensis* BolA binds to the promoter regions of ECH_1038 and the gene encoding tandem repeat protein 120 (TRP120), and transactivates the *lacZ* fusions**

E. coli BolA has a helix-turn-helix DNA-binding motif, and binds to the promoters of *mreB*, *dacA*, and *dacC*, which encode bacterial actin, PBP5 and PBP6, respectively, and proposed to act as a transcriptional regulator of these genes (Aldea *et al.*, 1989; Freire *et al.*, 2009; Guinote *et al.*, 2011). We found that *E. chaffeensis* BolA is also predicted as a DNA-binding protein by a bioinformatic analysis (Kumar *et al.*, 2007). To determine whether *E. chaffeensis* BolA is a transcriptional regulator, and identify the potential BolA regulon, we explored *E. chaffeensis* genes that are expressed at the late growth stage and related to bacterial morphology and outer membrane. TRP120 is a surface-exposed protein, highly expressed in DCs cultured in DH82 canine macrophages, and *E. coli* expressing *E. chaffeensis* TRP120 internalizes into HeLa cells (Popov *et al.*, 2000; Zhang *et al.*, 2007). Among bacteria isolated from infected THP-1 cells, DCs (<0.5 μ m) expressed TRP120 predominantly over RCs (>0.5 μ m) (Fig. 9A). Although major outer membrane proteins: P28 and OMP-1F are upregulated during the exponential growth stage (Kumagai *et al.*, 2008), ECH_1038, which is predicted to be an outer membrane protein by PSORT analysis and highly bacterial strain-variable (Miura and Rikihisa, 2007), was upregulated at the late stage of bacterial growth (Fig. 9B). Rabbit serum against recombinant His-*E. chaffeensis* ECH_1038 N-terminus (His-ECH1038N) recognized purified His-ECH1038N and native *E. chaffeensis* ECH_1038 (Fig. 9C), and preferentially stained individual bacteria smaller than 0.5 μ m (Fig. 9D), suggesting that ECH_1038 is expressed mostly by DC-form bacteria.

To determine whether *E. chaffeensis* BolA regulates the expression of *trp120* and *ECH_1038*, EMSA was performed using biotinylated probes derived from the promoter regions of *trp120* and *ECH_1038*. Upon incubation of these probes with GST-BolA, shifted bands were detected with both probes (Fig. 10A). The specificity of the binding was confirmed by the result that no band shift occurred when 50-fold of the corresponding unlabeled competitor was added (Fig. 10A). As a negative control, the *glnA* promoter probe did not shift upon incubation with GST-BolA (Fig. 10A). To measure K_d values of the GST-BolA binding, we varied the concentration of GST-BolA. The K_d value of GST-BolA binding to the *ECH_1038* promoter was calculated to be 10.8 ± 1.0 nM (Fig. 10B), which is similar to the K_d values of *E. coli* BolA binding to the promoters of *E. coli* *mreB* (6.9 nM), *dacA* (1.8 nM) and *dacC* (5.3 nM) determined by surface plasmon resonance analysis (Guinote *et al.*, 2011).

To confirm our BolA EMSA results, we incubated Alu I-digested bacterial DNA with GST-BolA. The PCR using the pulled down DNA as a template, detected the promoter regions of *trp120* and *ECH_1038* (Fig. 10C). None of these sequences were detected in the DNA pulled down with GST-CtrA (Fig. 10C).

We next examined whether BolA transactivates *trp120* and *ECH_1038*. The *trp120* and *ECH_1038-lacZ* fusions were transformed into *E. coli* BL21 (DE3) strain expressing recombinant His-*E. chaffeensis* BolA (His-BolA) or His-NtrYHKD. For each of the two fusions, His-BolA induction by IPTG resulted in a significant increase in β -galactosidase activity compared to His-NtrYHKD induction (Fig. 10D), demonstrating transactivation of the two different genes by BolA. The expression of His-BolA and His-NtrYHKD was confirmed by Western blot analysis (Fig. 10D). The *lacZ* fusion containing the *glnA* promoter was not transactivated despite the expression of His-BolA (Fig. 10D).

***E. chaffeensis* SurE confers stress resistance to *E. coli* and has phosphatase activity**

SurE homologs are distributed widely among bacteria, archaea, and eukaryotes (Iwasaki and Miki, 2007). SurE was named as a stationary phase survival protein, since an *E. coli* *surE* insertional knockout mutant survives poorly in the stationary phase, at elevated temperatures, and in high-salt media (Li *et al.*, 1994). *E. coli* BL21(DE3) strain expressing recombinant His-*E. chaffeensis* SurE (His-SurE) was more resistant to osmotic stress (0.5 M NaCl) than the strain expressing His-NtrYHKD, as a negative control (Fig. 11A), suggesting *E. chaffeensis* SurE also plays a role in bacterial growth under stress. The expression of His-SurE and His-NtrYHKD was confirmed by Western blot analysis (Fig. 11A).

E. chaffeensis SurE belongs to the COG0496 group of predicted acid phosphatases as *E. coli* SurE. Recombinant *E. coli* SurE shows phosphatase activity with broad substrate specificity: high affinity to 3'-AMP, 5'-dGMP, 5'-GMP, 3'-CMP and 5'-AMP (Proudfoot *et al.*, 2004). His-SurE had phosphatase activity to 5'-AMP in pH range of 6.8 to 7.5 (Fig. 11B) and in the presence of Co²⁺, Mg²⁺, or Ni²⁺ ions, but not in the presence of Ca²⁺, Cu²⁺, Mn²⁺, or Zn²⁺ ions (Fig. 11C). However, for the other phosphate esters we examined (5'-GMP, dNTP mixture, alpha-naphthyl phosphate and beta-glycerol phosphate), enzyme activity could not be detected. The calculated K_m and V_{max} of His-SurE to 5'-AMP were 0.40 ± 0.07 (mM) and 2.90 ± 0.13 ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein), respectively (Fig. 11D). As a negative control for potential *E. coli* phosphatase contamination in His-SurE, we examined phosphatase activity of His-NtrYHKD purified by the same methods, which did not show any activity (Fig. 11D).

Discussion

The present study showed that CtrA regulates the expression of several genes that are upregulated in *E. chaffeensis* during the differentiation of RCs to DCs, suggesting CtrA might coordinate this conversion. In particular, *E. chaffeensis* CtrA upregulates the expression of genes that might confer physicochemical stress resistance to DCs. In addition to resistance to sonication, the present study showed oxidative stress resistance of DCs, suggesting DC survival potential in the immune-inflammatory tissues.

In *E. coli*, OmpA is necessary for maintaining the integrity of the cell envelope and resistance to detergents or antibiotics (Cascales *et al.*, 2002), but how *ompA* is regulated in *E. coli* or in any other bacteria is unknown. An α -helical motif of *E. coli* OmpA (NX₂LSX₂RGX₂VX₃L), which binds to peptidoglycan to support the structure of the cell wall (Koebnik, 1995), is conserved in *E. chaffeensis* OmpA. However, as most genes for peptidoglycan biosynthesis are absent in *E. chaffeensis* (Dunning Hotopp *et al.*, 2006), the α -helical motif may interact with molecules other than peptidoglycan to support the cell-wall structure. The present study showed that OmpA is antigenic in *E. chaffeensis*-infected mammals, and its surface exposure on *E. chaffeensis* (Kumagai *et al.*, 2010) allows anti-OmpA IgG to inhibit bacterial binding to and infection of host cells *in vitro*. These results suggest that antibody against OmpA suppresses *E. chaffeensis* infection *in vivo*. Whether OmpA is directly involved in *E. chaffeensis* binding and internalization is, however, unknown. Although treatment of *E. chaffeensis* with a c-di-GMP antagonist reduces the amount of OmpA by protease-dependent degradation, the treatment does not reduce bacterial binding (Kumagai *et al.*, 2010), suggesting that perhaps inhibition by the anti-OmpA IgG is due to steric hindrance.

Induction of *bolA* in *E. coli* by various stresses is mainly controlled by RpoS, the starvation/stationary phase sigma factor σ^S (Santos *et al.*, 1999). It has been shown that the osmotic stress response regulator, OmpR, can bind to the *E. coli* *bolA* promoter (Yamamoto *et al.*, 2000). CtrA belongs to the OmpR family (Quon *et al.*, 1996), and the property of *bolA*

regulation by OmpR may have been conserved in *E. chaffeensis* CtrA to compensate the loss of σ^S in the ancestor of *E. chaffeensis*.

Although it has been shown that the stability of *E. chaffeensis* surface TRP120 is regulated by c-di-GMP (Kumagai *et al.*, 2010), little is known about transcriptional regulation of *trp120*. Our current results showed that the BolA protein can directly bind to the promoter regions of *trp120* and *ECH_1038*, and transactivate their expression. Because they are not found in the *E. coli* BolA regulon (Freire *et al.*, 2009; Guinote *et al.*, 2011), the BolA regulon in *E. chaffeensis* may have evolved during adaptation to the obligatory intracellular life style. *E. chaffeensis* CtrA-BolA transcriptional cascade is expected to amplify CtrA signal transduction, yet allowing differential control of the BolA regulon.

SurE regulation has not been reported in any bacteria and whether SurE is upregulated in the stationary phase is unknown. Therefore, the present study is the first example of SurE regulation. We did not examine *E. coli* *surE* mutant complementation with *E. chaffeensis* *surE*, because the original *surE* mutant contains an *rpoS* nonsense mutation, and a new *surE* null mutant strain that does not contain the *rpoS* mutation survives just as well as the parent strain under heat, osmotic and oxidative stresses (Visick *et al.*, 1998). However, we found overexpression of *E. chaffeensis* SurE in *E. coli* enhanced resistance to osmotic stress. The enzyme characteristics (K_m , V_{max} , optimum pH and metal-dependence except Mn^{2+}) of *E. chaffeensis* SurE were similar to those of *E. coli* SurE using 5'-AMP as a substrate (Proudfoot *et al.*, 2004). However, unlike *E. coli* SurE, which has a higher affinity to 5'-GMP than to 5'-AMP (Proudfoot *et al.*, 2004), *E. chaffeensis* SurE selectively dephosphorylates 5'-AMP but not 5'-GMP. SurE substrate specificity, pH optimum, and metal ion dependency are considered to be important for confining the enzyme activity and preventing collateral damage by SurE phosphatase activity, as suggested in *Thermotoga maritima* (Zhang *et al.*, 2001). It remains to be studied whether SurE enzyme activity is related to the bacterial stress resistance.

It has been shown that CtrA is autoregulated in *C. crescentus* (Domian *et al.*, 1999), and *B. abortus* CtrA binds to its own promoter region (Bellefontaine *et al.*, 2002). Additionally, consensus CtrA-binding motifs are found in the promoter regions of *R. capsulatus* *ctrA* and *S. meliloti* *ctrA* (Lang and Beatty, 2000; Barnett *et al.*, 2001). Similarly, we found one 9-mer and one 8-mer consensus CtrA-binding motif in the promoter region of *E. chaffeensis* *ctrA*, and our EMSA results support autoregulation of *E. chaffeensis* *ctrA*. Other than autoregulation, it remains unknown how *E. chaffeensis* CtrA is regulated. Among 9 proteins known to directly regulate the cellular level of *C. crescentus* CtrA [CtrA, GcrA, DnaA, CcrM, ClpXP, CpdR, PopA, RcdA and SciP (Quon *et al.*, 1996; Ryan *et al.*, 2002; Holtzendorff *et al.*, 2004; Gorbatyuk and Marczynski, 2005; Collier *et al.*, 2006; Iniesta *et al.*, 2006; McGrath *et al.*, 2006; Gora *et al.*, 2010)], only CtrA, DnaA and ClpXP have been detected in the *E. chaffeensis* proteome (Dunning Hotopp *et al.*, 2006; Lin *et al.*, 2011). In addition to CtrA, DnaA and ClpXP homologs, *R. capsulatus* has GcrA, and *B. abortus* has GcrA and CcrM, but they lack the rest of the *C. crescentus* CtrA regulatory proteins. Thus, the amounts of CtrA in the three bacterial species (*E. chaffeensis*, *R. capsulatus*, and *B. abortus*) are likely regulated by mechanisms different from those found in *C. crescentus*. Fifteen residues in the *C. crescentus* CtrA C-terminus are important for the proteolysis of CtrA (Ryan *et al.*, 2002). The CtrA C-terminal extensions identified among members of the family *Anaplasmataceae*, including *E. chaffeensis*, may be involved in the stability of CtrA.

The EMSA results with the *ompA* and *surE* promoter probes suggest that some of the 1-bp mismatch *C. crescentus* consensus CtrA-binding motifs are functional in *E. chaffeensis*, which was also reported in *B. abortus* (Bellefontaine *et al.*, 2002). *E. chaffeensis* CtrA-regulated genes, *ompA*, *bolA* and *surE*, found in this study don't belong to *C. crescentus*

CtrA regulon. Similarly, CtrA regulon in *B. abortus* is different from that in *C. crescentus* (Bellefontaine *et al.*, 2002). Moreover, unlike *C. crescentus*, CtrA is not required for viability of *R. capsulatus* (Lang and Beatty, 2000). It appears that adaptation of these α -proteobacteria to respective environmental niches led to the emergence of unique CtrA regulon, and in some bacteria *ctrA* became dispensable.

We used GST-*E. chaffeensis* CtrA for our EMSA study. GST-*C. crescentus* CtrA can not be phosphorylated by *E. coli* EnvZ fused to maltose-binding protein *in vitro*, which can phosphorylate His-*C. crescentus* CtrA (Reisenauer *et al.*, 1999). After incubation with acetyl-phosphate, which is a small molecule phosphodonator and can phosphorylate His-*C. crescentus* CtrA *in vitro* (Quon *et al.*, 1996), the affinity of GST-*E. chaffeensis* CtrA to the *bolA* promoter did not increase (data not shown), suggesting that GST-*E. chaffeensis* CtrA also can not be phosphorylated *in vitro*. GST-*C. crescentus* CtrA forms an active dimer in solution and has high affinity to CtrA-binding motifs (in the same range of phosphorylated His-*C. crescentus* CtrA) (Spencer *et al.*, 2009). The K_d value of GST-*E. chaffeensis* CtrA to the *bolA* promoter was in the similar range of those of GST-*C. crescentus* CtrA and phosphorylated His-*C. crescentus* CtrA to CtrA-binding motifs (Spencer *et al.*, 2009), suggesting GST-*E. chaffeensis* CtrA is also an active form.

In summary, this work began to uncover the CtrA-dependent genetic network in obligatory intracellular bacteria. Our results indicate that *E. chaffeensis* CtrA is a functional transcriptional regulator and suggest it plays an essential role in pathogenesis of obligatory intracellular *E. chaffeensis* infection by regulating intracellular development and extracellular survival, particularly development of bacterial stress resistance in the environment. Thus further studies on CtrA, the CtrA regulon and functions of the CtrA regulon will help to identify targets for development of new treatments and preventive measures for HME by inhibiting pathogen survival.

Experimental procedures

Bacterial strains and culture

E. chaffeensis Arkansas (Dawson *et al.*, 1991) was propagated in THP-1 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2mM L-glutamine at 37°C in 5% CO₂ and 95% air. *E. coli* strains NovaBlue (Novagen, San Diego, CA) and BL21 (DE3) (Novagen) for DNA cloning and protein expression, and MC1061 (*bolA* wt), SK6519 (+*bolA*) and SK6583 (Δ *bolA*) were cultured in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989). When required, the medium was supplemented with ampicillin (50 μ g ml⁻¹) (Fisher Bioreagents, Pittsburgh, PA), kanamycin (30 μ g ml⁻¹) (Fisher Bioreagents), chloramphenicol (34 μ g ml⁻¹) (Sigma, St Louis, MO) and streptomycin (25 μ g ml⁻¹) (Sigma).

Treatment of host cell-free *E. chaffeensis* with paraquat

Host cell-free *E. chaffeensis* was prepared as described (Lin *et al.*, 2002). Isolated bacteria were suspended in 350 μ l SPK buffer (200 mM sucrose, 50 mM potassium phosphate buffer, pH 7.4) supplemented with 1 mM L-glutamine. Each aliquot (100 μ l) was incubated with 5 or 20 μ M paraquat (Sigma) (1 mM stock solution in RPMI medium) or RPMI medium alone as a negative control at 37°C for 15 min. Treated bacteria were washed, and stained with a LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Eugene, OR). The fluorescent images were analyzed using a Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments Inc., Melville, NY).

Synchronous culture of *E. chaffeensis*

E. chaffeensis was synchronously cultured as described (Cheng *et al.*, 2008). After incubation of isolated bacteria with THP-1 cells, the bacteria-host cell mixture was washed with cold 2× phosphate buffered saline (2 × PBS: 274 mM NaCl, 5.4 mM KCl, 20 mM Na₂HPO₄, 4 mM KH₂PO₄, pH 7.4) three times (2000 × g, 5 min) to remove the unbound bacteria, and then incubated in culture medium at 37°C. Samples were collected at 0, 24, 48, and 72 h p.i. by centrifugation and total RNA was isolated from each sample. The amount of *E. chaffeensis* 16S rRNA as well as the mRNA level of human *gapdh* and *E. chaffeensis* *ctrA*, *ompA*, *bolA*, *surE* and *ECH_1038* was determined by quantitative RT-PCR using specific primers (Cheng *et al.*, 2006; Zhang *et al.*, 2007) (Table S2). Based on determination of the amount of bacterial 16S rRNA normalized against the amount of host cell *gapdh* RNA, the number of live bacteria in host cells at each time point was calculated. The transcription level of *E. chaffeensis* genes at each time point was normalized against bacterial 16S rRNA. To determine the amount of CtrA and OmpA at each time point, protein samples corresponding to the same numbers of live bacteria as determined by bacterial 16S rRNA, were subjected to SDS-PAGE (12% acrylamide) and transferred onto a nitrocellulose membrane. CtrA and OmpA were detected using rabbit anti-CtrA serum (Cheng *et al.*, 2006) and anti-OmpA IgG (Huang *et al.*, 2008), respectively, and then horseradish peroxidase-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MA). All sera were pre-adsorbed with uninfected THP-1 cells and used at 1:1,000 dilutions. The membranes were visualized with ECL Western blotting detection reagents (Amersham, Piscataway, NJ) and an LAS-3000 luminescent image analyzer (Fujifilm, Stamford, CT). The density of the band was analyzed using a Multi Gauge V3.0 software (Fujifilm).

Search for putative *E. chaffeensis* CtrA regulon

The entire *E. chaffeensis* genome sequence (NC_007799) and a list of the start site and direction of each ORF were obtained from the NCBI website. The promoter sequence of each ORF was extracted from the genome sequence using Visual Basic on Excel: We defined the promoter region of each ORF as -400 to -50 from the translational start site. If one ORF is on the plus strand and starts at X on the genome, the promoter region was extracted by following formula: start of promoter = X - 400; end of promoter = X - 50. If one ORF is on the minus strand and starts at Y on the genome, the promoter region was extracted by following formula: start of promoter = Y + 400; end of promoter = Y + 50. Then we searched the 9-mer TTAAN₇TTAAC and the 8-mer TTAACCAT *C. crescentus* consensus CtrA-binding motif (Quon *et al.*, 1996; Laub *et al.*, 2002) in the promoter sequences obtained above in Excel using the “search” command, which indicates the motif position from the beginning of the promoter sequence of each ORF. The results were verified with pDRAW (AcaClone software, Frederiksberg, Denmark). If a CtrA-binding motif was found in the promoter region of downstream tandem genes with less than 50 bp of intergenic region separating them, then the downstream gene was excluded from the list of potential CtrA regulon, as this is likely a part of an operon.

Expression and purification of recombinant proteins, generation of antibodies and Western blot analysis

The DNA fragments encoding full-length CtrA, OmpA, BolA, SurE and the N-terminal region of ECH_1038 (ECH1038N, amino acid position 57 to 707) were amplified by PCR using *E. chaffeensis* chromosomal DNA as a template and specific primers (Table S3). The amplified fragments were digested with restriction enzymes and ligated into restriction enzyme-digested pET-41a(+) (for GST-BolA and GST-CtrA) and pET-33b(+) (for His-CtrA, His-OmpA, His-BolA, His-SurE and His-ECH1038N). *E. coli* NovaBlue cells were transformed and cultured in LB broth supplemented with kanamycin (30 µg ml⁻¹) at 37°C overnight. The plasmids were extracted using a QuickClean 5M Miniprep kit (GenScript,

Piscataway, NJ). The cloned fragments were confirmed by DNA sequencing. *E. coli* BL21 (DE3) cells were transformed with the plasmids and induced to express the recombinant proteins with IPTG. For EMSA, GST-BolA and GST-CtrA which contain 6 × His downstream of GST were Ni-affinity purified from *E. coli* soluble fraction (Kumagai *et al.*, 2006). The purified proteins were dialyzed against EMSA binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol). His-OmpA and His-ECH1038N were purified from *E. coli* inclusion bodies (Cheng *et al.*, 2006). Purified His-ECH1038N was subcutaneously injected into the back of a New Zealand White female rabbit 3 times in every other week. For the first injection, 0.3 mg purified protein was injected with TiterMaxGold (TiterMax USA, Norcross, GA) as an adjuvant. For the second and third injections, 0.4 mg and 0.5 mg purified protein, respectively, were mixed with Freund's incomplete adjuvant (Sigma). One month after the third injection, 30 ml total blood was collected from the rabbit to prepare antiserum. For OmpA antibody detection, purified His-OmpA subjected to SDS-PAGE (12% acrylamide) was transferred to a PVDF membrane and incubated with sera from *E. chaffeensis*-infected dogs (3918815, CTUALJ, 1425, or OH001) (Huang *et al.*, 2008), rabbit anti-OmpA IgG as a positive control, or normal dog serum as a negative control. After being washed, the membranes were incubated with the secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-dog IgG (KPL).

Immunofluorescence and immunogold labeling

Host cell-free bacteria derived from nonsynchronous culture were cytocentrifuged onto glass slides and fixed with methanol at -20°C for 5 min. Bacteria were incubated with dog anti-*E. chaffeensis* serum and rabbit sera against CtrA, ECH1038N and TRP120 (Zhang *et al.*, 2007) and anti-OmpA IgG. After being washed, the cells were incubated with the secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and Texas Red-conjugated goat anti-dog IgG (Rockland, Gilbertsville, PA). All antisera used were pre-adsorbed with uninfected THP-1 cells. Immunogold labeling was performed as previously described using 15-nm gold-conjugated goat anti-rabbit IgG, EMS-Aurion (Electron Microscopy Sciences, Hatfield, PA) (Popov *et al.*, 2000). All antisera were used at 1:100 dilutions.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described (Cheng *et al.*, 2008). Biotinylated DNA fragments were prepared using specific primers (Table S4). For consensus CtrA-binding motifs, 24-bp biotinylated single strand oligonucleotides were synthesized (Sigma) and annealed. GST-CtrA or GST-BolA (20 nM) was incubated with biotinylated DNA probe (2 nM) in a 50-μl reaction containing 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 2.5% (v/v) glycerol, and 50 ng μl⁻¹ Poly (dI-dC) on ice for 30 min. As a control for binding specificity, a separate reaction was prepared containing the above components plus a 50-fold excess of the corresponding unlabeled DNA competitor. For dose-dependent experiments, biotinylated DNA probe (2 nM) was incubated with GST-CtrA or GST-BolA (1 nM – 200 nM). As a control for binding specificity, a separate reaction was prepared using GST protein (200 nM). Samples were subjected to native polyacrylamide gel electrophoresis (5% acrylamide), transferred to a nylon membrane and UV cross-linked. The biotinylated DNA fragments were detected using a LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL). To determine the dissociation constant (K_d value), the band density of the shifted promoter probe was plotted against the GST-CtrA or GST-BolA concentration, and K_d was calculated by non-linear least-squares analysis using the GraphPad Prism software (version 5.04, GraphPad Software, San Diego, CA).

Pull-down assay

DNA extracted from host cell-free bacteria were digested with Alu I at 37°C overnight, then purified with a QuickClean 5M PCR Purification kit (GenScript). Purified DNA was incubated with 15 µg of GST-CtrA or GST-BolA in a 200 µl reaction containing 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂ and 2.5% (v/v) glycerol on ice for 30 min. MagneHis Ni-Particles (Promega, Madison, WI) (50 µl) pre-equilibrated with reaction buffer were added to the DNA-protein mixture and incubated on ice for 30 min. The particles were washed three times with 500 µl reaction buffer at 4°C to remove unbound DNA fragments. The DNA-protein complex was eluted with 100 µl reaction buffer containing 500 mM imidazole at 4°C and used as a template for PCR with specific primers (Table S5). The PCR fragments were sequenced to confirm the results of pull-down assays.

Construction of *lacZ* fusions

The *lacZ* fusion was constructed as described (Cheng *et al.*, 2008). Briefly, DNA fragments were amplified by PCR with specific primers (Table S6) and inserted upstream of the promoterless *lacZ* gene in pACYC184 (New England Biolabs). BL21 (DE3) strains containing pET-33b(+) encoding His-CtrA, His-BolA, or His-NtrYHKD as a negative control were transformed with the *lacZ* fusions. After inducing the recombinant proteins with 0.05 mM IPTG, β-galactosidase activity was measured (Wang *et al.*, 2007). For each *lacZ* fusion construct, plasmids were extracted from the same number of *E. coli*, subjected to agarose gel electrophoresis and stained with ethidium bromide. The relative copy number of each plasmid was determined based on the DNA band density to confirm that *E. coli* expressing His-CtrA or His-BolA, and His-NtrYHKD contained the same copy number of pACYC184. Recombinant protein expression was confirmed by Western blot analysis using anti-His-tag antibody (Sigma).

Effects of anti-OmpA IgG on *E. chaffeensis* binding to and infection of THP-1 cells

Host cell-free bacteria were freshly prepared from 10⁶ heavily infected THP-1 cells (>90% cells infected). For binding analysis, isolated bacteria were incubated in 200 µl RPMI medium with various concentrations (20, 100, 500 µg ml⁻¹) of either pre-immune rabbit IgG or rabbit anti-OmpA IgG on a Heto RK10-VS rotator (Heto-Holten A/S, Denmark) at set 20 at room temperature for 30 min. Each mixture was then added to 10⁶ uninfected THP-1 cells in 300 µl culture medium. After incubation at room temperature for 15 min with rotation, each mixture was incubated at 37°C for 1 h. Cells were harvested by centrifugation at 750 × g for 5 min and washed three times with 2× PBS to remove the unbound bacteria. The mixture was cytocentrifuged onto glass slides and fixed with methanol at -20°C for 5 min and sequentially incubated with dog anti-*E. chaffeensis* serum and Texas Red-conjugated goat anti-dog IgG.

For analysis of infection, isolated bacteria were incubated in 200 µl RPMI medium with either pre-immune rabbit IgG, rabbit anti-OmpA IgG (500 µg ml⁻¹) on the rotator at room temperature for 30 min. Each mixture was added to 2 × 10⁶ uninfected THP-1 cells in 5 ml culture medium and incubated at 37°C for 48 h. Total RNA was extracted from 10⁶ infected cells in each sample and the number of live bacteria was determined by quantitative RT-PCR based on bacterial 16S rRNA.

E. coli complementation and growth under stresses

The *E. coli* *bolA* promoter region was inserted upstream of the promoterless *E. chaffeensis* *bolA* gene in pUC19 (New England Biolabs) by overlap extension PCR using specific primers (Table S7) (Ho *et al.*, 1989). SK6583 (*ΔbolA*) strain was transformed with this plasmid or pUC19 as a negative control. *E. coli* strains MC1061 (wt), SK6519 (+*bolA*),

SK6583 and transformed SK6583 strains were cultured in LB broth at 37°C overnight, then diluted 50 times in LB broth with or without 0.4% (w/v) SDS and cultured at 37°C for 24 h (Freire *et al.*, 2006). Bacterial growth was monitored by colony forming unit (CFU) ml⁻¹ assay. The expression of *E. chaffeensis* *bolA* in *E. coli* was confirmed by RT-PCR using specific primers (Table S2).

E. coli BL21 (DE3) strains containing pET-33b(+) encoding His-SurE and His-NtrYHKD were cultured in LB broth at 37°C for overnight, then diluted 50 times in LB broth and cultured at 37°C to OD₆₀₀ 0.2. IPTG (0.1 mM) was added to induce the expression of recombinant proteins. After OD₆₀₀ reached 0.3, 0.5 M NaCl (final concentration) was added to the cultures. After being cultured at 37°C for 3 h, bacterial growth was monitored by CFU ml⁻¹ assay.

Phosphatase assay

For phosphatase enzymatic assay, His-SurE was purified as described (Mura *et al.*, 2003). Briefly, the bacterial lysate was clarified over a DE52 column pre-equilibrated with binding buffer [20 mM HEPES, pH 7.8, 1.5 M NaCl, 0.5% (v/v) Triton X-100, 30 mM PMSF]. The flow-through fraction was applied to a Ni column pre-equilibrated with binding buffer. The recombinant protein was eluted from the column with an imidazole gradient (100 mM to 400 mM), and then dialyzed against storage buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA). Phosphatase activity of His-SurE was determined as described (Zhang *et al.*, 2001) with minor modifications. Briefly, a 250-μl reaction mixture of 0.1 M buffer (sodium acetate for pH 4.5-6.3 or Tris-HCl for pH 6.8-9.0), 1 mM MgSO₄ (or 0.1 mM CaCl₂, CoCl₂, CuSO₄, MnSO₄, NiSO₄, ZnSO₄), 1 mM 5'-AMP (or 5'-GMP, dNTP mixture, alpha-naphthyl phosphate and beta-glycerol phosphate) (Sigma) and either His-SurE, His-NtrYHKD or storage buffer as a negative control, was incubated at 37°C for 30 min. Phosphate released from the substrates was measured with an EnzChek system (E-6646) (Molecular Probes, Eugene, OR) (Zhang *et al.*, 2001). For determination of *K_m* and *V_{max}*, the phosphatase assays contained 5'-AMP at concentrations of 0.3-3 mM. Kinetic parameters were determined by GraphPad Prism software.

Statistical analysis

Statistical analyses were performed using analysis of variance and Student's *t*-test, and a *P* value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Sidney R. Kushner at University of Georgia (Athens, GA) for kindly providing *E. coli* strains (MC1061, SK6519 and SK6853). We appreciate Ms. Julie Wen and Dr. Xue-jie Yu at the University of Texas Medical Branch at Galveston (Galveston, TX) for excellent help with electron microscopy and providing rabbit anti-TRP120 serum, respectively. This work was supported by the National Institutes of Health grant R01 AI054476.

References

- Aldea M, Garrido T, Hernandez-Chico C, Vicente M, Kushner SR. Induction of a growth-phase-dependent promoter triggers transcription of *bolA*, an *Escherichia coli* morphogene. *EMBO J.* 1989; 8:3923–3931. [PubMed: 2684651]
- Antunes LC, Ferreira RB, Buckner MM, Finlay BB. Quorum sensing in bacterial virulence. *Microbiology.* 2010; 156:2271–2282. [PubMed: 20488878]

- Babior BM. NADPH oxidase: an update. *Blood*. 1999; 93:1464–1476. [PubMed: 10029572]
- Barnett MJ, Hung DY, Reisenauer A, Shapiro L, Long SR. A homolog of the CtrA cell cycle regulator is present and essential in *Sinorhizobium meliloti*. *J Bacteriol*. 2001; 183:3204–3210. [PubMed: 11325950]
- Bastedo DP, Marczyński GT. CtrA response regulator binding to the *Caulobacter* chromosome replication origin is required during nutrient and antibiotic stress as well as during cell cycle progression. *Mol Microbiol*. 2009; 72:139–154. [PubMed: 19220749]
- Bellefontaine AF, Pierreux CE, Mertens P, Vandenhaute J, Letesson JJ, De Bolle X. Plasticity of a transcriptional regulation network among alpha-proteobacteria is supported by the identification of CtrA targets in *Brucella abortus*. *Mol Microbiol*. 2002; 43:945–960. [PubMed: 11929544]
- Bird TH, Mackrell A. A CtrA homolog affects swarming motility and encystment in *Rhodospirillum centenum*. *Arch Microbiol*. 2011; 193:451–459. [PubMed: 21243338]
- Braeken K, Moris M, Daniels R, Vanderleyden J, Michiels J. New horizons for (p)ppGpp in bacterial and plant physiology. *Trends Microbiol*. 2006; 14:45–54. [PubMed: 16343907]
- Brassinga AK, Siam R, McSween W, Winkler H, Wood D, Marczyński GT. Conserved response regulator CtrA and IHF binding sites in the alpha-proteobacteria *Caulobacter crescentus* and *Rickettsia prowazekii* chromosomal replication origins. *J Bacteriol*. 2002; 184:5789–5799. [PubMed: 12270838]
- Cascales E, Bernadac A, Gavioli M, Lazzaroni JC, Lloubes R. Pal lipoprotein of *Escherichia coli* plays a major role in outer membrane integrity. *J Bacteriol*. 2002; 184:754–759. [PubMed: 11790745]
- CDC. Final 2009 reports of nationally notifiable infectious diseases. *MMWR Morb Mortal Wkly Rep*. 2010; 59:1025–1039.
- Cheng Z, Kumagai Y, Lin M, Zhang C, Rikihisa Y. Intra-leukocyte expression of two-component systems in *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* and effects of the histidine kinase inhibitor closantel. *Cell Microbiol*. 2006; 8:1241–1252. [PubMed: 16882029]
- Cheng Z, Wang X, Rikihisa Y. Regulation of type IV secretion apparatus genes during *Ehrlichia chaffeensis* intracellular development by a previously unidentified protein. *J Bacteriol*. 2008; 190:2096–2105. [PubMed: 18192398]
- Collier J, Murray SR, Shapiro L. DnaA couples DNA replication and the expression of two cell cycle master regulators. *EMBO J*. 2006; 25:346–356. [PubMed: 16395331]
- Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS. ppGpp conjures bacterial virulence. *Microbiol Mol Biol Rev*. 2010; 74:171–199. [PubMed: 20508246]
- Dawson JE, Anderson BE, Fishbein DB, Sanchez JL, Goldsmith CS, Wilson KH, Duntley CW. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. *J Clin Microbiol*. 1991; 29:2741–2745. [PubMed: 1757543]
- Domian IJ, Quon KC, Shapiro L. Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell*. 1997; 90:415–424. [PubMed: 9267022]
- Domian IJ, Reisenauer A, Shapiro L. Feedback control of a master bacterial cell-cycle regulator. *Proc Natl Acad Sci USA*. 1999; 96:6648–6653. [PubMed: 10359766]
- Driskell LO, Yu XJ, Zhang L, Liu Y, Popov VL, Walker DH, et al. Directed mutagenesis of the *Rickettsia prowazekii* *pld* gene encoding phospholipase D. *Infect Immun*. 2009; 77:3244–3248. [PubMed: 19506016]
- Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and ‘HGE agent’ as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol*. 2001; 51:2145–2165. [PubMed: 11760958]
- Dunning Hotopp JC, Lin M, Madupu R, Crabtree J, Angiuoli SV, Eisen JA, et al. Comparative genomics of emerging human ehrlichiosis agents. *PLoS Genet*. 2006; 2:e21. [PubMed: 16482227]
- Freire P, Vieira HL, Furtado AR, de Pedro MA, Arraiano CM. Effect of the morphogene *bolA* on the permeability of the *Escherichia coli* outer membrane. *FEMS Microbiol Lett*. 2006; 260:106–111. [PubMed: 16790025]

- Freire P, Moreira RN, Arraiano CM. BofA inhibits cell elongation and regulates MreB expression levels. *J Mol Biol.* 2009; 385:1345–1351. [PubMed: 19111750]
- Gora KG, Tsokos CG, Chen YE, Srinivasan BS, Perchuk BS, Laub MT. A cell-type-specific protein-protein interaction modulates transcriptional activity of a master regulator in *Caulobacter crescentus*. *Mol Cell.* 2010; 39:455–467. [PubMed: 20598601]
- Gorbatyuk B, Marczyński GT. Regulated degradation of chromosome replication proteins DnaA and CtrA in *Caulobacter crescentus*. *Mol Microbiol.* 2005; 55:1233–1245. [PubMed: 15686567]
- Guinote IB, Matos RG, Freire P, Arraiano CM. BofA affects cell growth, and binds to the promoters of penicillin-binding proteins 5 and 6 and regulates their expression. *J Microbiol Biotechnol.* 2011; 21:243–251. [PubMed: 21464593]
- Hackstadt T, Baehr W, Ying Y. *Chlamydia trachomatis* developmentally regulated protein is homologous to eukaryotic histone H1. *Proc Natl Acad Sci USA.* 1991; 88:3937–3941. [PubMed: 2023942]
- Hallez R, Bellefontaine AF, Letesson JJ, De Bolle X. Morphological and functional asymmetry in alpha-proteobacteria. *Trends Microbiol.* 2004; 12:361–365. [PubMed: 15276611]
- Hassan HM, Fridovich I. Paraquat and *Escherichia coli*. Mechanism of production of extracellular superoxide radical. *J Biol Chem.* 1979; 254:10846–10852. [PubMed: 227855]
- Hassett DJ, Britigan BE, Svendsen T, Rosen GM, Cohen MS. Bacteria form intracellular free radicals in response to paraquat and streptonigrin. Demonstration of the potency of hydroxyl radical. *J Biol Chem.* 1987; 262:13404–13408. [PubMed: 2820968]
- Heinzen RA, Hackstadt T. A developmental stage-specific histone H1 homolog of *Coxiella burnetii*. *J Bacteriol.* 1996; 178:5049–5052. [PubMed: 8759877]
- Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* 1989; 77:51–59. [PubMed: 2744487]
- Holtzendorff J, Hung D, Brende P, Reisenauer A, Viollier PH, McAdams HH, Shapiro L. Oscillating global regulators control the genetic circuit driving a bacterial cell cycle. *Science.* 2004; 304:983–987. [PubMed: 15087506]
- Huang H, Lin M, Wang X, Kikuchi T, Mottaz H, Norbeck A, Rikihisa Y. Proteomic analysis of and immune responses to *Ehrlichia chaffeensis* lipoproteins. *Infect Immun.* 2008; 76:3405–3414. [PubMed: 18490460]
- Huynen MA, Spronk CA, Gabaldón T, Snel B. Combining data from genomes, Y2H and 3D structure indicates that BofA is a reductase interacting with a glutaredoxin. *FEBS Lett.* 2005; 579:591–596. [PubMed: 15670813]
- Iniesta AA, McGrath PT, Reisenauer A, McAdams HH, Shapiro L. A phospho-signaling pathway controls the localization and activity of a protease complex critical for bacterial cell cycle progression. *Proc Natl Acad Sci USA.* 2006; 103:10935–10940. [PubMed: 16829582]
- Iwasaki W, Miki K. Crystal structure of the stationary phase survival protein SurE with metal ion and AMP. *J Mol Biol.* 2007; 371:123–136. [PubMed: 17561111]
- Koebnik R. Proposal for a peptidoglycan-associating alpha-helical motif in the C-terminal regions of some bacterial cell-surface proteins. *Mol Microbiol.* 1995; 16:1269–1270. [PubMed: 8577259]
- Kumagai Y, Cheng Z, Lin M, Rikihisa Y. Biochemical activities of three pairs of *Ehrlichia chaffeensis* two-component regulatory system proteins involved in inhibition of lysosomal fusion. *Infect Immun.* 2006; 74:5014–5022. [PubMed: 16926392]
- Kumagai Y, Huang H, Rikihisa Y. Expression and porin activity of P28 and OMP-1F during intracellular *Ehrlichia chaffeensis* development. *J Bacteriol.* 2008; 190:3597–3605. [PubMed: 18359808]
- Kumagai Y, Matsuo J, Hayakawa Y, Rikihisa Y. Cyclic di-GMP signaling regulates invasion by *Ehrlichia chaffeensis* of human monocytes. *J Bacteriol.* 2010; 192:4122–4133. [PubMed: 20562302]
- Kumar M, Gromiha MM, Raghava GP. Identification of DNA-binding proteins using support vector machines and evolutionary profiles. *BMC Bioinformatics.* 2007; 8:463–472. [PubMed: 18042272]
- Lang AS, Beatty JT. Genetic analysis of a bacterial genetic exchange element: the gene transfer agent of *Rhodobacter capsulatus*. *Proc Natl Acad Sci USA.* 2000; 97:859–864. [PubMed: 10639170]

- Laub MT, Chen SL, Shapiro L, McAdams HH. Genes directly controlled by CtrA, a master regulator of the *Caulobacter* cell cycle. *Proc Natl Acad Sci USA*. 2002; 99:4632–4637. [PubMed: 11930012]
- Li C, Ichikawa JK, Ravetto JJ, Kuo HC, Fu JC, Clarke S. A new gene involved in stationary-phase survival located at 59 minutes on the *Escherichia coli* chromosome. *J Bacteriol*. 1994; 176:6015–6022. [PubMed: 7928962]
- Lin M, Zhu MX, Rikihisa Y. Rapid activation of protein tyrosine kinase and phospholipase C- γ 2 and increase in cytosolic free calcium are required by *Ehrlichia chaffeensis* for internalization and growth in THP-1 cells. *Infect Immun*. 2002; 70:889–898. [PubMed: 11796624]
- Lin M, Rikihisa Y. Degradation of p22phox and inhibition of superoxide generation by *Ehrlichia chaffeensis* in human monocytes. *Cell Microbiol*. 2007; 9:861–874. [PubMed: 17087735]
- Lin M, Kikuchi T, Brewer HM, Norbeck AD, Rikihisa Y. Global proteomic analysis of two tick-borne emerging zoonotic agents: *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*. *Front Microbiol*. 2011; 2:24. [PubMed: 21687416]
- Maeda K, Markowitz N, Hawley RC, Ristic M, Cox D, McDade JE. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. *N Engl J Med*. 1987; 316:853–856. [PubMed: 3029590]
- Martinez-Hackert E, Stock AM. The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. *Structure*. 1997; 5:109–124. [PubMed: 9016718]
- McGrath PT, Iniesta AA, Ryan KR, Shapiro L, McAdams HH. A dynamically localized protease complex and a polar specificity factor control a cell cycle master regulator. *Cell*. 2006; 124:535–547. [PubMed: 16469700]
- Miura K, Rikihisa Y. Virulence potential of *Ehrlichia chaffeensis* strains of distinct genome sequences. *Infect Immun*. 2007; 75:3604–3613. [PubMed: 17438035]
- Mura C, Katz JE, Clarke SG, Eisenberg D. Structure and function of an archaeal homolog of survival protein E (SurE α): an acid phosphatase with purine nucleotide specificity. *J Mol Biol*. 2003; 326:1559–1575. [PubMed: 12595266]
- Paddock CD, Childs JE. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. *Clin Microbiol Rev*. 2003; 16:37–64. [PubMed: 12525424]
- Paul-Satyaseela M, Karched M, Bian Z, Ihalin R, Borén T, Arnqvist A, et al. Immunoproteomics of *Actinobacillus actinomycetemcomitans* outer-membrane proteins reveal a highly immunoreactive peptidoglycan-associated lipoprotein. *J Med Microbiol*. 2006; 55:931–942. [PubMed: 16772422]
- Popov VL, Chen SM, Feng HM, Walker DH. Ultrastructural variation of cultured *Ehrlichia chaffeensis*. *J Medical Microbiol*. 1995; 43:411–421.
- Popov VL, Yu X, Walker DH. The 120 kDa outer membrane protein of *Ehrlichia chaffeensis*: preferential expression on dense-core cells and gene expression in *Escherichia coli* associated with attachment and entry. *Microb Pathog*. 2000; 28:71–80. [PubMed: 10644493]
- Proudfoot M, Kuznetsova E, Brown G, Rao NN, Kitagawa M, Mori H, et al. General enzymatic screens identify three new nucleotidases in *Escherichia coli*. *Biochemical characterization of SurE, YfbR, and YjgG*. *J Biol Chem*. 2004; 279:54687–54694. [PubMed: 15489502]
- Quon KC, Marczynski GT, Shapiro L. Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell*. 1996; 84:83–93. [PubMed: 8548829]
- Reisenauer A, Quon K, Shapiro L. The CtrA response regulator mediates temporal control of gene expression during the *Caulobacter* cell cycle. *J Bacteriol*. 1999; 181:2430–2439. [PubMed: 10198005]
- Rikihisa Y. *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*: subversive manipulators of host cells. *Nat Rev Microbiol*. 2010; 8:328–339. [PubMed: 20372158]
- Ryan KR, Judd EM, Shapiro L. The CtrA response regulator essential for *Caulobacter crescentus* cell-cycle progression requires a bipartite degradation signal for temporally controlled proteolysis. *J Mol Biol*. 2002; 324:443–455. [PubMed: 12445780]
- Sambrook J, Fritsch EF, Maniatis T, Irwin N. *Molecular Cloning: a Laboratory Manual*. 2nd. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
- Santos JM, Freire P, Vicente M, Arraiano CM. The stationary-phase morphogene *bolA* from *Escherichia coli* is induced by stress during early stages of growth. *Mol Microbiol*. 1999; 32:789–798. [PubMed: 10361282]

- Spencer W, Siam R, Ouimet MC, Bastedo DP, Marczynski GT. CtrA, a global response regulator, uses a distinct second category of weak DNA binding sites for cell cycle transcription control in *Caulobacter crescentus*. J Bacteriol. 2009; 191:5458–5470. [PubMed: 19542275]
- Thomas S, Popov VL, Walker DH. Exit mechanisms of the intracellular bacterium *Ehrlichia*. PLoS One. 2010; 5:e15775. [PubMed: 21187937]
- Visick JE, Ichikawa JK, Clarke S. Mutations in the *Escherichia coli* *surE* gene increase isoaspartyl accumulation in a strain lacking the *pcm* repair methyltransferase but suppress stress-survival phenotypes. FEMS Microbiol Lett. 1998; 167:19–25. [PubMed: 9785447]
- Wang X, Kikuchi T, Rikihisa Y. Proteomic identification of a novel *Anaplasma phagocytophilum* DNA binding protein that regulates a putative transcription factor. J Bacteriol. 2007; 189:4880–4886. [PubMed: 17483233]
- Wuichet K, Cantwell BJ, Zhulin IB. Evolution and phyletic distribution of two-component signal transduction systems. Curr Opin Microbiol. 2010; 13:219–225. [PubMed: 20133179]
- Yamamoto K, Nagura R, Tanabe H, Fujita N, Ishihama A, Utsumi R. Negative regulation of the *bolA1p* of *Escherichia coli* K-12 by the transcription factor OmpR for osmolarity response genes. FEMS Microbiol Lett. 2000; 186:257–262. [PubMed: 10802181]
- Zhang JZ, Popov VL, Gao S, Walker DH, Yu XJ. The developmental cycle of *Ehrlichia chaffeensis* in vertebrate cells. Cell Microbiol. 2007; 9:610–618. [PubMed: 16987329]
- Zhang K, Shan L, Rahman MS, Unruh H, Halayko AJ, Gounni AS. Constitutive and inducible thymic stromal lymphopoietin expression in human airway smooth muscle cells: role in chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol. 2007; 293:L375–382. [PubMed: 17513456]
- Zhang RG, Skarina T, Katz JE, Beasley S, Khachatryan A, Vyas S, et al. Structure of *Thermotoga maritima* stationary phase survival protein SurE: a novel acid phosphatase. Structure. 2001; 9:1095–1106. [PubMed: 11709173]

Fig. 1. DCs are more resistant to oxidative stress than RCs

The percentage of viable bacteria in two size groups: >0.5 μm and <0.5 μm corresponding to RCs and DCs, respectively after the oxidative stress induced by paraquat treatment. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test. Images show the viability of individual bacteria of diverse sizes: green, live bacteria; red, dead bacteria. White arrowheads point to live DCs (<0.5 μm). Bar = 1 μm.

Fig. 2. CtrA is upregulated at the late stage of *E. chaffeensis* intracellular growth and in DCs

A. RNA samples prepared from synchronously cultured *E. chaffeensis* in THP-1 cells at different time points were subjected to quantitative RT-PCR analysis. The value reflects bacterial 16S rRNA normalized against human *gapdh* mRNA, relative to the amount determined at 0 h p.i. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates.

B. Upper panel: RNA samples prepared from synchronously cultured *E. chaffeensis* in THP-1 cells at different time points were subjected to quantitative *ctrA* RT-PCR analysis and normalized against bacterial 16S rRNA. Relative values to the amount at 0 h p.i. are shown. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) compared to the value at 0 h p.i. by analysis of variance. Lower panel: CtrA amounts in samples at different time points were determined by Western blot analysis. All samples were normalized against bacterial 16S rRNA as determined by quantitative RT-PCR. The sample at 72 h p.i. was diluted 10 times. The numbers below the panel indicate relative densities of the protein bands. #Estimated band density without dilution.

C. The percentage of CtrA-positive bacteria in two size groups: >0.5 μ m and <0.5 μ m. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test. Immunofluorescence

images show labeled individual bacteria in different sizes. Host cell-free bacteria were double labeled with dog anti-*E. chaffeensis* serum (Ech, red) and rabbit anti-CtrA serum (CtrA, green). The merged image (merge) is viewed with green and red filters. White arrowheads point to CtrA-positive DCs ($<0.5 \mu\text{m}$). Bar = $1 \mu\text{m}$.

D. Immunogold labeling of CtrA of *E. chaffeensis* in the inclusions of THP-1 cells. Ultrathin sections of infected cells were labeled with rabbit anti-CtrA or pre-immune serum followed by gold-conjugated goat anti-rabbit IgG. DCs and RCs are indicated by arrowheads and arrows respectively. Bar = $1 \mu\text{m}$.

E. cha	MRILLIEDDIAACAKAVEASLSSEGHFCETMTSAQDCY	SSIIPKNDYDVVLI	DIHFP	GKIDG	YDILV	KLR	RESGI	KVPVLI	80		
B. abo	MRVLLIEDDSAI	AQSI	ELMLKSESFNVYTTDL	GEE-	-GI	DLG	KLYDYDI	LLDLNLP-	DMSGYEVLRTRLRSKVKTPIL	77	
C. cre	MRVLLIEDDSATAQT	ELMLKSEGFNVYTTDL	GEE-	-GVDL	GKI	YDYDI	LLDLNLP-	DMSGI	DVLRTRLRVAKINTPIM	77	
R. cap	MRILLVEDDPTTSR	SI	ELMLTHANLVNYCTDL	GEE-	-GI	DLAKLYDYDI	LLDLNLP-	DMNGLEVL	RQLRLARVDTPI	77	
R. cen	MRVLLVEDDPSMAKS	SI	ELMLNSEGYVDSTEL	GED-	-GLEI	GKLYDYDI	LLDLNLP-	DI	DGYEVLRLRSARVTTPI	77	
S. mel	MRVLLIEDDSATAQS	SI	ELMLKSESFNVYTTDL	GEE-	-GVDL	GKLYDYDI	LLDLNLP-	DMSGYEVLRTRLRSKVKTPIL	77		
E. cha	LSGIS	SSVSHKVKGLYYGAD	YVTKPFHKSELLARI	KAI	VRRTRGHPESI	VRV	GNI	AINLDHRCV	ECNGEIVHLTKKEYGM	160	
B. abo	LSGMAGI	EDKVRGLGFGAD	YMTKPFHKDELI	ARI	HAI	VRRSKGHAQSVI	TTGDL	VVNLDAKT	VEVAGQRVHLTGKEYQM	157	
C. cre	LSGSSEI	DTKYKTFAGGAD	YMTKPFHKDEMI	ARI	HAVVRRSKGHAQSVI	KTGDI	VVNLDAKT	VEVNGNRVHLTGKEYQM	157		
R. cap	LTGADDTENKI	KGFGE	GADYMTKPFHREELVARI	HAI	IRRSKGHSQSI	RTGKI	SVNLDAKT	VEVGGKPVHLTGKEYQM	157		
R. cen	LSGLSEL	DHKIKGLGVGAD	YMTKPFHKDELVARI	HAI	VRRSKGHSQSI	RTGKLT	VNLDAKT	VEVDGQPLHLTGKEYGI	157		
S. mel	LSGMAGI	EDKVRGLGFGAD	YMTKPFHKDELI	ARI	HAI	VRRSKGHAQSVI	STGELI	VNLDAKT	VEVGGQRVHLTGKEYQM	157	
E. cha	ELLSSRLGTVLT	KEMFLSHLYRELDEP	SDDKI	I	DVFMCKLRKKLVANN	GKNI	ETVWGRGYVL	KECVDEDESDDRGGY	240		
B. abo	LELLSLRKGTTLT	KEMFLNHL	YGGMDER	ELKI	I	DVFI	CKLRKKLDVSGNOSYI	ETVWGRGYVLREPD		225	
C. cre	LELLSLRKGTTLT	KEMFLNHL	YGGMDER	ELKI	I	DVFI	CKLRKKLAASAHGKHHI	ETVWGRGYVL	RDPN		225
R. cap	LELLSLRKGTTLT	KEMFLNHL	YGGMDER	ELKI	I	DVFI	CKLRKKLAEVTGGENYI	ETVWGRGYVL	RDPDGGDL		229
R. cen	LELLSLRKGTTLT	KEMFLNHL	YGGMDER	ELKI	I	DVFI	CKLRKKLTATKGENYI	ETVWGRGYVL	RDPTEVQ		228
S. mel	LELLSLRKGTTLT	KEMFLNHL	YGGMDER	ELKI	I	DVFI	CKLRKKLANAAGGANYI	ETVWGRGYVL	REREG		226
E. cha	RWDHGGANVDSI	YGQEE	DKKYVEEA							266	
B. abo		AEMRESA								232	
C. cre		EQVNAA								231	
R. cap		DRRMVGA								237	
R. cen		KQVVAS								234	
S. mel		SDYLETA								233	

Fig. 3. Comparison of *E. chaffeensis* CtrA with CtrAs from other members of α -proteobacteria
Alignment of CtrA of *E. chaffeensis* (*E. cha*) (GenBank accession number, YP_507798), *B. abortus* (*B. abo*) (AAL86376), *C. crescentus* (*C. cre*) (NP_421829), *R. capsulatus* (*R. cap*) (ADE85408), *R. centenum* (*R. cen*) (YP_002297962), and *S. meliloti* (*S. mel*) (AAG01017). Identical amino acids are shaded. The helix-turn-helix DNA-binding motif is boxed by a dashed line and the sequence for DNA-sequence-recognition α 3 helix is boxed by a solid line. Amino acid numbers of each CtrA are shown on the right.

Fig. 4. *ompA*, *bolA*, and *surE* are upregulated at the late stage of intracellular growth

RNA samples prepared from synchronously cultured *E. chaffeensis* in THP-1 cells at different time points were subjected to quantitative RT-PCR analysis and normalized against bacterial 16S rRNA. Relative values to the amount at 0 h p.i. are shown. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates.

*Significantly different ($P < 0.05$) compared to the value at 0 h p.i. by analysis of variance.

Fig. 5. *E. chaffeensis* CtrA binds to the promoter regions of *ctrA*, *ompA*, *bolA*, and *surE*, and transactivates the promoter-lacZ fusions in *E. coli*

A. EMSA for GST-CtrA binding to the promoter regions of *ompA*, *bolA*, *surE*, and *ctrA*. The length (bp) of the probe is shown above each panel. For each panel: biotinylated DNA probe (2 nM) was incubated alone (lane 1), with GST-CtrA (20 nM, lane 2), or GST-CtrA in the presence of 50-fold excess of the corresponding unlabeled DNA competitor (lane 3). Shifted bands are indicated by arrowheads.

B. EMSA for GST-CtrA binding to the consensus CtrA-binding motifs in the *E. chaffeensis ctrA* promoter. The name of each probe (24 bp) is shown above. For each probe: biotinylated DNA probe (2 nM) was incubated alone (lane 1), with GST (20 nM, lane 2), GST-CtrA (20 nM, lane 3), or GST-CtrA in the presence of 50-fold excess of the corresponding unlabeled DNA competitor (lane 4). Shifted bands are indicated by an arrowhead.

C. EMSA for dose-dependent binding of GST-CtrA to the *bolA* promoter. The length of the probe derived from the *bolA* promoter region is 171 bp. Biotinylated DNA probe (2 nM) was incubated alone (lane 1), with GST (200 nM, lane 2), or GST-CtrA at different concentrations (lanes 3-8, 1, 2, 6, 20, 60, and 200 nM, respectively). Shifted band is indicated by an arrowhead.

D. Alu I-digested *E. chaffeensis* DNA was pulled down with GST-CtrA. The PCR was performed using the pulled down DNA as a template. The promoter region and the length (bp) amplified by specific primers are shown above each panel. For each panel: bacterial

total DNA (lane 1, positive control), DNA pulled down with GST-CtrA (lane 2), and DNA pulled down with GST-BolA (lane 3, negative control) were used as templates. *E. coli* strains containing pET-33b(+) encoding His-CtrA and His-NtrYHKD were transformed with the *ompA*, *bolA*, *surE*, or *glnA-lacZ* fusions. The fusions contain the promoter regions of *ompA* (368 bp), *bolA* (171 bp), *surE* (430 bp), or *glnA* (243 bp). After inducing the His-CtrA or His-NtrYHKD with IPTG, β -galactosidase activity was measured. Upper panel: β -galactosidase activity (Miller units). His-NtrYHKD, *E. coli* strain expressing His-NtrYHKD; His-CtrA, *E. coli* strain expressing His-CtrA. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test. Lower panel: Western blot analysis of samples from the β -galactosidase assays was performed using monoclonal antibody against the His-tag to verify the expression of His-CtrA and His-NtrYHKD. A representative of three independent experiments is shown. Each lane received 1 μ g protein.

Fig. 6. OmpA expression is upregulated at the late stage of intracellular growth and in DCs

A. OmpA amounts in synchronously cultured *E. chaffeensis* in THP-1 cells at different time points were determined by Western blot analysis. All samples were normalized against bacterial 16S rRNA as determined by quantitative RT-PCR.

B. The percentage of OmpA-positive bacteria in two size groups: >0.5 μm and <0.5 μm . Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test.

Immunofluorescence images show labeled individual bacteria of different sizes. Host cell-free bacteria were double labeled with dog anti-*E. chaffeensis* serum (Ech, red) and rabbit anti-OmpA IgG (OmpA, green). The merged image (merge) is viewed with green and red filters. White arrowheads point to OmpA-positive DCs (<0.5 μm). Bar = 1 μm .

C. Immunogold labeling of OmpA in *E. chaffeensis* DCs in THP-1 cells. Ultrathin section of infected cells was labeled with rabbit anti-OmpA IgG followed by gold-conjugated goat anti-rabbit IgG. Bar = 1 μm .

Fig. 7. *E. chaffeensis* animals develop antibodies against OmpA, and anti-OmpA IgG reduces bacterial binding and infection

A. *E. chaffeensis* infection induces antibody against OmpA *in vivo*. Purified His-OmpA (1 µg) was subjected to SDS-PAGE (12% acrylamide) and transferred to a PVDF membrane, which was then incubated with rabbit anti-OmpA IgG (lane 1), sera from *E. chaffeensis*-infected dogs (3918815, CTUALJ, 1425, and OH001) (lane 2-5, respectively) or normal dog serum (lane 6). His-OmpA is indicated by an arrow.

B. Inhibition of *E. chaffeensis* binding to THP-1 cells with rabbit anti-OmpA IgG. Host-cell free bacteria were pre-incubated with culture medium, or 20, 100, 500 µg ml⁻¹ of pre-immune IgG or anti-OmpA IgG, and then incubated with THP-1 cells. Bound bacteria were counted in 100 THP-1 cells. Data indicate mean values ± standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) compared to medium alone or pre-immune IgG controls by analysis of variance.

C. Inhibition of *E. chaffeensis* infection of THP-1 cells with rabbit anti-OmpA IgG. The value reflects bacterial 16S rRNA under the indicated conditions relative to culture medium. Data indicate mean values ± standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) compared to medium alone or pre-immune IgG controls by analysis of variance.

Fig. 8. *E. chaffeensis bolA* complements an *E. coli bolA* mutant

Upper panel: the percentage of bacterial growth (CFU ml⁻¹) after 24 h treatment with 0.4% SDS relative to the detergent-less culture, and the proportion of round (length to width ratio < 1.5) bacteria in total 100 bacteria after the detergent stress. wt, wild-type strain; +*bolA*, strain overexpressing *E. coli* BolA; Δ *bolA*, *bolA* knockout mutant. pUC19-*Ech bolA*, pUC19 containing *E. chaffeensis bolA* gene downstream of the *E. coli bolA* promoter. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test. Lower panels: bacteria after detergent stress stained with Diff-Quik. Bar = 2 μ m.

Fig. 9. The expression of TRP120 and ECH_1038 is upregulated at the late stage of intracellular growth and in DCs

A. The percentage of TRP120-positive bacteria in two size groups: $>0.5 \mu\text{m}$ and $<0.5 \mu\text{m}$. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test. Immunofluorescence images show labeled individual bacteria of different sizes. Host cell-free bacteria were double labeled with dog anti-*E. chaffeensis* serum (Ech, red) and rabbit anti-TRP120 serum (TRP120, green). The merged image (merge) is viewed with green and red filters. White arrowheads point to TRP120-positive DCs ($<0.5 \mu\text{m}$). Bar = $1 \mu\text{m}$.

B. RNA samples prepared from synchronously cultured *E. chaffeensis* in THP-1 cells at different time points were subjected to quantitative *ECH_1038* RT-PCR analysis and normalized against bacterial 16S rRNA. Relative values to the amount at 0 h p.i. are shown. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) compared to the value at 0 h p.i. by analysis of variance.

C. Purified His-ECH1038N and antibody specificity. Purified His-ECH1038N (0.5 µg) was subjected to SDS-PAGE (10% acrylamide) followed by Coomassie brilliant blue staining (lane 1) and Western blot analysis using rabbit anti-ECH1038N serum (lane 2). His-ECH1038N is indicated by an arrowhead. The lysates prepared from uninfected (lane 3) and *E. chaffeensis*-infected (Lane 4) THP-1 cells (7.5×10^5) were subjected to SDS-PAGE (6% acrylamide) followed by Western blot analysis using rabbit anti-ECH1038N serum. Native ECH_1038 is indicated by an arrow.

D. The percentage of ECH_1038-positive bacteria in two size groups: >0.5 µm and <0.5 µm. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test. Immunofluorescence images show labeled individual bacteria in different sizes. Host cell-free bacteria were double labeled with dog anti-*E. chaffeensis* serum (Ech, red) and rabbit anti-ECH1038N serum (ECH_1038, green). The merged image (merge) is viewed with green and red filters. White arrowheads point to ECH_1038-positive DCs (<0.5 µm). Bar = 1µm.

Fig. 10. *E. chaffeensis* BolA binds to the promoter regions of *trp120* and *ECH_1038*, and transactivates the promoter-*lacZ* fusions in *E. coli*

A. EMSA for GST-BolA binding to the promoter regions of *trp120* and *ECH_1038*. The length (bp) of the probe is shown above each panel. For each panel: biotinylated DNA probe (2 nM) was incubated alone (lane 1), with GST-BolA (2 nM, lane 2), or GST-BolA in the presence of 50-fold excess of the corresponding unlabeled DNA competitor (lane 3). Shifted bands are indicated by arrowheads.

B. EMSA for dose-dependent binding of GST-BolA to the *ECH_1038* promoter. The length of the probe derived from the *ECH_1038* promoter region is 196 bp. Biotinylated DNA probe (2 nM) was incubated alone (lane 1), with GST (200 nM, lane 2), or GST-BolA in different concentrations (lanes 3-8: 1, 2, 6, 20, 60, and 200 nM, respectively). Shifted band is indicated by an arrowhead.

C. Alu I-digested *E. chaffeensis* DNA was pulled down with GST-BolA. The PCR was performed using the pulled down DNA as a template. The promoter region and the length (bp) amplified by specific primers are shown above each panel. For each panel: bacterial total DNA (lane 1, positive control), DNA pulled down with GST-BolA (lane 2), and DNA pulled down with GST-CtrA (lane 3, negative control) were used as templates.

D. *E. coli* strains containing pET-33b(+) encoding His-BolA or His-NtrYHKD were transformed with the *trp120* and *ECH_1038-lacZ* fusions. The fusions contain the promoter regions of *trp120* (245 bp) and *ECH_1038* (199 bp). After inducing His-BolA and His-NtrYHKD with IPTG, β -galactosidase activity was measured. Upper panel: β -galactosidase activity (Miller units). His-NtrYHKD, *E. coli* strain expressing His-NtrYHKD; His-BolA, *E. coli* strain expressing His-BolA. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test. Lower panel: Western blot analysis of samples from the β -galactosidase assays was performed using monoclonal antibody against the His-tag to verify the expression of His-BolA and His-NtrYHKD. A representative of three independent experiments is shown. Each lane received 1 μ g protein.

Fig. 11. *E. chaffeensis* SurE confers stress resistance to *E. coli* and has phosphatase activity

A. Upper panel: the percentage of bacteria growth (CFU ml^{-1}) after 3 h treatment with 0.5 M NaCl relative to that without NaCl. His-NtrYHKD, *E. coli* strain expressing His-NtrYHKD; His-SurE, *E. coli* strain expressing His-SurE. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates. *Significantly different ($P < 0.05$) by Student's *t*-test. Lower panel: Western blot analysis of samples under stress treatment was performed using monoclonal antibody against the His-tag to verify the expression of His-SurE and His-NtrYHKD. A representative of three independent experiments is shown. Each lane received 1 μg protein.

B. pH dependence of His-SurE phosphatase activity with 5'-AMP as a substrate. The reaction mixture contained 1 mM 5'-AMP, 1 mM Mg^{2+} , 0.1 μg His-SurE. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates.

C. Metal dependence of His-SurE phosphatase activity with 5'-AMP as a substrate. The reaction mixture contained 1 mM 5'-AMP, 0.1 μg His-SurE, 1 mM Mg^{2+} or 0.1 mM other metal as indicated, 0.1 mM Tris-HCl, pH 6.8. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates.

D. Phosphatase activity of His-SurE and His-NtrYHKD (a negative control) dose response with 5'-AMP as a substrate. The reaction mixture contained 1 μg His-SurE or His-NtrYHKD, 1 mM Mg^{2+} , 0.1 mM Tris-HCl, pH 6.8. Data indicate mean values $\pm$ standard deviations from three independent experiments performed in triplicates.