Transcription of *Ehrlichia chaffeensis* Genes Is Accomplished by RNA Polymerase Holoenzyme Containing either Sigma 32 or Sigma 70

Huitao Liu, Tonia Von Ohlen, Chuanmin Cheng, Bonto Faburay, Roman R. Ganta* 

Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas, United States of America

**Abstract**

Bacterial gene transcription is initiated by RNA polymerase containing a sigma factor. To understand gene regulation in *Ehrlichia chaffeensis*, an important tick-transmitted rickettsiae responsible for human monocytic ehrlichiosis, we initiated studies evaluating the transcriptional machinery of several genes of this organism. We mapped the transcription start sites of 10 genes and evaluated promoters of five genes (*groE*, *dnaK*, *hup*, *p28-Omp14* and *p28-Omp19* genes). We report here that the RNA polymerase binding elements of *E. chaffeensis* gene promoters are highly homologous for its only two transcription regulators, sigma 32 and sigma 70, and that gene expression is accomplished by either of the transcription regulators. RNA analysis revealed that although transcripts for both sigma 32 and sigma 70 are upregulated during the early replicative stage, their expression patterns remained similar for the entire replication cycle. We further present evidence demonstrating that the organism’s -35 motifs are essential to transcription initiations. The data suggest that *E. chaffeensis* gene regulation has evolved to support the organism’s growth, possibly to facilitate its intraphagosomal growth. Considering the limited availability of genetic tools, this study offers a novel alternative in defining gene regulation in *E. chaffeensis* and other related intracellular pathogens.

**Citation:** Liu H, Von Ohlen T, Cheng C, Faburay B, Ganta RR (2013) Transcription of *Ehrlichia chaffeensis* Genes Is Accomplished by RNA Polymerase Holoenzyme Containing either Sigma 32 or Sigma 70. PLoS ONE 8(11): e81780. doi:10.1371/journal.pone.0081780

**Editor:** Kelly A. Brayton, Washington State University, United States of America

**Received** September 3, 2013; **Accepted** October 22, 2013; **Published** November 21, 2013

**Copyright:** © 2013 Liu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work is supported by the National Institutes of Health, USA grant # AI070908. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** RG is a PLOS ONE Editorial Board member. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: rganta@vet.ksu.edu

**Introduction**

Several *Anaplasmataceae* family pathogens have been identified in recent years as the causative agents of important diseases in people and various vertebrate animals [1-3]. The limited availability of genetic tools to study these primarily obligate intraphagosomal pathogens hampers our understanding of the molecular mechanisms of pathogenesis. *E. chaffeensis*, a member of the *Anaplasmataceae* family, is a tick-transmitted pathogen responsible for an important emerging disease, human monocytic ehrlichiosis (HME) [4,5]. HME is an acute flu-like illness with symptoms including fever, headache, myalgia, anorexia and chills and is frequently accompanied by leukopenia, thrombocytopenia, anemia, and upgraded levels of serum hepatic aminotransferases [3]. This pathogen also infects several vertebrate animals, including white-tailed deer, dogs, goats and coyotes [6-8]. One of the significant features of *E. chaffeensis* infection, like other members of the *Anaplasmataceae* family, is prolonged persistence in vertebrate and tick hosts [9-12]. *E. chaffeensis* may have evolved specific strategies to establish persistent infections so they can successfully complete their lifecycles in dual hosts. Global host-specific differences in the transcription and expressed proteins of *E. chaffeensis* have been reported [13,14]. The host cell-specific differences in gene expression support the hypothesis that *E. chaffeensis* utilizes novel strategies to adapt and persist in both vertebrate and tick hosts, but the exact molecular mechanism of adaptation is unclear.

Gene expression in bacteria is accomplished by regulating transcription by RNA polymerase (RNAP). RNAP activity in bacteria is often regulated by altering transcription from a gene to adapt to different host environments [15,16]. A typical bacterial RNAP consists of a core RNA polymerase and a transcription regulator, a sigma (σ) factor. The core enzyme typically contains four or five different subunits: two α subunits and one each of β and β’ subunits, and some organisms contain a ω subunit [17]. Binding of a sigma factor to a core RNAP (referred to as an RNAP holoenzyme) enables specific recognition of a promoter element and the transcription...
initiation. Recognition of a specific promoter by RNAP holoenzyme is one of the important mechanisms that regulates gene expression in bacteria [18-20]. The primary housekeeping sigma factor, sigma 70 (σ70), in Escherichia coli (E. coli) and its homologs in other bacteria are shown to control the transcription of most of the genes during exponential growth of bacterial cells. Alternative sigma factors generally regulate transcription triggered by a specific stress environment or during developmental conversions [21]. The number of alternate sigma factors differs in different bacteria; for example, the E. coli genome has 7 sigma factors [22], whereas 65 sigma factors are found in Streptomyces coelicolor [23]. The E. chaffeensis genome, however, has only two sigma factor genes; rpoD (the predicted primary housekeeping σ70 gene) and rpoH (the predicted alternate σ32 gene) [24] (GenBank # NC_007799.1). Both σ32 and σ70 are conserved in most proteobacteria [25].

Transcription from a gene promoter by a σ32- or σ70-bound RNAP typically involves recognition of and binding to two DNA motifs located upstream from the transcription start site (TSS) [26]. These include the -35 motif, located about 35 bp upstream of the TSS [20], and the -10 motif which is present at about 10 bp upstream of TSS [20]. The conserved -35 region of promoters recognized by σ70 transcription regulator in E. coli (TTGAAA) is analogous to the -35 motif recognized by its σ70 (TTGACA) [22,27]; however, -10 motifs of σ32 promoters in E. coli (CCATNT) are markedly different from those recognized by σ32 (TATAAT) [27,28].

We recently mapped the promoters of two outer membrane protein genes (p28-Omp14 and p28-Omp19) of E. chaffeensis that are transcribed by its σ70 transcription regulator [29] and reported that the -35 motif is highly homologous to the consensus E. coli -35 motif and that it is required to initiate the transcription. Furthermore, we reported that the -10 motifs of E. chaffeensis genes are not homologous to the E. coli sequences and are also nonessential for the promoter-specific transcription [29].

In the current study, we used molecular approaches to map several gene promoters of E. chaffeensis. We present evidence demonstrating that the σ32 and σ70 binding motifs share extensive homology for genes likely to be transcribed by the two sigma factors and that the promoter-specific transcription is accomplished by either of the transcription regulators.

Materials and Methods

Bioinformatics

Promoter sequences upstream to the transcription start sites (experimentally determined) of genes were evaluated to identify -10 and -35 motifs using WCONSENSUS version 5c (http://stormo.wustl.edu/consensus/cgi-bin/Server/Interface/wconsensus.cgi) [30] and WebLogo (weblogo.berkeley.edu) [31,32] programs. Multiple DNA alignments were done using Clustal X version 2.0 with default parameters [33].

E. coli strains and plasmids

E. coli strains used in this study were TOP10 (Invitrogen Technologies, Carlsbad, CA), BL21(DE3)pLysS (Novagen, San Diego, CA) and CAG57101[34]. Genetic makeup of CAG57101 is included in Table 1. Several plasmid constructs used in this study were obtained from commercial sources or recombinantly modified from one or more existing plasmids described in the literature. They include pCR2.1 TOPO (Invitrogen Technologies), pET32a (Novagen) and the derivatives of pSAKT32 [35], pQF50K [35] and pMT504 [36]. Genetic makeup details of the original plasmids and their derivatives are included in Table 1 for all plasmids except those obtained from commercial sources. The plasmid pSAKT32, with a p15A origin of replication and containing an ampicillin resistance gene, has E. coli rpoH under the control of an IPTG inducible wild-type Pgal promoter [34,35]. The E. coli rpoH from this plasmid was replaced with the E. chaffeensis rpoH (Ech_rpoH). Cloning was accomplished by digesting the pSAKT32 with Afl II and Sal I to remove the E. coli rpoH, blunt ending with Klenow DNA polymerase (BioLabs, Ipswich, MA), then ligating with the Ech_rpoH sequence. Ech_rpoH was generated by PCR from genomic DNA using Pfu DNA polymerase (Promega, Madison, WI). The final derived plasmid is referred as the pSAKT32-Ech_rpoH. All E. chaffeensis rpoH variants (substitutions within the 4.2 region of E. chaffeensis σ32) were constructed with a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA). The names of the modified pSAKT32-Ech_rpoH are provided in Table 1.

The plasmid pQF50K, with a pMB1 origin of replication and carrying a kanamycin resistance gene cassette, contains the β-galactosidase coding sequence (lacZ) driven by E. coli groE promoter for use in assessing the promoter’s function [35]. It was modified to replace with E. chaffeensis promoters of groE, hup or dnaK genes in front of the lacZ coding sequence. The full length groE promoter of E. chaffeensis or the promoter lacking the -35 motif or the promoter lacking the -35 motif and the entire sequence upstream to it were amplified using the gene-specific primers and the organism’s genomic DNA as the template. Similarly, hup and dnaK promoter segments or those lacking -35 motifs were amplified with Sph I and Xba I restriction enzyme sites engineered to facilitate directional cloning. The promoter segments were cloned upstream to the lacZ coding sequences after deleting the E. coli groE promoter. The promoter derivatives with deletion of -35 motifs were constructed in the pQF50K-Ech_promoter plasmids by using a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). The names of all engineered plasmids are listed in (Table 1).

The pET32a plasmid vector (Novagen) encoding E. chaffeensis σ32 and σ70 were prepared and used to prepare purified recombinant proteins. The cloning and purification of E. chaffeensis σ32 is reported earlier [37]. E. chaffeensis σ32 was cloned similarly into pET32a vector and was utilized to prepare recombinant protein as in [37].

Full-length E. chaffeensis promoter segments of dnaK, hup and groE were also cloned into the plasmid pMT504 at the EcoR V site for use in the in vitro transcription analysis (described below). The promoter inserts were also cloned in opposite orientation to serve as negative controls to demonstrate promoter-specific in vitro transcription. The pMT504 has a G-less cassette to serve as the transcription
Table 1. Bacterial strain and plasmids used in this study.

| Name | Description | Reference |
|------|-------------|-----------|
| **E. coli** | | |
| CAG57101 | MG1655 △lacX74, P<sub>BAD</sub>-groESL::cat, △rpoH::<i>aadA</i>, Cm<sup>R</sup>, Sp<sup>R</sup> | [34] |
| **Plasmids** | | |
| pSAKT32 | p15A ori, <i>E. coli</i> pKmR, lacZ, Amp<sup>R</sup> | [35] |
| pSAKT32-Ech<sub>l</sub>_p28-Omp19 | pMT504 constructs lacking pSAKT32-Ech<sub>rpoH</sub>_R<sub>269</sub> | This study |
| pQF50K-groE | For <i>E. chaffeensis</i> σ<sub>32</sub> expression | This study |
| pQF50K-GroESL::cat | promoter, lacI, groESL::cat, Sp<sup>R</sup> | This study |
| pMT504-p28-Omp19 | Reverse orientation | This study |
| pMT504-p28-Omp19-35 | p28-Omp19 promoter with -35 motif deletion | This study |
| pMT504-P28-Omp14 | Reverse orientation | This study |
| pMT504-P28-Omp14-35 | p28-Omp14 promoter with -35 motif deletion | This study |
| pQF50K-groE | promoter, lacI | This study |
| pQF50K-Ech<sub>l</sub>_p28-Omp19 | pMT504 constructs lacking pQF50K-groE | This study |
| pQF50K-Ech<sub>l</sub>_hup | promoter | This study |
| pQF50K-Ech<sub>l</sub>_dnaK-35del | promoter with -35 motif deletion | This study |
| pQF50K-Ech<sub>l</sub>_hup-35del | promoter with -35 motif deletion | This study |
| pQF50K-Ech<sub>l</sub>_groE-35del | promoter with -35 motif deletion | This study |
| pQF50K-Ech<sub>l</sub>_groE-35delupd | promoter with -35 motif deletion | This study |
| pMT504 | Amp<sup>R</sup>, as templates of in vitro transcription | [36] |
| pMT504-p28-Omp19 | E. <i>chaffeensis</i> p28-Omp19 promoter in pMT504 | [37] |
| pMT504-p28-Omp19-R | Reverse orientation. E. <i>chaffeensis</i> P28-Omp19 promoter | [37] |
| pMT504-p28-Omp19-35 | E. <i>chaffeensis</i> p28-Omp19 promoter with -35 motif deletion | This study |
| pMT504-p28-Omp14 | Reverse orientation. E. <i>chaffeensis</i> P28-Omp14 promoter | [37] |
| pMT504-p28-Omp14-R | Reverse orientation. E. <i>chaffeensis</i> P28-Omp14 promoter | [37] |
| pMT504-p28-Omp14-35 | E. <i>chaffeensis</i> p28-Omp14 promoter with -35 motif deletion | This study |
| pMT504-Ech<sub>l</sub>_dnaK | E. <i>chaffeensis</i> dnaK promoter in pMT504 | This study |
| pMT504-Ech<sub>l</sub>_dnaK-R | Reverse orientation. E. <i>chaffeensis</i> dnaK promoter | This study |
| pMT504-Ech<sub>l</sub>_groE | E. <i>chaffeensis</i> groE promoter in pMT504 | This study |
| pMT504-Ech<sub>l</sub>_groE-R | Reverse orientation. E. <i>chaffeensis</i> groE promoter | This study |
| pMT504-Ech<sub>l</sub>_hup | E. <i>chaffeensis</i> hup promoter in pMT504 | This study |
| pMT504-Ech<sub>l</sub>_hup-R | Reverse orientation. E. <i>chaffeensis</i> hup promoter | This study |
| pET32-Ech<sub>l</sub>_rpoH | For overexpression of E. <i>chaffeensis</i> σ<sub>32</sub> | This study |
| pET32-Ech<sub>l</sub>_rpoD | For overexpression of E. <i>chaffeensis</i> σ<sub>70</sub> | [37] |

The PE products were detected after developing the film with a X-ray film. Plasmid DNA templates containing the respective gene segments were used in a Sanger’s DNA sequence reaction to generate the DNA sequence ladders to identify the transcription start sites (TSS) using a Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit (USB, Cleveland, OH). The PE products were detected after developing the film with a Konica film processor (Konica, Wayne, NJ).

by PCR cloning strategy using the previously prepared -35 deletion plasmids in pBlueTOPO [29] as templates. The predicted lengths of transcripts for the promoter segments groE, dnaK, hup, of p28-Omp14 and p28-Omp19 in pMT504 plasmid are 155, 156, 172, 162 and 162 nucleotides, respectively (as per the defined transcription start sites). Integrity of all cloned segments in the plasmid constructs described here was confirmed by automated DNA sequencing analysis using Beckman coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, CA). (All primers used for various plasmid manipulations were described in Table S1.)
5' Rapid Amplification of cDNA Ends (5'RACE)

5'RACE experiments were performed on E. chaffeensis RNA (1 μg) to map the TSSs using a 5' RACE kit version 2.0 (Invitrogen Technologies). The final products were resolved in a 1% agarose gel; the major amplicons were gel isolated and used to perform DNA sequencing analysis. (5'RACE primers are listed in Table S1.)

In vitro transcription assays

In vitro transcription reactions were performed in 10 μl reaction mixture containing 0.13 picomoles each of the supercoiled plasmid DNAs as templates and using RNAP holoenzymes containing either recombinant E. chaffeensis σ32 or σ70 as in [37]. The holoenzymes were prepared by incubating 0.5 μl of 1:10 diluted stock of E. coli core enzyme (Epicentre, Madison, WI) mixed with 10 fold molar excess of purified recombinant E. chaffeensis σ32 or σ70 on ice for 30 min. The transcription reactions were incubated at 37°C for 20 min, and the reactions were terminated by adding 7 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). Six microliters each of the samples were electrophoresed on a 6% polyacrylamide sequencing gel with 7 M urea, then gels were transferred to a Whatman paper, dried and transcriptions were visualized by exposing an X-ray film to the gels. Control reactions included only E. coli core enzyme or recombinant σ70 or σ32 in the absence of core enzyme. Additional controls included the use of promoter constructs prepared in reverse orientation or promoters lacking -35 motifs (promoters of p28-Omp14 and p28-Omp19 genes).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed with a LightShift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL) with minor modifications. Briefly, biotin labeled probes were prepared by PCR from E. chaffeensis genomic DNA as the template and using probe-specific oligonucleotide primers (one primer contained biotin at the 5’ end; the primers were listed in Table S1). The amplicons were purified using Qiagen DNA columns (Qiagen, Valencia, CA). Cold competitor probes were produced in the same manner, except that neither of the primers used in the PCRs contained biotin tags. All competitor DNAs were produced in 10-fold molar excess compared with the labeled probes. DNA–protein binding reactions were carried out at 30°C for 25 min in 20 μl volume containing 1x binding buffer [50 mM Tris-acetate (pH 8.0), 50 mM potassium acetate, 8.1 mM magnesium acetate, 27 mM ammonium acetate, 100 μg/ml BSA and 5% glycerol], 50 μg/ml poly dl-dc and 20 fmol each of a probe and RNAP holoenzyme. The holoenzyme was assembled with 0.25 μl E. coli RNAP core enzyme (Epicentre) and 10-fold molar excess of E. chaffeensis σ32 or σ70 relative to the E. coli RNAP core enzyme and incubated on ice for 30 min prior to use in the EMSAs. Control reactions included only E. coli core enzyme or recombinant σ70 or σ32 in the absence of core enzyme or used an open reading sequence probe of dnaK (dnaK-ORF). The reactions were stopped by adding 5 μl of gel loading buffer and electrophoresed in 4% native polyacrylamide gel in 0.5X TBE buffer at 80 V for 1.5 h, then the DNA and DNA–bound proteins were transformed to a nylon membrane by electrophoretic transfer. Biotinylated DNA fragments were detected by the chemiluminescence method (Pierce Biotechnology).

β-Galactosidase assays

Overnight E. coli cultures of CAG57101 were diluted 1:100 into a fresh medium containing appropriate antibiotics, grown to 0.6–0.8 optical density, and induced with 1 mM IPTG for 3 h before harvesting. E. coli lysates were then prepared and used to measure β-galactosidase activity using a β-gal assay kit (Invitrogen Technologies). IPTG-non induced cultures were used to serve as the controls. The experiment was performed three times with three independently grown cultures, and specific activity of β-galactosidase was calculated using the formula (specific activity = nmoles ONPG hydrolyzed/ min/mg protein).

Determination of the E. chaffeensis rpoD and rpoH Gene Expression by TaqMan-based Quantitative RT-PCR

DH82 macrophage culture-derived E. chaffeensis organisms from a nearly 100% infected culture flask was purified and used to infect flask containing naïve DH82 cultures. Total RNA was isolated from cell cultures several times post-inoculation (6 h to 84 h). Total RNA was also recovered from uninfected cultures to serve as a zero time point and also to serve as the negative control for the analysis. Total RNA was recovered using a Tri Reagent RNA isolation kit. The residual genomic DNA was eliminated from RNA preparations using a Turbo DNA-free kit (Invitrogen Technologies). Gene-specific primers targeting the rpoD or rpoH gene transcripts (Table S1) or the 16S rRNA (primers and probes were described earlier [40]) were used in real-time RT-PCR analysis using cDNAs as the templates and target-specific TaqMan probes. Total cDNA was synthesized from each RNA sample after annealing random oligonucleotides and by performing reverse transcription using a SuperScript III First Strand cDNA Synthesis System kit (Invitrogen Technologies). Concentrations of cDNAs were adjusted based on the initial PCR analysis targeting the 16S rRNA, and equal amounts of cDNAs were used to perform the TaqMan-based real-time PCR assays as in [40]. The Ct values were normalized based on 16S RNA transcripts and converted to fold change relative to zero time point RNA [41].

Statistical analysis

Statistical analyses were performed using Student's t-test, and a P-value <0.01 was considered significant.

Results

Mapping the transcription start sites (TSS) and locating the RNA polymerase binding motifs of E. chaffeensis genes

The E. chaffeensis contains only two genes encoding for sigma factors (GenBank # NC_007799.1) [24]; the primary housekeeping sigma factor, σ70, gene (rpoD) (genome...
and purified reconstitute the RNAP complex [37]. The promoter sequences resulted in generation of the predicted size transcripts for all (data not shown). Likewise, all five promoter segments cloned from the RNAP with additional negative controls for the promoters of outer membrane protein genes, p28-Omp14 and p28-Omp19, were consistent with our prior results [37] and demonstrated that these promoters were also recognized by the σ70. The abundance of transcripts, however, was higher with the RNAP containing recombinant σ70 compared to those observed for σ32.

Promoter activity of dnaK, groE and hup assessed in E. coli mutant for σ32 following induced expression of E. chaffeensis σ32

In vitro transcription analysis demonstrated that the E. chaffeensis transcription regulators, σ32 and σ70, were not exclusive in their recognition of the promoters, although their promoter recognition specificities differed. To further define the promoter activities of E. chaffeensis, we developed an assay in an E. coli strain, CAG57101, in which its chromosomal σ32 was inactivated [34] and E. chaffeensis σ32 protein was expressed from a plasmid under the control of IPTG-inducible Plac promoter. The E. coli strain was transformed with a second recombinant plasmid containing E. chaffeensis promoter segments of genes dnaK, groE or hup, which were cloned upstream to the promoterless β-galactosidase gene coding sequence. We reasoned that the E. coli RNAP core enzyme forms a functional holoenzyme complex with E. chaffeensis σ32 in vivo (similar to in vitro studies) when it is expressed. This experiment is similar to studies reported as characterizing the RNAP function of other bacterial organisms [34,35,43]. (Promoter segments and plasmids used for this assay system were described in Figure 4). Induction of the E. chaffeensis σ32 expression in E. coli resulted in the increase of β-galactosidase expression for two gene promoters (dnaK and groE) (Figure 5). The increase in β-galactosidase expression is significant and is about 60% and 45% above the background level observed for the dnaK and groE promoters, respectively. The β-galactosidase activity for the hup promoter remained unchanged in the presence or absence of E. chaffeensis σ32 expression. In controls where E. chaffeensis σ32 expression was not induced, β-galactosidase was also made from the dnaK and groE promoters; however, the enzyme activity was considerably low compared with that observed for the hup promoter which had about 6-10 times higher β-galactosidase activity compared with that observed for dnaK and groE promoters. Because the in vitro transcription analysis (described above) revealed that the hup is predominantly a σ70 specific promoter (Figure 3), we reasoned that the high hup promoter activity in E. coli is due to its recognition by the E. coli σ70. The transcription increase for dnaK and groE promoters in the E. coli strain following inducing the E. chaffeensis σ32 is consistent with the observations made from in vitro transcription analysis, thus validating that the dnaK and groE promoters have higher affinity for the σ32, whereas the hup promoter was predominantly recognized by the σ70. We reasoned that the low level promoter activity observed for dnaK and groE promoters in the absence of E. chaffeensis σ32 is also the result of the promoters’ recognition by the E. coli σ70. This
observation is also consistent with the prior study demonstrating that E. coli σ70-bound RNAP does serve as the surrogate in driving the gene expression from genes of rickettsial organisms, including from E. chaffeensis promoters [29,37,44,45].

Because our prior studies demonstrated that the -35 motif, but not the -10 motif, is critical to σ70 binding [29], we reasoned that the -35 motif is also similarly important to σ32 binding. We created constructs lacking the -35 motifs from all three gene promoter segments in the plasmids containing the dnaK, groE and hup promoters. The modified plasmids were then used to assess for the β-galactosidase expression (Figure 5). The -35 deletions from the promoters of dnaK and hup resulted in a considerable reduction of β-galactosidase expression, whereas the enzyme activity for the groE promoter -35 motif deletion increased significantly compared with the wild-type promoter.
and the higher promoter activity was independent of the IPTG induction (Figure 5). We found a second consensus -35 motif (TTGATA) immediately upstream from the deleted -35 motif for the groE promoter (Figure 4A). We reasoned that repositioning the second -35 motif into the deleted first -35 motif’s location kept the spacing between the -35 and -10 motifs almost constant (16 nucleotides in the first and 17 nucleotides in the second) and that repositioning of the second motif may have functioned as a new -35 motif (Figure 4A). This secondary -35 motif, however, was not responsive to the induced expression of the RNAP holoenzyme containing either σ70 or σ32 to promoter segment was further demonstrated by including additional controls; for example, holoenzyme containing σ32 or σ70 did not bind to a DNA fragment prepared from a coding sequence (dnak-ORF) (Figure 6B). Similarly, σ32 or σ70 alone did not bind to a promoter segment (assessed for dnak promoter) (Figure 6C). E. coli core enzyme, which is known to bind non-specifically to DNA [46,47], showed a minor gel shift (Figure 6C).

Substitutions in region 4.2 of E. chaffeensis σ32

Previous research in E. coli demonstrated that four conserved, charged amino acids within the region 4.2 of σ32, E265, R266, R268 and Q269, are essential for binding to the -35 motif of a promoter sequence [48]. To determine if the amino acids are similarly conserved, sequence alignment was performed for E. chaffeensis σ32 and σ70 with those of E. coli, which revealed extensive amino acid conservation among all four sequences. The conserved amino acids indeed included the four charged amino acids of the region 4.2 (Figure 7A). To assess if mutations in these four amino acids in E. chaffeensis gene similarly affect the promoter recognition, individual substitution mutations were made in the recombinant plasmid sequences spanning the four amino acids in the E. chaffeensis rpoH gene coding sequence and were used to express mutant proteins and their impact on the β-galactosidase expression driven from the wild-type dnaK promoter. Amino acid changes

and the higher promoter activity was independent of the IPTG induction (Figure 5). We found a second consensus -35 motif (TTGATA) immediately upstream from the deleted -35 motif for the groE promoter (Figure 4A). We reasoned that repositioning the second -35 motif into the deleted first -35 motif’s location kept the spacing between the -35 and -10 motifs almost constant (16 nucleotides in the first and 17 nucleotides in the second) and that repositioning of the second motif may have functioned as a new -35 motif (Figure 4A). This secondary -35 motif, however, was not responsive to the induced expression of the RNAP holoenzyme containing either σ70 or σ32 to promoter segment was further demonstrated by including additional controls; for example, holoenzyme containing σ32 or σ70 did not bind to a DNA fragment prepared from a coding sequence (dnak-ORF) (Figure 6B). Similarly, σ32 or σ70 alone did not bind to a promoter segment (assessed for dnak promoter) (Figure 6C). E. coli core enzyme, which is known to bind non-specifically to DNA [46,47], showed a minor gel shift (Figure 6C).

Substitutions in region 4.2 of E. chaffeensis σ32

Previous research in E. coli demonstrated that four conserved, charged amino acids within the region 4.2 of σ32, E265, R266, R268 and Q269, are essential for binding to the -35 motif of a promoter sequence [48]. To determine if the amino acids are similarly conserved, sequence alignment was performed for E. chaffeensis σ32 and σ70 with those of E. coli, which revealed extensive amino acid conservation among all four sequences. The conserved amino acids indeed included the four charged amino acids of the region 4.2 (Figure 7A). To assess if mutations in these four amino acids in E. chaffeensis gene similarly affect the promoter recognition, individual substitution mutations were made in the recombinant plasmid sequences spanning the four amino acids in the E. chaffeensis rpoH gene coding sequence and were used to express mutant proteins and their impact on the β-galactosidase expression driven from the wild-type dnaK promoter. Amino acid changes

and the higher promoter activity was independent of the IPTG induction (Figure 5). We found a second consensus -35 motif (TTGATA) immediately upstream from the deleted -35 motif for the groE promoter (Figure 4A). We reasoned that repositioning the second -35 motif into the deleted first -35 motif’s location kept the spacing between the -35 and -10 motifs almost constant (16 nucleotides in the first and 17 nucleotides in the second) and that repositioning of the second motif may have functioned as a new -35 motif (Figure 4A). This secondary -35 motif, however, was not responsive to the induced expression of the RNAP holoenzyme containing either σ70 or σ32 to promoter segment was further demonstrated by including additional controls; for example, holoenzyme containing σ32 or σ70 did not bind to a DNA fragment prepared from a coding sequence (dnak-ORF) (Figure 6B). Similarly, σ32 or σ70 alone did not bind to a promoter segment (assessed for dnak promoter) (Figure 6C). E. coli core enzyme, which is known to bind non-specifically to DNA [46,47], showed a minor gel shift (Figure 6C).

Substitutions in region 4.2 of E. chaffeensis σ32

Previous research in E. coli demonstrated that four conserved, charged amino acids within the region 4.2 of σ32, E265, R266, R268 and Q269, are essential for binding to the -35 motif of a promoter sequence [48]. To determine if the amino acids are similarly conserved, sequence alignment was performed for E. chaffeensis σ32 and σ70 with those of E. coli, which revealed extensive amino acid conservation among all four sequences. The conserved amino acids indeed included the four charged amino acids of the region 4.2 (Figure 7A). To assess if mutations in these four amino acids in E. chaffeensis gene similarly affect the promoter recognition, individual substitution mutations were made in the recombinant plasmid sequences spanning the four amino acids in the E. chaffeensis rpoH gene coding sequence and were used to express mutant proteins and their impact on the β-galactosidase expression driven from the wild-type dnaK promoter. Amino acid changes

Table 2. The TSS and -35 and -10 motifs of E. chaffeensis genes.

| Genes/operons* | Locus-tag | -35 motif | -10 motif | TSS mapped by |
|---------------|-----------|-----------|-----------|--------------|
| dnaK          | ECH_0471  | TTGTAACctgatcgtgctATATATCggtatA | PE |
| groE          | ECH_0364-0365 | TTGAAAAtagatcgtgctATATATgatT | PE |
| clpA          | ECH_0567  | TTATTTTcaacttatatttaattctTAGTGcttaggTTT | PE |
| hup           | ECH_0996-0997 | TTATACATctctctcaagttATAGAaagaG | PE |
| clpB          | ECH_0367  | TTATATTGttataattttcaattctTAGTaaapoT | PE |
| glyQ          | ECH_0023-0025 | TTGTAATacctcattlctctTATAATagcataA | 5 RACE |
| grpE          | ECH_0168  | TTGATAAATcctaaagtTlactTATAATcctcA | 5 RACE |

*Operons as listed in Figure 1. -35 and -10 (underlined text) motifs identified for each gene.

doi: 10.1371/journal.pone.0081780.t002

...and the higher promoter activity was independent of the IPTG induction (Figure 5). We found a second consensus -35 motif (TTGATA) immediately upstream from the deleted -35 motif for the groE promoter (Figure 4A). We reasoned that repositioning the second -35 motif into the deleted first -35 motif’s location kept the spacing between the -35 and -10 motifs almost constant (16 nucleotides in the first and 17 nucleotides in the second) and that repositioning of the second motif may have functioned as a new -35 motif (Figure 4A). This secondary -35 motif, however, was not responsive to the induced expression of the RNAP holoenzyme containing either σ70 or σ32. Complete deletion of the promoter region spanning from the first -35 motif to the entire upstream sequence of the groE promoter resulted in complete loss of promoter activity (Figure 5B).

Electrophoretic Mobility Shift assays (EMSA) to further assess the interactions of σ32 and σ70 with E. chaffeensis gene promoters

Shared recognition of E. chaffeensis promoters by both the transcription regulators (σ32 and σ70) (described above) validates our working hypothesis that although specificity differences exist, overlap in the recognition of -35 motifs of E. chaffeensis promoters by σ32 and σ70 is significant. In particular, the dnaK and groE promoters had greater specificity to the σ32, whereas the promoters of genes hup, p28-Omp14 and p28-Omp19 had higher affinity for the σ70. The shared recognition of E. chaffeensis promoters with altered specificities may have resulted due to differences in binding affinities of the sigma factors with core RNAP. To test this hypothesis, we performed EMSA analysis with probes prepared from the promoter segments of all five genes (dnaK, groE, hup, p28-Omp14 and p28-Omp19) and by incubating with the RNAP holoenzyme containing E. chaffeensis recombinant σ32 or σ70 (Figure 6A). The promoters with higher affinity for σ32, dnaK and groE, had stronger gel-shifted fragments in the presence of RNAP holoenzyme with σ32 compared with those observed for the σ70 dependent promoter segments (hup, p28-Omp14 and p28-Omp19). Similarly, shifted fragments were more abundant for the σ70-containing RNAP for the genes with higher affinity for it (hup, p28-Omp14 and p28-Omp19). The specificity of gel-shifted fragments was confirmed by adding cold competitors. Specific interaction of the RNAP holoenzyme containing either σ32 or σ70 to promoter segment was further demonstrated by including additional controls; for example, holoenzyme containing σ32 or σ70 did not bind to a DNA fragment prepared from a coding sequence (dnak-ORF) (Figure 6B). Similarly, σ32 or σ70 alone did not bind to a promoter segment (assessed for dnak promoter) (Figure 6C). E. coli core enzyme, which is known to bind non-specifically to DNA [46,47], showed a minor gel shift (Figure 6C).
at each of the four locations to the non-polar amino acid (alanine) resulted in about an 80% decline in transcription activity (assessed by β-galactosidase expression) compared with that observed for wild-type σ32 (Figure 7B).

Figure 2. RNAP binding motifs -35 and -10 of E. chaffeensis genes. RNAP binding motifs, -35 and -10, are identified for the 12 E. chaffeensis genes for which TSS were mapped (listed in Tables 2). The upper panel has the consensus motifs for the σ32 dependent gene promoters; the middle panel includes the σ70-dependent promoters and the lower panel includes the consensus motifs for all 12 genes assessed.

doi: 10.1371/journal.pone.0081780.g002

Expression patterns of E. chaffeensis rpoD and rpoH gene transcripts during the infection of mammalian cells are very similar

To assess the expression patterns of rpoH (σ32 gene) and rpoD (σ70 gene), E. chaffeensis RNA isolated from infected macrophage cultures at different times post infection was examined by TaqMan probe-based quantitative RT-PCR assay (Figure 8). The expression levels of both rpoD and rpoH were initially high following inoculation, with relatively higher expression from the rpoH gene at 6 h post-infection. Both the
gene transcripts decreased steadily, however, and remained low until 60 h post infection and slightly increased thereafter. No notable differences were found in the expression patterns for the \( \text{rpoD} \) and \( \text{rpoH} \) gene transcripts during the 84 h of assessment of the organism’s growth in macrophage cultures.

**Discussion**

Life for an intraphagosomal bacterium is complex because the organisms must adapt to the host’s phagosomal micro-environment, which is suboptimal for bacterial growth. The intraphagosomal bacterium, \( \text{E. chaffeensis} \), has an unusual developmental cycle requiring the growth and replication within phagosomes of eukaryotic cells of vertebrate and tick hosts. During its developmental cycle, \( \text{E. chaffeensis} \) exists in two distinct morphological forms: the elementary bodies (EBs) and the reticulate bodies (RBs) [49-51]. EB (the infectious form) transforms to metabolically active RB after entry into a host cell and replicates by binary fission [49-51]. Very little is known about how the organism overcomes the host-induced stress in support of its invasion and replication in host phagosomes and its subsequent release and reinfection of naïve host cells. Cheng et al. [52] recently presented the first evidence of the possible involvement of a response regulator, CtrA, of \( \text{E. chaffeensis} \) in human monocytes when the organism develops into EBs. The CtrA binds to the promoter regions of several genes activated during this stage of development; however, much remains to be understood about how \( \text{E. chaffeensis} \) regulates its gene expression in support of its growth in a host cell. Growth of the organism in vertebrate and tick host cells results in altering expression of many genes; recent studies revealed many host-specific differences in gene expression as
assessed by global changes in gene and protein expression [13,14]. It is entirely unknown how the organism senses the host environment and alters its gene expression. One possibility is that the differential expression may be accomplished by regulating gene expression by DNA binding proteins which influence the function of RNAP. This hypothesis remains to be tested.

Host adaptation of a bacterium requires altering its gene expression of many genes, including those encoding for stress response proteins and many outer membrane proteins. For example, the heat shock protein ClpB is an ATP-dependent molecular chaperone that reactivates aggregated proteins accumulating under stress conditions [53]. We recently presented the first evidence that clpB gene expression increases during replication stage of *E. chaffeensis* [54]. Similarly, we and others reported global macrophage and tick cell-specific protein and gene expression differences [13,14]; the differentially expressed proteins included various outer membrane proteins [11,13,55,56].

The sigma factor, σ70, is considered the chief regulatory protein of transcription of genes under stressful environments in bacteria, and genes expressed with the help of this regulator include many conserved heat-shock proteins that help bacteria resist stress by decreasing the accumulation of misfolded and aggregated proteins [57-59]. On the contrary, σ32 is considered the primary housekeeping sigma factor [20]. Considerable progress has been made in defining the contributions of alternate sigma factor (σ32) in support of stress response in *E. coli* [60]. Recent study on *Francisella tularensis* demonstrates that σ32 also contributes to the stress response in this intracellular pathogenic bacterium [61]; however, research is limited about understanding the role of sigma factors in intraphagosomal bacteria such as *E. chaffeensis* and other related rickettsiae organisms. Knowledge about how regulation of gene expression is accomplished by the pathogenic rickettsiae is similarly limited. In this study, we utilized multiple molecular tools to assess the functions of the only two sigma factors (σ32 and σ70) of *E. chaffeensis*. We mapped the transcription start sites of 12 genes of *E. chaffeensis*, which are likely recognized by the pathogen’s σ32 or σ70 (10 in the current study and two reported previously [29]). We examined the promoter activities of several *E. chaffeensis* genes in vitro using the *E. coli* expression system. We discovered that the first three nucleotides are identical for both the -10 and -35 motifs in the predicted σ32 and σ70 binding motifs (-10 and -35 motifs) of *E. chaffeensis* gene promoters; we presented evidence supporting the shared recognition of *E. chaffeensis* gene promoters by both σ32 and σ70, although the specificities of promoters for each sigma factor are different. For example, promoters of the predicted stress-response genes, *groE* and *dnaK*, are primarily recognized by the alternate sigma factor, σ32, whereas the *hup*, *p28-Omp14* and *p28-Omp19* gene promoters had higher affinity to σ70. Interestingly, the host cell-specific differentially expressed membrane protein genes, *p28-Omp14* and *p28-Omp19*, although predominantly transcribed by σ32, are also transcribed by the pathogen’s likely alternate sigma factor, σ32, at a relatively high rate. The shared recognition of the pathogen gene promoters by RNAP

---

**Figure 5.** *E. chaffeensis* promoter activities (A, *dnaK*; B, *groE*; and C, *hup*) assessed in *E. coli* by measuring the β-galactosidase expression. The β-galactosidase expression driven by *E. chaffeensis* promoters from the wild-type promoters (*dnaK*, *groE* or *hup*), promoters containing -35 motif deletion (*dnaK-35del*, *groE-35del* or *hup-35del*) or *groE* promoter having complete deletion from -35 to the entire upstream sequence (*groE-35updel*) were measured in the CAG57101 strain of *E. coli* before or after the induced expression of *E. chaffeensis* *rpoH*. The CAG57101 strain contained either promoterless pQF50K (control) or one of the pQF50K-Ech_promoter plasmids together with the pSAKT32-Ech_rpoH plasmid (described in Figure 4). Three independent experiments were performed; the error bars indicate standard deviation. Significant changes in the β-galactosidase activity were identified with double asterisks where the P values were <0.01.

doi: 10.1371/journal.pone.0081780.g005
containing either σ\(^{32}\) or σ\(^{70}\) in transcribing genes of E. chaffeensis is intriguing and suggests that its transcription system is evolved to be co-regulated by both sigma factors in controlling the gene expression. Our transcriptional analysis of the E. chaffeensis rpoH and rpoD genes revealed similar expression patterns of an initial burst during the first 6 h post-infection, then maintaining expression with a slow decrease in both transcripts from 12 h to 60 h post-infection, then a minor increase in transcription from 60-84 h post-infection. The observed changes in the gene expression of rpoD and rpoH probably parallels the organism’s transformation to RBs, their continued replication and then reversion into EBs [1]. The transcription regulators, σ\(^{32}\) or σ\(^{70}\), in E. chaffeensis may function as a team in regulating gene expression. Gene regulation in E. chaffeensis may also involve the contributions of transcription regulatory proteins, specifically to alter gene expression in support of adapting the organism to dual host environments. Progress described here in understanding gene regulation is the important first step, and continued research in this virtually unexplored territory is warranted.

In proteobacteria, regardless of α, β, γ and δ subdivision, the -35 motif of the σ\(^{32}\)-dependent genes shows the sequence conservation of the first three residues as ‘TTG’ [61-67]. The consensus -35 motifs in all assessed E. chaffeensis genes,
independent of their prediction as the $\sigma^{32}$ or $\sigma^{70}$ dependent genes, included the same three conserved nucleotides. In this study, we presented evidence that the deletion of the predicted -35 motifs of $dnaK$, $groE$ and $hup$ promoters significantly decreased promoter activities. We reported earlier that the deletion of -35 motifs in $p28-Omp14$ and $p28-Omp19$ gene promoters also results in a significant decline in promoter activity [29]. Experimental data presented here demonstrated that the binding of $\sigma^{32}$ to -35 motif of a promoter is mediated by the four well-conserved charged amino acids in region 4.2. Sigma factors are composed of a variable number of regions, each having a specific function in promoter recognition and region 4.2 in $E. coli$ $\sigma^{32}$ is shown to be involved in the base-specific interaction with the -35 motif. In particular, mutations causing substitutions in four conserved charged amino acids at E265, R266, R268 and Q269 to a non-polar amino acid, alanine, significantly reduce the promoter activity of $E. coli$ $\sigma^{32}$ [48]. Because these four amino acids are also conserved in $E. chaffeensis$ $\sigma^{32}$ and $\sigma^{70}$, mutational analysis in modifying the corresponding four conserved amino acids in the $rpoH$ gene of $E. chaffeensis$ to alanine similarly reduced the promoter activity of its $dnaK$ promoter. Together these results demonstrate that the -35 motifs are important to recognition of gene promoters by sigma factors in $E. chaffeensis$. Considering the shared recognition of $E. chaffeensis$ promoters by RNAP containing either $\sigma^{32}$ or $\sigma^{70}$ in transcribing the gene products, the pathogen’s gene regulation may have evolved requiring the interplay of both $\sigma^{32}$ and $\sigma^{70}$ and DNA binding proteins (transcription regulators) in support of its continued survival in vertebrate and tick hosts and in altering host cell-specific gene expression. This hypothesis, however, remains to be tested. We expect this study to lead the way in furthering our understanding of the regulation of gene expression in $E. chaffeensis$. This study also will aid future

Figure 7. Mutational analysis of $E. chaffeensis$ $rpoH$ gene spanning the conserved region 4.2. A) Protein sequence homology of $\sigma^{32}$ and $\sigma^{70}$ of $E. chaffeensis$ and $E. coli$ was assessed by Clustal X (version 2.0) for the entire sequence. The homology spanning region 4 was presented here. Numbers on the left indicate the amino acid position relative to the start codon of each protein. The four amino acids in $E. coli$, which are identified as critical for binding to the -35 motifs of $\sigma^{32}$ and $\sigma^{70}$, are also conserved in $E. chaffeensis$ (highlighted with an underlined text). Amino acids that are conserved in all four protein primary sequences are identified with asterisks; homology found only in two or three proteins was identified with a dot or colon, respectively. B) Mutational analysis of the four conserved amino acid residues predicted to be involved in the binding of $\sigma^{32}$ to the -35 motif. The amino acids at positions E266, R267, R269 and Q270 of $E. chaffeensis$ $\sigma^{32}$ were individually mutated to change the amino acids in the encoded proteins each to alanine. The mutant plasmids were used to assess the $E. chaffeensis$ $\sigma^{32}$ in driving the promoter activity of the wild-type $dnaK$ gene ($\beta$-galactosidase expression measured relative to the wild-type $E. chaffeensis$ $\sigma^{32}$). The experiment was performed three times, and average values were presented with error bars to show the standard deviation.

doi: 10.1371/journal.pone.0081780.g007
were used for plotting the graph.

normalizing the RNA levels used for the analysis relative to post-infection was assessed by quantitative RT-PCR after the experiment was performed three times, and the average values were used for plotting the graph.

doi: 10.1371/journal.pone.0081780.g008

research in defining the molecular mechanisms underlying the adaptation of *E. chaffeensis* to the host cell environment.

Conclusions

This study is the first to utilize various molecular approaches as useful in defining the promoters of *E. chaffeensis* genes. We mapped the transcription start sites of 8 *E. chaffeensis* genes and evaluated promoters of five genes; *groE, dnaK, hup, p28-Omp14* and *p26-Omp19* genes. We also presented evidence that the RNA polymerase binding motifs of *E. chaffeensis* gene promoters are highly homologous for its only two transcription regulators, sigma 32 and sigma 70, and that gene expression is accomplished by either of the transcription regulators. Evidence was also presented demonstrating that the sigma 32 and sigma 70 mRNAs are upregulated during the early replicative stage of the organism and the expression patterns remained similar for the entire replication cycle. Evidence was also presented demonstrating that the organism’s -35 motifs are essential to transcription initiations.

Supporting Information

Table S1. Oligonucleotides use in this study.

(DOCX)

Acknowledgements

We thank Dr. Pieter L. deHaseth of Case Western Reserve University, Cleveland, OH for providing pQF500GroE and pSAKT32 plasmids and Drs. Koo, Byoung-Mo and Carol A. Gross of University of California, San Francisco, CA for providing the *E. coli* CAG5T101 strain and Dr. Ming Tan of the University of California, Irvine, CA for providing the G-less cassette plasmid, pMT504. We also thank Ms. Mal Rooks Hoover for her help in preparing the figures. Publication of this article was funded in part by the Kansas State University Open Access Publishing Fund. This manuscript is contribution number 13-397-J from the Kansas Agricultural Experiment Station.

Author Contributions

Conceived and designed the experiments: RG HL. Performed the experiments: HL TV CC BF. Analyzed the data: HL RG CC. Contributed reagents/materials/analysis tools: HL CC BF RG. Wrote the manuscript: HL RG.

References

1. Rikihisa Y (2010) *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*: subversive manipulators of host cells. Nat Rev Microbiol 8: 328-339. doi:10.1038/nrmicro2318. PubMed: 20372198.
2. Walker DH, Dumler JS (1998) Emergence of the ehrlichioses as human health problems. Emerg Infect Dis 2: 18-29. doi:10.3201/eid0201.960102. PubMed: 8903194.
3. Walker DH, Paddock CD, Dumler JS (2008) Emerging and re-emerging tick-transmitted rickettsial and ehrlichial infections. Med Clin North Am 92: 1345-1361. doi:10.1016/j.mcna.2008.06.002. PubMed: 19061755.
4. Dawson JE, Anderson BE, Fishbein DB, Sanchez JL, Goldsmith CS et al. (1991) Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J Clin Microbiol 29: 2741-2745. PubMed: 1757543.
5. Fishbein DB, Sawyer LA, Holland CJ, Hayes EB, Okoroanyanwu W et al. (1987) Unexplained febrile illness after exposure to ticks: infection with *Ehrlichia*. JAMA 257: 3100-3104. doi:10.1001/jama.257.22.3100. PubMed: 3586228.
6. Dawson JE, Biggie KL, Warner CK, Cookson K, Jenkins S et al. (1996) Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human monocytic ehrlichiosis, in dogs, from southeast Virginia. Am J Vet Res 57: 1175-1179. PubMed: 8836370.
7. Dugan VG, Little SE, Stallknecht DE, Beall AD (2000) Natural infection of domestic goats with *Ehrlichia chaffeensis*. J Clin Microbiol 38: 448-449. PubMed: 10618139.
8. Kocan AA, Levesque GC, Whitworth LC, Murphy GL, Ewing SA et al. (2000) Naturally occurring *Ehrlichia chaffeensis* infection in coyotes from Oklahoma. Emerg Infect Dis 6: 477-480. doi:10.3201/eid0605.000505. PubMed: 10998377.
9. Davidson WR, Lockhart JM, Stallknecht DE, Howett ER, Dawson JE et al. (2001) Persistent *Ehrlichia chaffeensis* infection in white-tailed deer. J Wildl Dis 37: 538-546. doi:10.7589/0090-3558-37.3.538. PubMed: 11504227.
10. Dumler JS, Sutker WL, Walker DH (1993) Persistent infection with *Ehrlichia chaffeensis*. Clin Infect Dis 17: 903-905. doi:10.1093/clinids/17.5.903. PubMed: 8286638.
11. Unver A, Rikihisa Y, Stich RW, Ohashi N, Felek S (2002) The omp-1 major outer membrane multigene family of *Ehrlichia chaffeensis* is differentially expressed in canine and tick hosts. Infect Immun 70: 4701-4704. doi:10.1128/IAI.70.8.4701-4704.2002. PubMed: 12117987.
12. Zhang XF, Zhang JZ, Long SW, Ruble RP, Yu XJ (2003) Experimental *Ehrlichia chaffeensis* infection in beagles. J Med Microbiol 52: 1021-1026. doi:10.1099/jmm.0.05234-0. PubMed: 14532348.
13. Seo GM, Cheng C, Tomich J, Ganta RR (2008) Total, membrane, and immunogenetic proteomes of macrophage- and tick-derived *Ehrlichia chaffeensis* evaluated by liquid chromatography-tandem mass spectrometry and MALDI-TOF methods. Infect Immun 76: 4823-4832. doi:10.1128/IAI.00484-08. PubMed: 18710870.
14. Kuriakose JA, Miyashiro S, Luo T, Zhu B, McBride JW (2011) *Ehrlichia chaffeensis* transcriptome in mammalian and arthropod hosts reveals...
Extensive functional overlap between sigma factors in
Ehrlichia chaffeensis
441-466. doi: 10.1146/annurev.micro.57.030502.090913. PubMed: 11018136.

10.1186/1471-2180-9-99. PubMed: 19454021.

1365-2958.2009.06690.x. PubMed: 19400791.

1998.63.141. PubMed: 10384278.

16482227.

545-554. doi:10.1128/MMBR.00007-08. PubMed: 18772288.

16462227.

545-554. doi:10.1128/MMBR.00007-08. PubMed: 18772288.

5: 563-577. doi:10.1093/bioinformatics/15.7.563. PubMed: 10487864.

16818608.

2003) Convergence of transcriptional initiation and elongation in E. coli is highly variable and often rate limiting. Mol Cell 24: 747-757. doi: 10.1016/j.molcel.2006.10.030. PubMed: 17157257.

Tare P, China A, Naranja V (2012) Distinct and contrasting transcription Initiation patterns at Mycobacterium tuberculosis promoters. PLOS ONE 7: e43900. doi: 10.1371/journal.pone.0043900. PubMed: 22970148.

Barbet AF, Agnes JT, Moreland AL, Lundgren AM, Alleman AR et al. (2005) Identification of functional promoters in the msp2 expression loci of Anaplasma marginale and Anaplasma phagocytophilum. Gene 353: 89-97. doi:10.1016/j.gene.2005.03.036. PubMed: 15935572.

Long SW, Zhang XF, Qi H, Standaert S, Walker DH et al. (2002) Antigenic variation of Ehrlichia chaffeensis resulting from differential expression of the 28-kilodalton protein gene family. Infect Immunity 70: 1824-1831. doi: 10.1128/IAI.70.4.1824-1831.2002. PubMed: 11895944.

Ramirez-Romero MA, Masulis I, Cevallos MA, Gonzalez V, Dávila G (2006) The Rhizobium elijsigma70 (SagA) factor recognizes a lax consensus promoter. Nucleic Acids Res 34: 1470-1480. doi:10.1093/nar/gkj023. PubMed: 16528104.

Hinkle DC, Chamberlin MJ (1972) Studies of the binding of Ehrlichia coli RNA polymerase to DNA. I. The role of sigma subunit in site selection. J Mol Biol 70: 157-185. doi:10.1016/0022-2836(72)90531-1. PubMed: 4562312.

Koureennaiva OV, Tsujikawa L, Dehasest PL (2005) Mutational analysis of Escherichia coli heat shock transcription factor sigma32 reveals similarities with sigma 70 in recognition of the -10 promoter element and differences in promoter DNA melting and -10 recognition. J Bacteriol 187: 6762-6769. doi: 10.1128/JB.187.17.6762-6769.2005. PubMed: 16165359.

Popov VL, Chen SM, Feng HM, Walker DH (1995) Ultrasratral variation of cultured Ehrlichia chaffeensis. J Microbiol 43: 411-421. doi:10.1099/0222615-43-6-411. PubMed: 7467347.

Zhang JZ, Popov VL, Gao S, Walker DH, Yu XJ (2007) The developmental cycle of Ehrlichia chaffeensis in vertebrate cells. Cell Microbiol 9: 610-618. doi:10.1111/j.1462-5822.2006.00812.x. PubMed: 16987329.

DEDONDER SE, Cheng C, Willard LH, Boyle DL, Ganta RR (2012) Transmission electron microscopy reveals distinct macrophage- and tick cell-specific morphological stages of Ehrlichia chaffeensis. PLOS ONE 7: e36749. doi: 10.1371/journal.pone.0036749. PubMed: 22615806.

Cheng Z, Miura K, Popov VL, Kumagai Y, Rikihisa Y (2011) Insights into the CtrA regulon in development of stress resistance in obligatory intracellular pathogens, Ehrlichia chaffeensis. Mol Microbiol 82: 1217-1234. doi:10.1111/j.1365-2958.2011.07885.x. PubMed: 22014113.

Zolkiiewski M, Zhang T, Nagy M (2012) Aggregate reactivation mediated by the Hsp100 chaperones. Arch Biochem Biophys 520: 1-6. doi:10.1016/j.abb.2012.01.012. PubMed: 22242514.

Zhang T, Kedzierska-Mieszkowska S, Liu H, Cheng C, Ganta RR et al. (2013) Aggregate-reactivation activity of the molecular chaperone ClpB from Ehrlichia chaffeensis. PLOS ONE 8: e62454. doi:10.1371/journal.pone.0062454. PubMed: 23967479.
55. Singu V, Liu H, Cheng C, Ganta RR (2005) Ehrlichia chaffeensis expresses macrophage- and tick cell-specific 28-kilodalton outer membrane proteins. Infect Immun 73: 79-87. doi: 10.1128/IAI.73.1.79-87.2005. PubMed: 15618143.

56. Singu V, Peddireddi L, Sirigireddy KR, Cheng C, Munderloh U et al. (2006) Unique macrophage and tick cell-specific protein expression from the p28/p30-outer membrane protein multigene locus in Ehrlichia chaffeensis and Ehrlichia canis. Cell Microbiol 8: 1475-1487. doi: 10.1111/j.1462-5822.2006.00727.x. PubMed: 16922866.

57. Erickson JW, Vaughn V, Walter WA, Neidhardt FC, Gross CA (1987) Regulation of the promoters and transcripts of rpoH, the Escherichia coli heat shock regulatory gene. Genes Dev 1: 419-432. doi: 10.1101/gad.1.5.419. PubMed: 3158551.

58. Gamer J, Bujard H, Bukau B (1992) Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor σ32. Cell 69: 833-842. doi: 10.1016/0092-8674(92)90294-M. PubMed: 1534276.

59. Yura T, Nagai H, Mori H (1993) Regulation of the heat-shock response in bacteria. Annu Rev Microbiol 47: 321-350. doi: 10.1146/annurev.ml.47.100193.001541. PubMed: 7504905.

60. Yura T, Guisbert E, Portz M, Lu CZ, Campbell E et al. (2007) Analysis of sigma32 mutants defective in chaperone-mediated feedback control reveals unexpected complexity of the heat shock response. Proc Natl Acad Sci U S A 104: 17638-17643. doi: 10.1073/pnas.0708819104. PubMed: 17968012.

61. Grahl N, Livny J, Waldor M, Barel M, Charbit A et al. (2009) Pivotal role of the Francisella tularensis heat-shock sigma factor RpoH. Microbiology 155: 2560-2572. doi: 10.1099/mic.0.029058-0. PubMed: 19443547.

62. McGrath PT, Lee H, Zhang L, Iniesta AA, Hottes AK et al. (2007) High-throughput identification of transcription start sites, conserved promoter motifs and predicted regulons. Nat Biotechnol 25: 584-592. doi: 10.1038/nbt1294. PubMed: 17401361.

63. Green HA, Donohue TJ (2006) Activity of Rhodobacter sphaeroides RpoH, a second member of the heat shock sigma factor family. J Bacteriol 188: 5712-5721. doi: 10.1128/JB.00405-06. PubMed: 16885439.

64. Okamoto-Kainuma A, Ishikawa M, Nakamura H, Fukazawa S, Tanaka N et al. (2011) Characterization of rpoH in Acetobacter pasteurianus NBRC3283. J Biosci Bioeng 111: 429-432. doi: 10.1016/j.jbiosc.2010.12.016. PubMed: 21239225.

65. Gunasekere IC, Kahler CM, Powell DR, Snyder LA, Saunders NJ et al. (2006) Comparison of the RpoH-dependent regulon and general stress response in Neisseria gonorrhoeae. J Bacteriol 188: 4769-4776. doi: 10.1128/JB.01807-05. PubMed: 16788186.

66. Slamti L, Livny J, Waldor MK (2007) Global gene expression and phenotypic analysis of a Vibrio cholerae rpoH deletion mutant. J Bacteriol 189: 351-362. doi: 10.1128/JB.01297-06. PubMed: 17085549.

67. Ueki T, Lovley DR (2007) Heat-shock sigma factor RpoH from Geobacter sulfurreducens. Microbiology 153: 838-846. doi: 10.1099/mic.0.2006/000638-0. PubMed: 17322204.