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Sequence determinants spanning -35 motif and AT-rich spacer region impacting *Ehrlichia chaffeensis* Sigma 70-dependent promoter activity of two differentially expressed p28 outer membrane protein genes

Huitao Liu†, Laxmi U. M. R. Jakkula†, Tonia Von Ohlen†, and Roman R. Ganta*

Department of Diagnostic Medicine/Pathobiology, Center of Excellence for Vector-Borne Diseases, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA

*To whom correspondence should be addressed. Tel. +1 785 532 4612. Fax. +1 785 532 4851. Email: rganta@vet.ksu.edu

†The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

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Abstract

Ehrlichia chaffeensis is an obligate intracellular tick-borne bacterium which causes the disease, human monocytic ehrlichiosis. *Ehrlichia chaffeensis* contains only two sigma factors, σ^{32} and σ^{70}. It is difficult to study *E. chaffeensis* gene regulation due to lack of a transformation system. We developed an *Escherichia coli*-based transcription system to study *E. chaffeensis* transcriptional regulation. An *E. coli* strain with its σ^{70} repressed with *trp* promoter is used to express *E. chaffeensis* σ^{70}. The *E. coli* system and our previously established *in vitro* transcription system were used to map transcriptional differences of two *Ehrlichia* genes encoding p28-outer membrane proteins 14 and 19. We mapped the -10 and -35 motifs and the AT rich spacers located between the two motifs by performing detailed mutational analysis. Mutations within the -35 motif of the genes impacted transcription differently, while -10 motif deletions had no impact. The AT-rich spacers also contributed to transcriptional differences. We further demonstrated that the domain 4.2 of *E. chaffeensis* σ^{70} is important for regulating promoter activity and the deletion of region 1.1 of *E. chaffeensis* σ^{70} causes enhancement of the promoter activity. This is the first study defining the promoters of two closely related *E. chaffeensis* genes.

Key words: gene regulation, intracellular bacteria, Anaplasmataceae

1. Introduction

Human monocytic ehrlichiosis (HME) is caused by the tick-borne pathogen *Ehrlichia chaffeensis*.[1] HME is considered an emerging infectious disease in the USA and is also reported from several other parts of the world.[2] 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] Similarly,

several other *Anaplasmataceae* family pathogens, including the genera *Ehrlichia* and *Anaplasma*, have been identified in recent years as the causative agents of important emerging diseases in people and various vertebrate animals.[3–5] The limited availability of genetic tools to study obligate intra-phagosomal pathogens impacted our understanding of the molecular mechanisms of pathogenesis and the pathogen's prolonged persistence in vertebrate and tick hosts.[6–8] Host-specific differences in the gene expression of *E. chaffeensis* are also reported,[9,10] but it is entirely unknown how the organism accomplishes such changes in gene expression.

Transcriptional regulation in prokaryotes is accomplished by the action of RNAP holoenzyme. RNAP holoenzyme is a multi-protein complex composed of two alpha (α) subunits, two beta (β) subunits and a sigma (σ) factor.[11] Promoter specificity for an RNAP is accomplished by the inclusion of a sigma factor. *Ehrlichia chaffeensis* genome contains only two sigma factor genes; *rpoD* (ECH_0760) (the predicted primary housekeeping σ^{70} gene) and *rpoH* (ECH_0655) (the predicted alternate σ^{32} gene) (GenBank # NC_007799.1).[1] Both σ^{32} and σ^{70} are conserved in most proteobacteria and share extensive similarity at the amino acid level.[12] Transcription from a gene promoter by an RNAP typically involves the recognition of and binding to two DNA motifs located upstream from the transcription start site (TSS) of a gene; the motifs -10 and -35, which is a common occurrence for many bacteria.[13,14] The -10 motif interacts with the 2.3–2.4 region of a σ^{70} to bind RNA polymerase,[15–19] while the -35 motif is known to interact with the conserved 4.2 region.[20–22] Recent studies in *Escherichia coli* suggest that the spacer sequences located between the -35 and -10 motifs also contribute to transcription initiation and regulation.[23–25]

We recently mapped the promoters of several *E. chaffeensis* genes by performing *in vitro* transcription studies using the RNAP containing recombinant *E. chaffeensis* sigma factors.[26,27] RNA polymerase binding motifs of *E. chaffeensis* gene promoters are highly homologous for its only two sigma factors, σ^{32} and σ^{70}. The gene expression in this bacterium can also be accomplished by either of the two factors, but with varying affinities for different gene promoters.[27] We reported that the *E. chaffeensis* outer membrane protein genes encoding for p28-Omp14 and p28-Omp19 proteins (Ech_1136 and Ech_1143, respectively) are transcribed predominantly by σ^{70}. Our initial studies revealed that only the -35 motifs, but not -10 motifs, are required for transcription for these two genes.[26] The transcriptional assessment of *E. chaffeensis* genes requires additional investigations to define the contributions of the pathogen sigma factors for RNAP function, as prior studies were carried out with *E. coli* RNAP. Such studies are a challenge due to the lack of appropriate molecular tools for this organism.

Most of the current knowledge of bacterial gene regulation comes from studying the gene regulation of *E. coli*. Such knowledge is severely limited for other Gram-negative bacteria and more importantly, it is unclear how intracellular pathogens, such as *E. chaffeensis*, regulate gene expression to overcome the host stress. In the current study, we developed an *E. coli*-based promoter mapping system to study functions of two genes and validated the data using the *in vitro* transcription system. We took advantage of a previously developed *E. coli* strain in which the endogenous *rpoD* gene expression is controlled by the repressible *trp* promoter.[28] In this *E. coli*, we complemented *E. chaffeensis* σ^{70} after suppressing its native σ^{70}. This system was then used to systematically map sequence determinants spanning from the -10 to -35 motifs of two differentially expressed genes recognized primarily by *E. chaffeensis* σ^{70}. Together, the study allowed us to test the function of *E. chaffeensis* σ^{70} and its ability to regulate target genes. In view of the lack of a transformation system in *E. chaffeensis* and in other related tick-borne intracellular rickettsial pathogens, the assessment of *Ehrlichia* transcriptional machinery in the surrogate *E. coli* system along with the validation experiments carried out by *in vitro* transcription assays offer innovative means in studying gene expression in *E. chaffeensis* and other important intracellular rickettsial pathogens belonging to the *Anaplasmataceae* family.

2. Materials and methods

2.1. *Escherichia coli* strains and plasmids

Escherichia coli strains used in this study were TOP10 (Invitrogen Technologies, Carlsbad, CA), BL21(DE3) (Novagen, San Diego, CA) and CAG20177.[28,29] Several plasmid constructs used in this study were obtained from a commercial source or modified from one or more of the existing plasmids. They include pET32a (Novagen) and the derivatives of pSAKT32,[30] pQF50K[30] and pMT504.[31] Genetic makeup of the plasmids described in this study was included in Supplementary Table S1, except those obtained from a commercial source. The plasmid pSAKT32 containing a p15A origin of replication and an ampicillin resistance gene has *E. coli rpoH* gene under the control of IPTG inducible P_{lac} promoter.[30] The *E. coli rpoH* from this plasmid was replaced with the *E. chaffeensis rpoD* (Ech_*rpoD*) gene by digesting the plasmid with Afl II and Sal I, blunt ending the digested fragments with Klenow DNA polymerase (BioLabs, Ipswich, MA), and then ligating with the Ech_*rpoD* sequence. Ech_*rpoD* segment was generated by PCR from plasmid pET32-Ech_*rpoD*[32] using Pfu DNA polymerase (Promega, Madison, WI). The modified plasmid is referred to as the pSAKT32-Ech_*rpoD*. *Ehrlichia chaffeensis rpoD* variants with substitutions within the 4.2 region of σ^{70} were constructed by mutagenesis using a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA). *Ehrlichia chaffeensis rpoD* variant with deletion of 1.1 region of σ^{70} was also generated in it by using Q5 Site-Directed Mutagenesis Kit (New England Biolab, Inc, Ipswich, MA). The names of the modified pSAKT32-Ech_*rpoD* are provided in Supplementary Table S1.

The pQF50K plasmid with a pMB1 origin of replication and with a kanamycin resistance gene contains the β-galactosidase coding sequence (*lacZ*) driven by *E. coli groE* promoter.[30] The *groE* promoter in the plasmid was replaced with *E. chaffeensis p28-Omp14* or *p28-Omp19* gene promoters by employing directional cloning by taking advantage of existing restriction sites with the plasmid surrounding the insertion. The *E. chaffeensis* promoter segments were generated by PCR using Pfu DNA polymerase (Promega, Madison, WI). The promoter plasmids are referred to as pQF50K-p28-Omp14 and pQF50K-p28-Omp19, respectively. Mutations with deletion of -10 or -35 motifs of the promoters were generated from these plasmids using Q5 Site-Directed Mutagenesis Kit (New England Biolab, Inc, Ipswich, MA). Site directed mutagenesis at every nucleotide of the -35 motif of the promoters was also generated from the plasmids using the Quick-change Multisite Mutagenesis Kit (Agilent Technologies, La Jolla, CA). Mutations to modify the AT rich spacer sequence of the *p28-Omp14* promoter were generated by modifying the pQF50K-p28-Omp14 plasmid using Q5 Site-Directed Mutagenesis Kit. The names of all engineered plasmids are listed in Supplementary Table S1. Mutagenic oligonucleotides were described in the Supplementary Table S2.

The expression plasmids of *E. chaffeensis* wild-type (WT) σ^{70} or its variants were constructed for preparing purified recombinant

proteins using the *E. chaffeensis* σ^{70} plasmid reported earlier.[32] *Ehrlichia chaffeensis* σ^{70} variants within the 4.2 region of *E. chaffeensis* σ^{70} were constructed by modifying the plasmid pET32a-Ech_*rpoD* by using a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA) and the modified expression constructs were then used to prepare modified recombinant proteins. The names of the modified pET32-Ech_*rpoD* are provided in Supplementary Table S1.

For in vitro transcription analysis, *E. chaffeensis* promoter segments of *p28-Omp14* and *p28-Omp19* or their mutants were cloned in front of the G-less casette of pMT504 plasmid at the EcoR V site to serve as transcription templates.[31] The constructs with various mutations at -35 motif for the *p28-Omp14* and *p28-Omp19* promoters were generated by PCR using the -35 motif mutant-specific plasmids in pQF50K as the templates from the respective gene promoter plasmids. The lengths of transcripts for the various promoter segments of *p28-Omp14* and *p28-Omp19* genes are 162 nucleotides. Integrity of all cloned segments in the plasmid constructs was confirmed by automated DNA sequence analysis using CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

2.2. *Escherichia coli* growth conditions and β-galactosidase assays

The CAG20177 *E. coli* strain alone or with the recombinant plasmids was grown as described earlier.[28] Briefly, cultures were grown at 37 °C in Luria–Bertani medium with chloramphenicol (30 μg/ml) plus indole-3-acrylic acid (IAA) (0.2 mM) to maintain expression of endogenous σ^{70}. To express *E. chaffeensis* σ^{70} from plasmid pSKAT32-Ech_*rpoD* or its derivatives, *E. coli* CAG20177 strain containing the plasmid were grown with ampicillin overnight along with the IAA and chloramphenicol then diluted 1:100 into a fresh medium containing the same antibiotics, but without IAA to suppress the *E. coli* σ^{70} and to induce the expression of WT *E. chaffeensis* σ^{70} or its derivatives. Due to the leaky expression from the *lac* promoter, *E. chaffeensis* σ^{70} expression was adequate to sustain the bacterial growth in the absence of IPTG. Accordingly, all experiments were carried out without adding IPTG. To assess the functions and impact of various mutations within the promoter regions of genes encoding *p28-Omp14* and *19*, pQF50K plasmid containing the promoter segments were maintained by growing the *E. coli* cultures with the addition of kanamycin. The β-galactosidase assays were performed on the lysates prepared from the cultures grown until the OD at 600 nm reached to ∼0.6 using a β-gal assay kit (Invitrogen Technologies, Carlsbad, CA). The experiments were performed thrice with independently grown cultures; specific activity of β-galactosidase was calculated as outlined in the kit protocol.

2.3. *In vitro* transcription assays

In vitro transcription reactions were performed in 10 μl reaction mixture containing 0.13 pmol each of the supercoiled plasmid DNA as the template and using RNAP holoenzyme containing either recombinant *E. chaffeensis* σ^{70} *or its* derivatives.[32] The holoenzyme was prepared by mixing 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* σ^{70} *or its* derivatives and kept in ice for 30 min prior to using for the reactions. The transcription reactions were performed 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 resolved on a 6% polyacrylamide sequencing gel with 7 M urea, then gels were transferred to a Whatman paper, dried and 162 nucleotide transcripts were visualized by exposing an X-ray film to the gels. The transcripts were quantified using ImageJ software (http://rsb.info.nih.gov/ij).

2.4. Native PAGE analysis

The DNA promoter segments of *p28-Omp14* gene (222 bp) which included the AT-rich spacer (WT) or the modified spacers were generated by PCR from the *p28-Omp14* gene. The modified derivatives of the spacer containing complementary sequence (SP1), GC-rich spacer (SP2), or the *p28-Omp19* gene spacer inserted in place of the *p28-Omp14* spacer (SP3) cloned in plasmid pQF50K were used as templates for amplification using the primers, Gene14-up and Gene14-down (Supplementary Table S2). The PCR products were separated by electrophoresis at 4 °C in 0.5× TBE buffer on a non-denaturing 8% polyacrylamide gel. The DNA in the gel was stained with ethidium bromide and visualized by UV illumination and images captured using KODAK 1D Image Analysis system.

2.5. Modelling of DNA fragments *in silico*

The spacer sequence DNA segments (WT, SP1, SP2 and SP3) described above were assessed computationally using the online software, 'model.it' (http://hydra.icgeb.trieste.it/dna/model_it.html) using the parameter 'Electrophoresis (dinucleotide)' to predict the DNA structure. The resulting predicted structures were downloaded to pdb format and PyMOL was used to prepare figures.

2.6. Statistical analysis

Statistical analyses were performed using Student's *t*-test, and a *P*-value <0.05 was considered significant. *P*-values between 0.05 and 0.01 are identified with a single asterisk and *P*-value <0.01 are identified with double asterisks.

3. Results

3.1. *Ehrlichia chaffeensis* genes encoding for the p28-Omp14 and 19 proteins recognized by σ^{70} require the -35 motif, but not -10 motif, for transcription

Our prior studies demonstrated that the -35 motif, but not the -10 motif, is critical to *E. chaffeensis* promoter activity.[26] That study was carried out using the *E. coli* σ^{70} containing RNAP holoenzyme and thus the data may not be a true reflection of the outcome from the RNAP of the pathogen. To validate the data, we developed an *E. coli* surrogate system expressing *E. chaffeensis* σ^{70} by taking advantage of a previously described *E. coli* strain (CAG20177) in which the endogenous σ^{70} gene (*rpoD*) expression is controlled by the repressible *trp* promoter.[28] In particular, the expression of chromosomally encoded *E. coli* σ^{70} requires IAA for optimal growth, as it relieves the tryptophan repression (Supplementary Fig. S1). In the absence of IAA, the *E. coli* growth is significantly inhibited (e.g. ∼6-fold difference between the cultures with IAA or without IAA at 3 h). The inhibition was also significantly releaved when complemented by another related σ^{70}, as we observed with the introduction of the plasmid expressing the *E. chaffeensis* σ^{70} gene (Ech-*rpoD*) from the *lac* promoter in the presence of IPTG (Supplementary Fig. S1). This modified *E. coli* expressing Ech-*rpoD* is then used for studying the pathogen gene promoters. We used this *E. coli* system to map promoters of two *E. chaffeensis* genes; Ech_1136 and Ech_1143 encoding for the proteins p28-Omp14 and p28-Omp19, respectively.

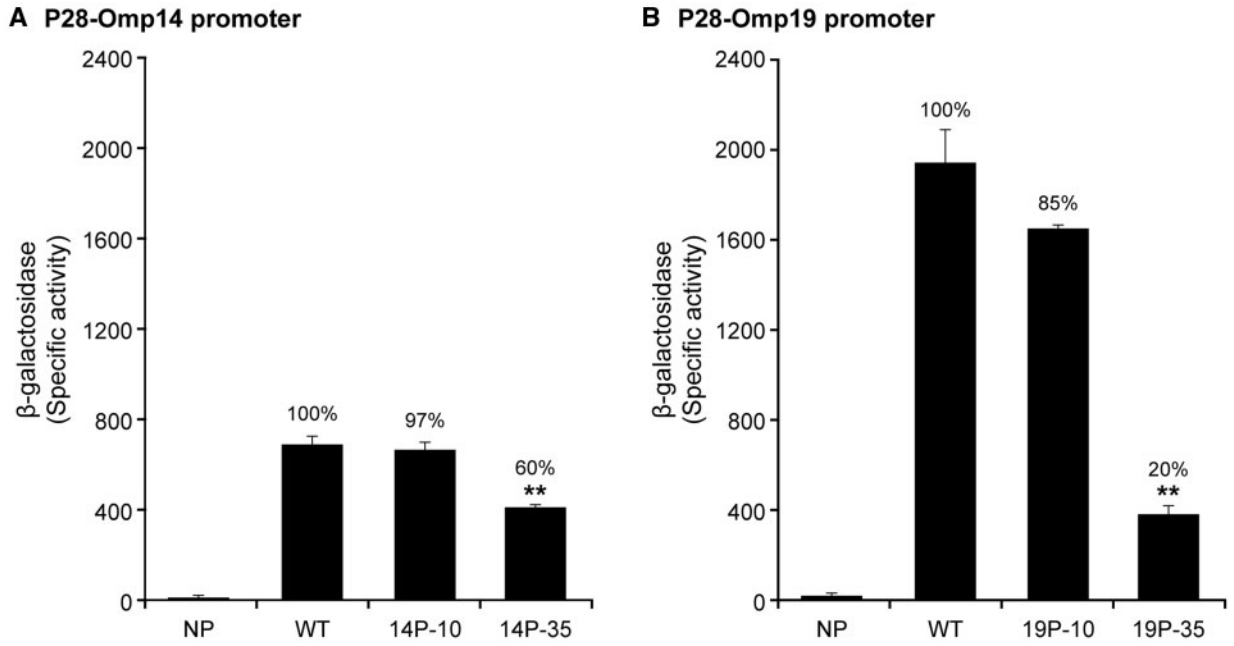

Figure 1. Importance of -10 and -35 motifs of two *Ehrlichia chaffeensis* gene promoters assessed in the surrogate system of *E. coli* strain CAG20177. The β-galactosidase expression, driven by *E. chaffeensis p28-Omp14* (A) or *p28-Omp19* (B) gene promoters or from the promoters containing deletion mutations at -10 and -35, was assessed relative to no promoter controls. (Constructs; NP, no promoter; WT, wild-type promoter; 14P-10 and 19P-10 represent deletions at -10 motifs and 14P-35 and 19P-35 refer to deletion constructs with -35 motif deletions.) Significant changes in the β-galactosidase activity were identified compared with the data observed for the WT constructs.

These genes were previously identified as transcribed by the *E. chaffeensis* σ^{70}.[32] These gene promoter segments, cloned in front of a reporter gene for β-galactosidase in a plasmid, were used to transform the modified CAG20177 strain of *E. coli* (Supplementary Fig. S2). Our initial experiments in media lacking IAA tested to assess differences in transcription with or without the induction of *E. chaffeensis* σ^{70} by IPTG (Supplementary Fig. S3). As the β-galactosidase activity is also observed for the induced bacteria that is not significantly different from the non-induced, due to the leaky expression from *lac* promoter,[28] all subsequent assessments were carried out without adding IPTG to the culture media. The *p28-Omp19* gene promoter induced ~3-fold more β-galactosidase compared with that found for the promoter of *p28-Omp14* (Fig. 1). The complete deletion of -35 motifs from promoters of the genes encoding *p28-Omp14* and *p28-Omp19* caused a 40% and 80% reduction of the promoter activity, respectively ($P \leq 0.005$), while deletion of -10 motifs from these two promoters resulted in non-signifant change (Fig. 1).

3.2. Identifying the critical sequence determinants of -35 motifs in *E. chaffeensis* genes recognized by σ^{70}

The -35 motifs are extensively conserved for *E. chaffeensis* genes; its consensus sequence is TTGWNW.[27] Further, this motif is identical for *p28-Omp14* and *p28-Omp19* genes (TTGCTT) (Supplementary Fig. S2A). To define the critical sequence determinants for the promoter activity, substitutions at each base of the six nucleotide motifs were made in the *p28-Omp14* and *19* gene promoters and the impact of mutations was assessed by changes in the β-galactosidase expression with *E. chaffeensis* σ^{70} in the *E. coli* surrogate system (Fig. 2A and B). For each base pair substitution, the combination of letters and numbers indicates a specific substitution mutation. For example, T1A indicates a change from T to A transversion at the first position in the -35 motif. The mutations in the first three nucleotides (TTG) had a significant impact in reducing the promoter activities of both the genes. The impact of mutations was also gene-specific. For *p28-Omp14* gene promoter, substitution at the first position T to any other nucleotide resulted in ~40% decline in the promoter activity. Mutations in *p28-Omp19* gene at this position also caused a decline in the promoter activity, however, the nucleotide changes caused a greater decline which ranged from 55% to 86%. The promoter activity for this gene is also different for different substitutions; T1C had the greatest impact. Mutations in the second T for both genes had lesser impact compared with the first position mutations. T2A mutation in both genes had an approximately equal amount of decline in the promoter activities (22–25% decline), whereas the T2C mutation caused slightly variable declines in the promoter activities (31% for *p28-Omp14* and 46% for *p28-Omp19*) and T2G had an opposite effect trend for the two gene promoters; this mutation resulted in decline in promoter activity for gene 14 and enhancement for gene 19 promoter. The G3 position resulted in the strongest reduction of promoter activity of both the genes; 39–57% for *p28-Omp14* and 46–66% for *p28-Omp19*. Substitutions in the fourth position also caused significant variations in the promoter activities; C4A mutation in *p28-Omp14* and C4T in *p28-Omp19* caused increases in the respective promoter activites by ~35%, whereas no significant change was observed for the C4A mutation for *p28-Omp19* and for the C4T mutation for *p28-Omp14*. C4G transversions for both genes resulted in the promoter activities decline to 52% and 36%, respectively. Substitutions in the fifth position T to A caused a substantial enhancement of the promoter activities for both the genes (26% and 75%, respectively). T5C mutation had no significant effect for both the gene promoters, whereas T5G caused about a 63% increase for *p28-Omp19* promoter and had no significant for *p28-Omp14* promoter. Mutations in the sixth position had no

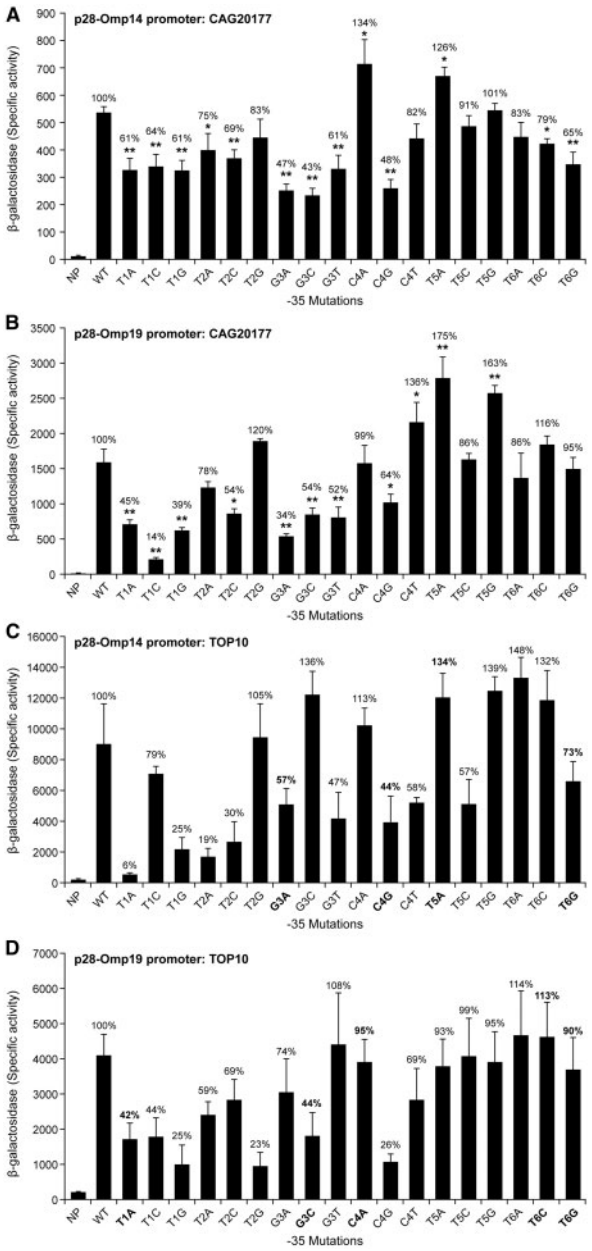

Figure 2. Mapping the sequence determinants of -35 motifs in *Ehrlichia chaffeensis* genes. The β-galactosidase expression driven by *E. chaffeensis* promoters constructs containing point mutations at each of the six nucleotide positions of the -35 motifs of genes encoding *p28-Omp14* (A) and *p28-Omp19* (B) were measured in the CAG20177 strain of *E. coli* expressing *E. chaffeensis* σ^{70}. The experiment included the no promoter (NP) and wild-type promoter (WT) controls. Each mutation is identified with a change of the nucleotide at each position to the modified nucleotide. β-galactosidase expression was presented relative to the respective wild-type promoters. The β-galactosidase expression driven by *E. chaffeensis* promoters constructs containing point mutations at each of the six nucleotide positions of the -35 motifs of genes encoding *p28-Omp14* (C) and *p28-Omp19* (D) also were measured in the TOP10 strain of *E. coli* expressing its native chromosomally expressed σ^{70} with only the promoter plasmid pQF50K-p28-Omp14 or pQF50K-p28-Omp19 . The experiment also included the no promoter (NP) and wild-type promoter (WT) controls. Only four substitutions in *p28-Omp14* gene promoter and five substitions in *p28-Omp19* gene promoter correlated well in altering the promoter activities when using σ^{70} of *E. chaffeensis* and *E. coli* (within ~10% variations); these mutations were identified in this figure with bold text.

significant impact for *p28-Omp19* promoter, but notable declines in the promoter activities were observed for the T6C and T6G mutations for the *p28-Omp14* promoter (21% and 35% declines, respectively). The extensive mutational analysis spanning all six positions of the -35 were also assessed for both the gene promoters in an *E. coli* strain (TOP10) with its native σ^{70} (Fig. 2C and D). The data revealed that the *E. chaffeensis* σ^{70} differed considerably compared with the *E. coli* σ^{70} in responding to various point mutations assessed. In particular, only four substitutions in *p28-Omp14* gene promoter and five substitions in *p28-Omp19* gene promoter correlated well in altering the promoter activities when using σ^{70} of *E. chaffeensis* and *E. coli* (within ~10% variations). Mutations that correlated well in altering the promoter activity with σ^{70} of *E. coli* and *E. chaffeensis* were identified with bold text in Fig. 2C and D. These data suggest that, while the *E. coli* σ^{70} may complement the function of *E. chaffeensis* σ^{70}, the promoter specificities the two sigma factors are distinct in recognizing the *Ehrlichia* promoters.

3.3. *In vitro* transcription for the sequence determinants of -35 motif by recombinant *E. chaffeensis* σ^{70}

To validate the results of -35 motif mutational analyses in CAG20177, we tested several promoter mutations by performing in vitro transcription assays with the holoenzyme reconstituted with recombinant *E. chaffeensis* σ^{70}.[32] We randomly selected five mutants of genes encoding *p28-Omp14* and *p28-Omp19* and the mutant promoters were re-cloned into the G-less cassette and used as the transcription templates of *in vitro* transcription assays (Fig. 3). The mutants causing decline in the transcriptional activity in *E. coli* surrogate system also yielded reduced levels of *in vitro* transcripts and likewise the mutants which caused an enhancement of transcription also resulted in the increased synthesis of *in vitro* transcripts. We also noted minor bands migrating slightly larger than the predicted transcripts in two mutnats (T2A and G3A); it is possible that these products may have generated by the RNAP binding to other sites near the promoter in the absence of specific binding. As we previously described,[32] the recombination σ^{70} alone or *E. coli* core enzyme without the sigma factor did not generate *in vitro* transcripts (data not shown).

3.4. Substitutions in region 4.2 of *E. chaffeensis* σ^{70}

A conserved region near the C-terminus of the *E. coli* sigma factor is identified as essential for DNA binding and promoter activity, specifically to the -35 motif,[22,28,34] which includes four amino acids in the 4.2 regions of *E. coli* σ^{70} and are also conserved in *E. chaffeensis* σ^{70}.[27] In *E. chaffeensis* σ^{70}, the conserved amino acids are; glutamic acid at 597, two arginines at 598 and 600, and glutamine at 601. To evaluate if mutations in these four amino acids in *E. chaffeensis* σ^{70} would affect the promoter activity, individual substitution mutation were created to modify these four amino acids in the *E. chaffeensis* σ^{70} gene coding sequence in the expression plasmid to alanine. Transcriptional activities of the modified sigma factors were assessed with the WT promoters of *p28-Omp14* and *p28-Omp19* in the *E. coli* surrogate system (CAG20177). Mutations at all four locations for both the gene promoters resulted in significant reduction of the promoter activity (~48–57% decline) (Fig. 4A and B). To verify these data, we also performed *in vitro* transcription assays with the *E. chaffeensis* σ^{70} mutants where arginine at position 598 and 600 was modified to alanine using the WT promoters of both *p28-Omp14* and *p28-Omp19* genes (Fig. 4C and D). The *in vitro*

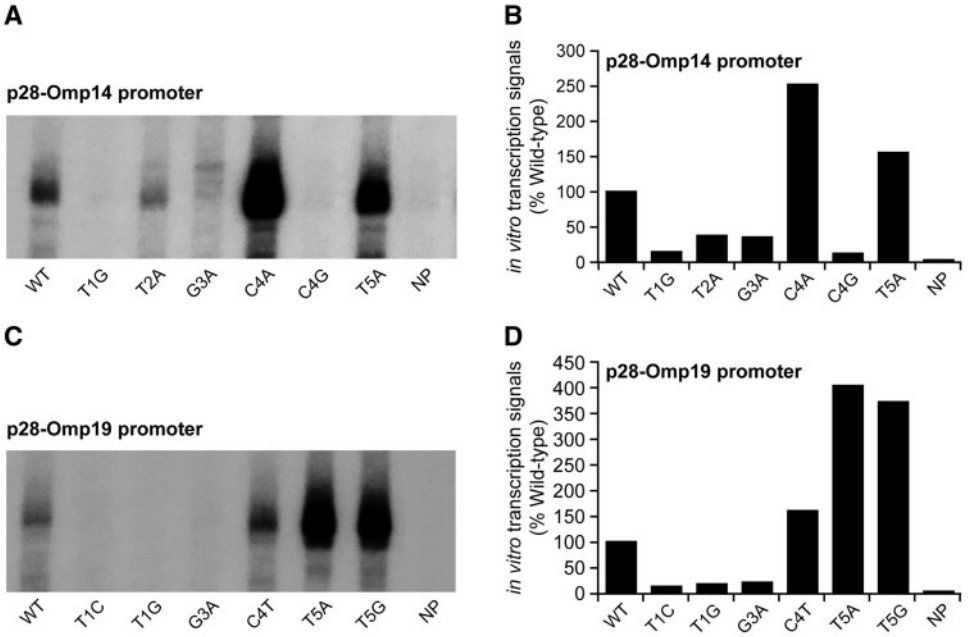

Figure 3. *In vitro* transcription analysis validating the *Ehrlichia chaffeensis* gene promoter mutants spanning the -35 motifs. Six each of the randomly selected mutations at -35 motifs of *p28-Omp14* (A and B) and *p28-Omp19* (C and D) were exmined by *in vitro* transcription assays using RNAP holoenzyme containing *E. chaffeensis* recombinant σ^{70}. The abundance of transcripts for each gene was captured from the ^{32}P incorporation. The intensity of a band signals in a gel for *in vitro* transcriptions made for the wild-type and mutant promoters was determined using the software ImageJ. Panels A and C have the image data, and panels B and D included the quantitative data collected from the image signals. The bars show the relative transcription products of mutant promoters as the percentage of transcripts compared with the wild-type promoter for σ^{70}. (NP is a construct without a promoter; WT refers to a wild-type promoter, and various mutant promoter constructs are identified as in Fig. 2).

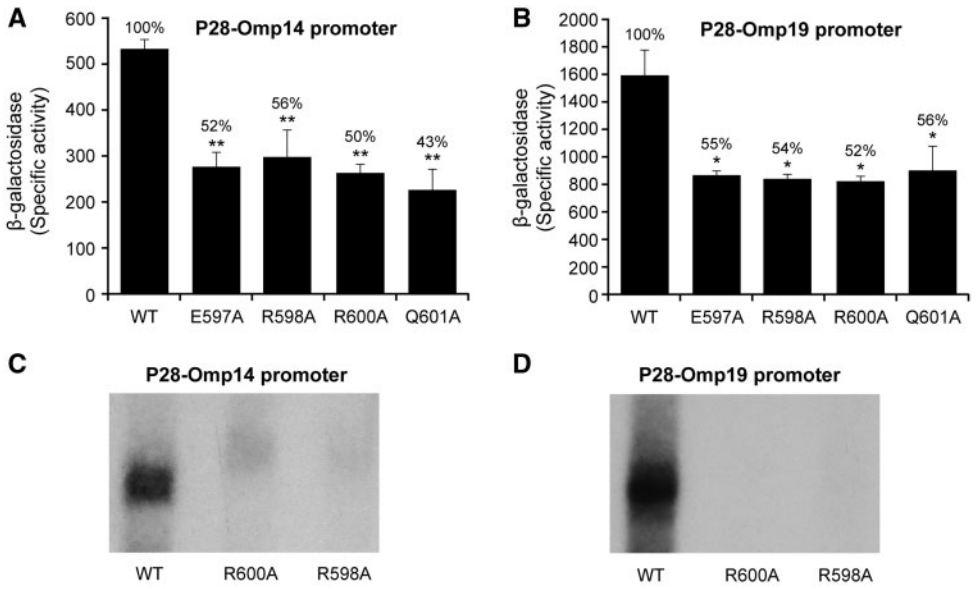

Figure 4. Substitutions in region 4.2 of *Ehrlichia chaffeensis* σ^{70} influence the promoter activity of the wild-type *E. chaffeensis* promoters (A, *p28-Omp14* and B, *p28-Omp19*). Mutations to change amino acids to alanine at four conserved residues (E597, R598, R600 and Q601) of *E. chaffeensis* σ^{70} were assessed with the wild-type promoters; *p28-Omp14* (A) and *p28-Omp19* (B). β-galactosidase expression was measured for the mutant proteins relative to the wild-type (WT) *E. chaffeensis* σ^{70}. Mutations in the conserved amino acids of *E. chaffeensis* σ^{70} 4.2 region also cause reduction in the *in vitro* transcript synthesis from the wild-type promoters (C and D). *In vitro* transcription analysis was performed using RNAP holoenzyme containing *E. chaffeensis* recombinant wild-type σ^{70}, or with its mutants R598A or R600A and with wild-type *p28-Omp14* (C) and *p28-Omp19* (D) promoters.

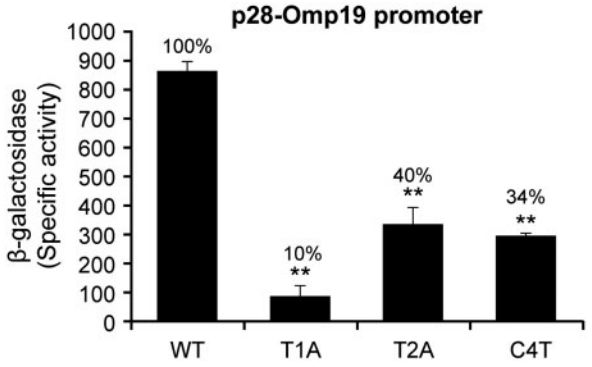

Figure 5. A change in a conserved amino acid of *Ehrlichia chaffeensis* σ^{70} 4.2 region further reduced the promoter activity in -35 motif mutants. The activities for the -35 motif mutants T1A, T2A and C4T of *p28-Omp19* promoter were assessed with *E. chaffeensis* σ^{70} mutant (E597A) by measuring changes in the β-galactosidase expression in the *E. coli* strain CAG20177. The reduction of the enzyme activity was expressed relative to the wild-type promoter.

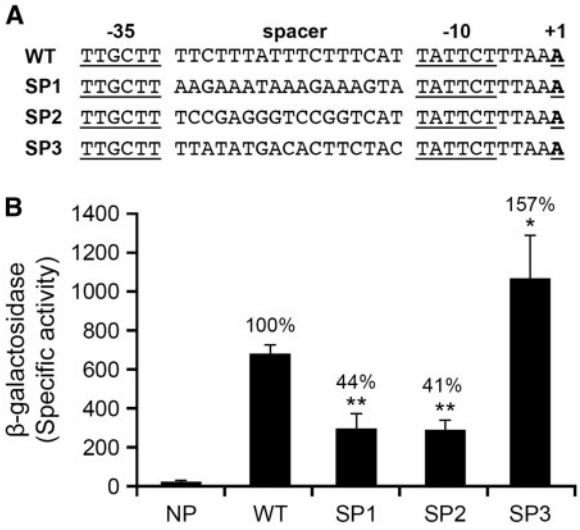

Figure 6. AT-rich spacer sequence located between -10 and -35 motif contributes to altering the promoter activity of *Ehrlichia chaffeensis* genes. Promoter fragments used in the assays are as in Supplementary Fig. S2 for wild-type *p28-Omp14* gene. (A) sequence spanning from +1 to -35 motif and the AT-rich spacer sequence is presented for the wild-type construct (WT) and for the constructs with modified spacer sequences which included replacing the AT-rich spacer with complementary sequence (SP1), with GC-rich spacer sequence (SP2) or with the *p28-Omp19* gene spacer sequence (SP3). (B) The β-galactosidase expression driven by promoters of WT, SP1, SP2 and SP3 in *E. coli* (CAG20177) expressing *E. chaffeensis* σ^{70} was measured and the data were presented. The assay also included the data generated from the promoterless construct control (NP).

transcription also was reduced for the mutants. We also tested the ability of one of the mutant forms (E579A mutation) of *E. chaffeensis* σ^{70} in driving transcription from three mutant promoters of *p28-Omp19* with substitutions T1A, T2A and C4T in *E. coli* surrogate system (Fig. 5). The promoter activities for all three mutations in the of *p28-Omp19* promoter caused a further decline of 90%, 60% and 66% relative to the WT promoter, respectively.

3.5. The spacer sequences affect promoter activity

The extensive experimental analysis described above revealed that the -35 motif, but not -10, impacted the *E. chaffeensis* transcription driven by its σ^{70} for the two genes assessed. The deletion of -35 motifs caused significant decline of the promoter activities, but did not completely abolish the transcription, suggesting that the promoter function requires the contributions of additional sequences. In particular, we hypothesized that the sequences other than the -35 motifs also contribute to the differences in the promoter activities of the two genes. In *E. coli*, the length of a spacer sequence between the -10 and -35 motifs influences promoter activity.[33,35] Previous studies in *E. coli* also demonstrated that the nucleotide differences within the spacer sequences also influence the promoter activity.[23–25] We investigated if spacer sequences also similarly influence the promoter activity of *E. chaffeensis* genes by modifying the spacer sequences. We constructed three mutants to modify the spacer region of the *p28-Omp14* gene promoter: (i) the AT-rich spacer sequence of the gene is replaced with complementary nucleotides at each position of the spacer (SP1) to keep the AT and GC content constant, (ii) the sequence is replaced with a sequence having GC-rich spacer sequence (SP2), or (iii) the spacer sequence of *p28-Omp14* gene promoter is replaced with the *p28-Omp19* gene promoter spacer sequence (SP3) (Fig. 6A). The SP3 construct is included to test if replacing the spacer sequence of *p28-Omp14* gene with *p28-Omp19* gene is sufficient in enhancing the promoter activity to that observed for the *p28-Omp19* gene promoter, as both the promoters have identical -35 motifs. The WT and the modified constructs were tested in the *E. coli* system by measuring the β-galactosidase activity; the SP1 and SP2 caused the reduction of the enzyme activity by 56% and 59%, respectively (Fig. 6B). The substitution with the *p28-Omp19* gene spacer (SP3) caused enhancement of the promoter activity by ~1.6-fold (Fig. 6B). These results suggested that the spacer sequences of the *E. chaffeensis* gene promoters play an important role in transcriptional variations and may also account for some of the differences in the activities of *p28-Omp14* and *p28-Omp19* gene WT promoters.

Previous studies in *E. coli* revealed that the 1.1 region of its σ^{70} contributes to the promoter activity by modulating the formation of stable polymerase and promoter complexes.[24,36,37] Deletion of this region causes enhanced or decreased promoter activity depending on a promoter.[24,36] The impact of 1.1 deletion is also variable for different spacer sequences for the promoters containing the same -35 and -10 motifs, as evidenced for P_{tac} and $P_{uvsX/sigma}$.[36] As *E. chaffeensi p28-Omp14* and *p28-Omp19* promoters have the identical -35, and that -10 was found to be less important for the transcription from these two gene promoters, we investigated if the 1.1 deletion *E. chaffeensis* σ^{70} also cause variations in the promoter activities if we modify the spacer sequences. The deletion of 1.1 region in *E. chaffeensis* σ^{70} led to significant enhancement of the promoter activity when assessed for the WT *p28-Omp14* promoter segment. Modified spacer sequences containing the complementary sequence (SP1) or GC sequence (SP2) or the replacement of the spacer sequence with WT *p28-Omp19* promoter spacer sequence (SP3) also resulted in the enhancement of promoter activities (Fig. 7).

The changes of base sequence for the spacer DNA fragments with identical length possibly render different conformations or curvatures to a DNA molecule[24,25] and may aid in altering the affinities of RNAP binding and transcription. DNA conformational changes can also impact migration patterns in a polyacrylamide gel (PAG).[24,25] To test this, we compared the mobility of DNA fragments of *p28-*

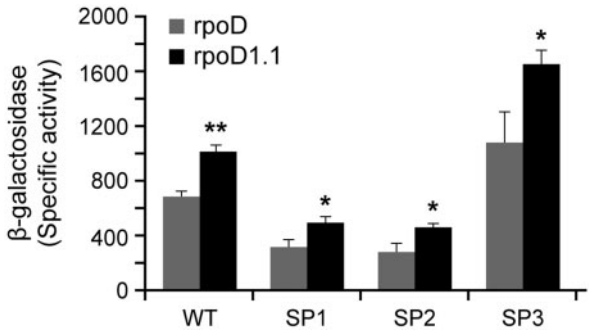

Figure 7. WT and SP1, SP2 and SP3 constructs were assessed for their promoter activities in *E. coli* expressing wild-type *Ehrlichia chaffeensis* σ^{70} from *rpoD* gene or from its mutant having deletion at 1.1 region (*rpoD* 1.1). The β-galactosidase expression was significantly higher for all four promoters when assessed with *rpoD* 1.1 compared with the wild-type *rpoD*.

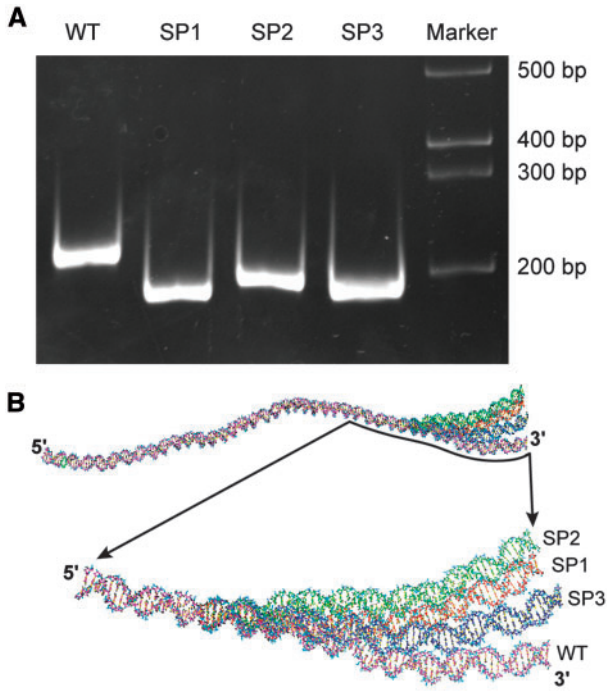

Figure 8. Changes in the AT-rich spacer sequence impact DNA gel migration and conformation. (A) Wild-type *p28-Omp14* gene promoter and with modified spacers; SP1, SP2 and SP3 (described in Fig. 6) had variable migration patterns when resolved in a non-denaturing PAGE. (B) Topology of the wild-type and the three spacer modified promoter segments revealed conformational changes when assessed by the prediction program;[38] WT, pink; SP1, red; SP2, green and SP3, blue.

Omp14 promoter segments, WT, SP1, SP2 and SP3, by subjecting to electrophoresis in a non-denaturing PAG (Fig. 8A). The gel migration patterns are different for all four DNAs. Consistent with these results, the predicted DNA structures have different topologies as judged by the 'model it' program (Fig. 8B).[38]

4. Discussion

It is unclear how *E. chaffeensis* and the related *Anaplasmataceae* family pathogens transmitted from ticks regulate their gene expression in vertebrate and tick hosts. *Ehrlichia chaffeensis* genome contains genes only for two sigma factors (σ^{32} and σ^{70}) and for very few predicted transcriptional regulators (GenBank # NC_007799.1).[1] To understand how the *Anaplasmataceae* family pathogens adapt to their vertebrate and tick hosts and sense nutrient and starving environments within an infected host cell requires a detailed knowledge about the pathogens' gene regulation. Studying the regulation of gene expression is also important in defining the molecular basis for the conversions to the pathogens' infectious form (dense core cells) and replicating form (reticulate cells) within a phagosome of an infected host cell. Our recent data support the hypothesis that the *E. chaffeensis* sigma factors, σ^{32} and σ^{70}, function cooperatively in transcribing pathogen genes.[27] The current study is the first to undertake a detailed investigation at the gene level to map differences in gene expression accomplished by two distinct and closely related genes; Ech_1136 and Ech_1143, of the pathogen encoding for the proteins; p28-Omp14 and p28-Omp19, respectively.

In the absence of a genetic transformation system, researchers investigating the gene expression of intracellular *Chlamydia* species pathogens relied on the use of *in vitro* transcription method to study the bacterial gene regulation and to define the transcriptional mechanisms.[31,39–41] *In vitro* transcription assays are proven the most valuable in defining the transcriptional machinery of several *Chlamydia* genes.[40–46] *Ehrlichia* species research is also challenging due to lack of a well-established genetic transformation system and the lack of natural plasmids in them further complicates the research focused on studying gene regulation. In the current study, we developed and utilized the *E. coli* surrogate system to map the DNA binding domains involved in regulating the gene expression in *E. chaffeensis*. Further, we used the *in vitro* transcription system to validate the data. The approaches also aided in determining the molecular basis for differences in gene expression from two closely related genes.

Escherichia coli transcriptions for housekeeping genes are driven by RNAP holoenzyme containing σ^{70} which recognizes two highly conserved motifs; referred as -10 and -35 motifs.[13] The consensus motif sequences are TATAAT and TTGACA, respectively. The σ^{70} homologs are also extensively conserved in several other Gram-negative bacteria.[12] We recently reported that the consensus -10 and -35 motifs for *E. chaffeensis* σ^{70} are TATTNT and TTGNTT, respectively.[27] We also reported that the -10 and -35 motifs for the alternative sigma factor, σ^{32}, in *E. chaffeensis* (TATATN and TTGAAA, respectively) are very similar to σ^{70} consensus sequences for the genes we assessed.[27] The -10 motif of *E. chaffeensis*, however, differs considerably from the *E. coli* σ^{32} consensus -10 motif (CCCCATNT), while the consensus -35 motif is identical (TTGAAA).[47,48] Consistent with the extensive homology of σ^{32} and σ^{70} consensus -10 and -35 motifs, *E. chaffeensis* genes can also be transcribed by both the sigma factors, but with varying affinities.[27] *Ehrlichia chaffeensis* has two morphological forms; dense core and reticulate cells[49,50] and it is entirely unknown how the organism and the related rickettsial organisms having two distinct morphological forms and also having the ability to adapt to dual hosts regulate their gene expression. Considering the lack of genetic tools and transformation system, the methods described in the current study will be valuable in defining the gene regulation in this organism, the related *Anaplasmataceae* family organisms, and in extending studies to other intracellular Gram-negative pathogens having two distinct morphological forms, such as *Chlamydia* species and *Coxiella* burnetii.[51,52]

The consensus -35 motifs in all mapped *E. chaffeensis* genes, independent of a gene primarily transcribed by σ^{32} or σ^{70}, contain the extensively conserved first three nucleotides at the 5′ end.[27] In this study, we presented data demonstrating that the -10 motifs are not critical for the gene activities of two closely related outer membrane protein genes (*p28-Omp14* and *p28-Omp19*) driven by its primary sigma factor, σ^{70}. At this time, it is not clear if -10 motif is similarly less important for other pathogen genes. This hypothesis needs further investigation. We also presented evidence that the -35 motifs are critical for the σ^{70} function for the two genes assessed. Further, we reported that any changes to the first three nucleotides of the -35 motif, TTG, result in significant decline in the promoter activities, despite different degrees of variations observed for the two gene promoters. The TTG in -35 motifs is conserved in most of the *E. chaffeensis* genes suggesting that its interactions with σ^{70} may be vital for its function, although the remaining three nucleotides on the -35 motif may also play a critical role for the gene-specific transcription. The TTG is also found to be important for σ^{70} gene promoters of *E. coli*.[53] It is well known that sigma factors possess variable numbers of DNA binding regions.[12,54] Each region holds a specific role in promoter recognition. For example, the region 4 located in the C-terminus contains a helix-turn-helix (HTH) motif of known DNA-binding protein.[55] Previous studies revealed that the 4.2 region in *E. coli* σ^{70} is involved in the base-specific recognition with the -35 motif.[15,20] Moreover, the substitutions in four conserved charged amino acids at E265, R266, R268 and Q269 in *E. coli* σ^{32} to a nonpolar amino acid, alanine, cause reduction of the promoter activity.[22] Sequence allignment revealed that the *E. chaffeensis* σ^{32} has the same four amino acids as conserved and mutating these amino acids to alanine also resulted in the reduction of its function in driving the promoter activities of the genes recognized by it.[27] These four amino acids are also conserved in *E. chaffeensis* σ^{70} and that the mutations in these amino acids to alanine also negatively impacted the promoter activity. In Fig. 5, when combined E579A substitution in σ^{70} and T1A, T2A and C4T substitution in the *p28-Omp19* promoter, respectively, the lower activity of promoter was observed compared with WT promoter. The results suggest that the E579 may not interact with these bases of the -35 motif for the *p28-Omp19* promoter, as reported previously for *E. coli*.[22] Additional experimental analysis is necessary to test this hypothesis and to evaluate if this domain in *E. chaffeensis* is also involved in base-specific recognition.

It is well demonstrated in *E. coli* that the length of a spacer sequence between the -10 and -35 motifs influences promoter activity.[33,35] Recent studies also suggest that the kind of specific nucleotides present within a spacer region also influence the promoter activity.[23–25] In the current study, we investigated the role of spacer sequences for *E. chaffeensis* RNAP function and their contributions to differences in transcription levels of two closely related genes, as both the genes have different nucleotide sequences in the spacers while the lengths remain the same. Indeed, our data demonstrated that modifying the spacer sequence with complementary sequence in the *p28-Omp14* gene promoter or by replacing with a randomly selected GC-rich spacer sequence caused significant reduction in the promoter activity. Interestingly, replacing the WT *p28-Omp14* spacer with the spacer from the *p28-Omp19* gene promoter enhanced the promoter activity by ~1.6-fold. The *p28-Omp19* gene promoter is ~3-fold stronger than the *p28-Omp14* gene promoter, as evidenced by the 3-fold higher β-galactosidase expression observed in the *E. coli* surrogate system. The 1.6-fold enhancement of the *p28-Omp14* gene promoter activity when replaced with the *p28-Omp19* spacer suggests that the spacer sequence is a major contributer for the differences in the promoter activities of the two colesely related outer membrane protein genes. As reported earlier for an *E. coli* gene,[24] the data for *E. chaffeensis* genes also demonstrate that the variations in spacer sequences influence in altering the promoter activity of a gene, possibly due to differing conformations or curvatures. In particular, we present the evidence that the nucleotide differences within a spacer sequence are important contributors in influencing the promoter strengths, possibly due to altering the curvature of a promoter leading to altered interactions with RNAP. Previous studies in *E. coli* demonstrated that the spacer sequences affect the RNA polymerase binding affinity.[56] This hypothesis remains to be tested for *E. chaffeensis*.

Depending on the promoter assessed, the loss of region 1.1 within the *E. coli* σ^{70} protein can influence the promoter activity of a gene positively or negatively or can cause no impact.[36] For example, Hook-Barnard[24] reported that the deletion of region 1.1 domain within the *E. coli* σ^{70} protein increases the transcription by ~2-fold from P_{min7} gene promoter. On the contrary, its deletion has no significant effect on the amount of mRNA made from the $P_{min7/GC}$ or $P_{min/comp}$ promoters when assessed with modified spacer sequences (GC-rich spacer or complementary spacer).[24] It is reported that region 1.1 at the N-terminal of σ^{70} of *E. coli* affect spacer-mediated changes in transcriptional initiation via converting the trajectory of the spacer of promoter.[24] In the current study, we presented evidence that *E. chaffeensis* σ^{70} with a mutation of region 1.1 significantly enhances the activity of WT *p28-Omp14* gene promoter and the promoter with mutant spacer sequences (SP1, SP2 and SP3).

This work is the first to utilize various molecular approaches in defining the -10 and -35 motifs and the AT-rich spacer sequences located between the two motifs of two closely related *E. chaffeensis* genes encoding for differentially expressed proteins; *p28-Omp14* and *p28-Omp19*. The differences in the spacer sequences alone are sufficient in altering the gene transcription by 1.6-fold. In particular, we presented the first evidence demonstrating that the difference in transcription by ~50% from two closely related genes can be accounted due to differences in their AT-rich spacer sequences. DNA binding proteins may be additional contributors in influencing the gene expression. Previous studies by Cheng *et al.*[57] using the *E. coli* RNAP holoenzyme demonstrated that an *E. chaffeensis* DNA regulator, EcxR, serves as an activator in promoting the gene expression of several type IV secretion system genes of the pathogen. The role of DNA transcription regulators remain to be investigated for their contributions to differential expression from *p28-Omp* genes. The *E. coli* surrogate system described in the current study can facilitate greatly in evaluating the DNA transcription regulators of *E. chaffeensis*. We believe that the current study will also be valuable for furthering our understanding of the regulation of gene expression in *E. chaffeensis* and in defining the detailed molecular basis of differential gene expression and its contributions to the pathogen adaptations to dual hosts and in sensing the distinct host cell environments. The molecular methods described here are also valuable for studies focused on understanding the gene regulation in other related rickettsial pathogens.

In summary, we developed an *E. coli* surrogate system and used it to extensively map *E. chaffeensis* two σ^{70} gene promoters. The *E. coli* system was also used to present evidence that the loss of -10 motifs has no role for the gene expression for the two genes assessed in the current study. We also mapped the critical determinants of the -35 motif by performing mutational analysis. Further, we demonstrated that the AT-rich sequences are involved in contributing to promoter-specific variations in the gene transcriptions.

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Supplementary data

Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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