H-NS Counter-silencing as a Primary Regulatory Mechanism of Curli Gene Expression in *Salmonella* Typhimurium

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Abstract

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Incorporation of horizontally acquired genes into transcriptional networks is essential for regulated expression of virulence in the bacterial pathogen *Salmonella enterica* serovar Typhimurium. The nucleoid-associated protein H-NS is able to recognize and bind AT-rich horizontally acquired genes, such as the *csg* regulon required for curli synthesis, and silence their expression. While previous studies have implicated a role of the alternative sigma factor σ^S in directly disrupting H-NS silencing, our system reveals that σ^S plays a more indirect role, acting through transcriptional regulators such as CsgD, IHF, and MlrA. I have identified an essential role of CsgD as a direct counter-silencer to H-NS silencing. Using a suite of footprinting techniques *in vitro*, I was able to determine that CsgD causes a topological distortion to disrupt the H-NS nucleofilament and reinstate RNA polymerase open complex formation. While σ^S appears to be required for *csgD* expression *in vivo*, *csgD* is σ^S-independent *in vitro.* Analysis of

regulators further upstream in the pathway suggest that both MlrA and IHF are required for *csgD* expression *in vivo* and are also dependent on σ^S. Initial studies *in vitro* suggest that MlrA may serve as a counter-silencer of H-NS, although the mechanism for this process awaits further characterization. Additionally, we identified a previously uncharacterized input for biofilm regulation. Vitamin B_{12} likely serves as an allosteric regulator of MlrA and may serve as a photosensor to trigger biofilm formation in response to harmful UV irradiation. Characterization of counter-silencers in *Salmonella* can help us understand mechanisms of gene regulation that can be extrapolated to other organisms and provide new insights into the evolution of regulatory networks in bacteria.

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Acknowledgements

My experience in graduate school was a kind of war of attrition, in which the emotional ups and downs of the science gradually wore away at me, and like most graduate students, I think there were plenty of moments in which I doubted my ability to finish what I had started. However, while the work presented in this thesis is my own, I think the only way it came to fruition was through the efforts of many, and as a result, I must acknowledge their contributions.

First and foremost, I need to thank Ferric. Ferric to me was always this kind of impossible human being that could be on top of everything professionally and still have an enriched personal life, and his ability to be exceptional at so many things was inspiring to me because it emulated the kind of personal-professional balance I hope to have and know is possible. His blanket knowledge of the field, clinically and scientifically, was useful in directing my project and placing it into a context that previously I didn't fully appreciate, and his perserverance in all facets of research will serve as a model for me as I go forward.

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Finally, I need to acknowledge my parents, Jim and Colleen Newman, to whom this dissertation is dedicated. Growing up, I know that I didn't appreciate them nearly as much as they deserved. They have forever and always been my biggest champions. They have

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Dedication

To my parents, James and Colleen Newman

Chapter 1: Introduction

I. Horizontal Gene Transfer as a Driver of Evolution

Horizontal gene transfer, also referred to as lateral gene transfer, is broadly defined as the process by which genetic material is non-genealogically transmitted from one organism to another (1). This process is a potent driver of evolution among all biological kingdoms, including eukaryotes (2), plants (3) and bacteria (4), occurring both among and between these domains (5,6). However, this process is most significant and best understood in bacteria, in which the horizontal acquisition of new traits accounts for up to a third of all genes in some bacterial species (7). The significance of horizontal gene acquisition first came to light in the 1950s when multi-drug resistant bacteria began to emerge worldwide (8). Heavy antibiotic use following the discoveries of penicillin and sulfonamides was followed by the emergence of resistant strains. Newly acquired genes allow bacteria to adapt to ever-changing environments and exploit new ecological niches. Gene acquisition and loss promote speciation over time.

Horizontally transferred genes may be acquired through transformation, conjugation, or transduction (9). Transformation allows an organism to take up naked DNA from the environment. In transduction, genes are transferred from one bacterium to another by bacterial viruses, referred to as bacteriophages, and the DNA is subsequently integrated into the chromosome of the recipient. Conjugation requires direct contact between two viable bacteria. A mating bridge forms between the bacteria, and DNA in the form a plasmid is transferred across this bridge. Horizontally transferred genes exhibit both genetic and functional biases, and these biases facilitate the identification of horizontally-acquired genes. Within the genome, these genes are often integrated near tRNA loci or found in close proximity to phage/transposon genes (7).

Recently, horizontally acquired genes have been shown to exhibit promoter architecture distinct from that of classically regulated core genes (10). Horizontally acquired genes may exhibit unusual codon usage or exhibit a nucleotide compositional bias, often being relatively rich in AT-content compared to the host genome (11). In *Salmonella*, the AT-content of the genome is approximately 48%, whereas the AT-content of horizontally-acquired genomic islands and plasmids ranges from 53% to 63% (12) (Table 1.1).

While there are multiple mechanisms that mediate DNA uptake, novel genes are infrequently incorporated into a host genome (13). Genes that confer new traits such as virulence, stress resistance or cell surface alterations may be more likely to be retained. Some horizontally-acquired elements include genes that aid in integration and self-regulation, which are found in some of the *Salmonella* pathogenicity islands. A classic example is the *csg* locus. Organized as two divergently transcribed operons, the *csg* region consists of *csgBA*, which encodes the major structural subunits of the fibrous polymers, and *csgDEFG*, which encodes the major biofilm transcriptional regulator (CsgD) and the protein components required for secretion and assembly of the curli structures (CsgEFG). However, for most horizontally-acquired genes, incorporation into existing regulatory networks to allow coordinately regulated gene expression within the context of the existing genome is essential. The acquisition of horizontally-acquired genes drives both evolution and genetic diversity. Examination of horizontally-acquired genes has provided insights into the evolution of regulatory networks over time.

II. *Salmonella* as a Model Organism

Salmonella enterica has in particular served as a useful model organism for understanding the regulation of horizontally-acquired genes. This gastrointestinal Gram-negative pathogen is of global importance, most commonly causing enteric fever (typhoid) and enterocolitis/diarrhea, resulting in as many as 1.3 billion cases of infection and over 3 million deaths annually (14). *Salmonella* genetics are often compared to those of the related bacterium *Eschericia coli*. However, given that between 10-20% of the *Salmonella* genome consists of material absent from *E. coli*, it is believed that most of the phenotypic traits that distinguish the two are acquired through lateral gene transfer (15).

Unlike *E. coli*, which can serve as a gut commensal, all *Salmonella* are pathogenic, able to invade the intestinal epithelium and multiply within gut-associated lymphoid tissues (16,17). Virulence determinants acquired over time in the form of pathogenicity islands, mobile genetic elements and plasmids (Figure 1.1), distinguish *Salmonella* from its ancestors and generated diversity. For instance, *Salmonella* Pathogenicity Island-1 (SPI-1) has enabled *Salmonella* to invade epithelial cells (18), while SPI-2 has facilitated intracellular survival (19).

Oftentimes, the incorporation of newly-acquired genes has required integration into existing regulatory networks to coordinate appropriate expression. For instance, the magnesium ion-sensing PhoPQ system controls SPI-1 (20), while the stationary phase-associated sigma factor RpoS regulates expression of genes on the *Salmonella* virulence plasmid (21). Conservation of these common regulators across many gram-negative species has facilitated transfer events and increases the likelihood that new bacterial lineages may arise via horizontal gene transfer.

Current studies are attempting to better understand the mechanisms of *Salmonella* virulence, with a particular focus on pathogen evolution through horizontal gene transfer. Understanding this process in *Salmonella* will have broad implications for other bacterial species.

III. Basic Prokaryotic Transcriptional Regulation

Gene expression is a fundamental and universal process, allowing specific genetic programs to respond to changing environmental and growth conditions. In bacteria, transcription is a multi-step process with RNA polymerase (RNAP) as the primary driver of classical gene activation. While recognition of specific promoter sequences by RNAP regulates transcriptional initiation at the most basic level, a combination of transcription factors and other regulatory proteins act at promoter regions to silence or enhance transcription in response to environmental and biochemical cues, creating a system of integrated regulatory networks. Basal levels of transcription occur when RNAP coupled to a sequence-specific sigma factor recognizes and binds a promoter sequence to initiate transcription through the formation of an open complex (22). Transcription can be inhibited by binding of a repressor protein or enhanced through directed binding of a transcriptional activator that promotes RNAP-DNA interactions.

Gene transcription is also extensively affected by DNA supercoiling. The ability of RNAP to interact with a promoter to form an open complex is dependent on DNA topology and the constraints created therein (23-25). The superhelical density of DNA is regulated by topoisomerases I and II (gyrase), which can relax DNA or induce negative supercoils, respectively (26,27). Introduction of negative supercoils causes DNA twisting around the helical axis and DNA writhe around the superhelical axis (28). Writhe promotes long-range DNA

interactions, facilitating proximal positioning of regulatory proteins and genes with their promoter targets (29). Twisting may induce the formation of single-stranded bubbles, which enable the movement of RNAP from a closed to an open transcriptional initiation complex to enhance promoter clearance and transcriptional levels at certain genes (30,31). Environmental and growth conditions influence topoisomerase activity such that the superhelical density of the chromosome at any given time is highly dynamic (23). In addition to affecting RNAP-binding preferences, topological structure may also influence binding preferences by important regulatory proteins such as nucleoid-associated proteins (32).

IV. Nucleoid-Associated Proteins

Global gene regulation is best understood as an integrated model that includes elements of nucleoid architecture in addition to regulatory proteins. Bacteria must package their DNA in a way that is compact yet allows the DNA to be accessible for gene expression and regulation. Lacking a nucleus, prokaryotes package their DNA within the confines of the "nucleoid," a macromolecular complex in which genetic material and associated proteins are located (33-35). Compaction within this structure is implemented via three main mechanisms including molecular crowding (36,37), negative supercoiling (38) and interaction of DNA with nucleoid-associated proteins (39). Additional superstructures, including 400 looped domains and six macrodomains, provide further organization (40). Dynamic nucleoid architecture allows adaptation to modifications introduced by newly-acquired bacterial genes, influencing their expression and ability to become incorporated.

Nucleoid-associated proteins (NAPs) are integral to the regulation of existing and newly-acquired genes. This family of proteins exerts pleiotropic effects on gene expression through reversible local modulation of chromatin structure (41,42) as well as through direct promoter binding. Sensitivity to environmental and growth conditions allows tuned temporal regulation of gene expression. Factor for inversion stimulation (Fis) is the most prominent NAP during exponential phase, serving to up-regulate or repress many genes directly through promoter binding and indirectly through interaction with the topoisomerase genes (43,44). During post-exponential growth, levels of Fis decline due to auto-regulation, making way for other prominent NAPs in stationary phase, including DNA protection during starvation protein (Dps), and the histone-like nucleoid-structuring protein (H-NS). Interplay between NAPs adds an additional layer of regulatory complexity and greater precision. Fis and heat-unstable protein (HU) antagonize the activities of H-NS (45), while integration host factor (IHF) is required for *dps* transcription (46). Because NAPs have low DNA-sequence specificity, they are well-suited to the regulation of new horizontally-acquired genes, as foreign promoters do not necessarily contain specific regulatory sequences that are recognized by existing transcriptional regulators in the new host. One NAP that has been recognized to play an essential role in this process is H-NS, which is able to silence recently-acquired DNA.

V. H-NS

At about 20,000 copies per cell (47), H-NS is one of the most abundant chromatin-associated proteins. It binds DNA ubiquitously, controlling expression of over 200 genes through interaction with DNA topology, environmental cues, and other regulatory proteins. At a structural level, H-NS has two discrete and functionally independent domains, separated by a

flexible linker. The C-terminal domain mediates DNA-binding, while the N-terminal domain is essential for H-NS oligomerization (48). H-NS primarily forms tetramers, but higher levels of oligomerization may occur following binding and nucleation of DNA (49). DNA-binding by H-NS is specified by AT content. AT-rich tracts are recognized by a structural Q/RGR motif in H-NS that targets the minor groove of DNA (50). Oligomerization in response to divalent cation concentrations can lead to either DNA stiffening, wherein the H-NS-DNA complex forms a rigid nucleoprotein filament, or bridging, wherein relatively distant regions of H-NS-bound DNA interact, forming a DNA loop (51). This function is theorized to play a role in trapping RNAP (52). However, the stiffening mode of oligomerization has increasingly been associated with transcriptional silencing *in vivo* (53,54).

A strong correlation between H-NS binding and AT-rich horizontally acquired genes suggests a mechanism to allow self/non-self DNA-discrimination in bacteria (55,56). In a process called "xenogeneic silencing" (55), newly-acquired genes are silenced to protect a cell from potentially detrimental effects of unregulated expression, which can be relieved under appropriate conditions by other regulatory proteins. H-NS-mediated repression primarily occurs through direct transcriptional silencing (57,58) as well as through the modulation of DNA topology (32) (Figure 1.2). H-NS nucleation and oligomerization along a promoter region may be sufficient to occlude RNAP binding, thereby preventing transcription (10). In other instances, H-NS binds to regulatory regions both upstream and downstream of a promoter and alters superhelicity by constraining negative supercoils (59). In this scenario, RNAP is still able to bind but is unable to form an open complex. In some cases, RNAP may bind and form an open complex when H-NS is present, but structural loops formed by bridging prevent clearance of the promoter and thus

inhibit transcriptional elongation (52,60,61). H-NS is also able to bend DNA during bridging (62), bringing otherwise distant segments of DNA into close proximity. This may not only affect downstream topology, but may also facilitate silencing.

VI. Counter-silencing

In order to be useful to the recipient cell, newly-integrated DNA sequences silenced by H-NS must also be able to be appropriately expressed. Disruption of H-NS-mediated silencing by counter-silencing is a means by which repression can be reversed (Figure 1.2) (54,55). Oftentimes, counter-silencing factors consist of proteins that act as transcriptional activators at other loci, which are already able to respond to environmental cues. Exploitation of existing regulatory networks is one way in which horizontally-acquired genes can be regulated under appropriate conditions. Environmental changes in temperature, pH, and osmolarity can have a profound impact on DNA curvature, and at certain promoters, have been shown to affect H-NS binding (63).

Other regulatory proteins may also disrupt DNA-binding by H-NS. Fis is able to compete for binding sites and disrupt H-NS-stabilized complexes, promoting a switch from repression to stimulation at certain loci (64). In some instances, one protein may mediate both counter-silencing of H-NS and direct transcriptional activation. SsrB, a response regulator in the *Salmonella* SsrAB two-component system, both directly competes with H-NS for binding at virulence gene promoters and also induces a DNA conformational change that is unfavorable for H-NS-mediated stiffening (53). Other proteins may work in concert to achieve counter-silencing. For example, the MarR family regulatory protein SlyA competes with H-NS for DNA-binding

and alters the structure of H-NS bound DNA to allow another regulatory protein, PhoP, to bend DNA and facilitate open complex formation (10). H-NS homologs may serve as counter-silencers by disrupting the oligomerization capabilities of H-NS. H-NST, for example, is a truncated form of H-NS that lacks a DNA-binding domain but dimerizes with full-length H-NS molecules to disrupt their DNA-binding activity (65). More recently, a possible role of alternative sigma factors as counter-silencers has also been suggested (66-68).

VII. Scope of this dissertation

The overall goal of this dissertation is identify the scope of counter-silencing in the context of curli gene regulation. Chapter 2 contains specific details of the methods used throughout the dissertation. In Chapter 3, I consider a long-held hypothesis regarding the role of the alternative sigma factor σ^S as a potential counter-silencer. Other researchers have hypothesized a role for this sigma factor as an H-NS counter-silencer at the *spv* and *csg* loci, but this possibility has only been tested indirectly in *in vivo* studies. For the first time, I have used recently developed tools to directly characterize the nature of σ^S-H-NS regulatory interactions at these loci *in vitro*. Contrary to previous findings, I have found that σ^S lacks counter-silencing activity and have been able to demonstrate that σ^S exerts its actions indirectly via regulation of upstream pathways. In Chapter 4, I identify CsgD as the protein that is directly responsible for counter-silencing of the *csgBA* operon. I provide both *in vivo* and *in vitro* evidence for this finding and further define the mechanism of CsgD-mediated counter-silencing through DNase I and $KMnO_4$ footprinting. In Chapter 5, I describe studies to characterize the direct site of σ^S regulation, with a particular focus on the *csgD* locus. I show that σ^S is not directly required for transcription or counter-silencing of *csgD*, but rather, the σ^S-controlled regulators MlrA and IHF are responsible for

relieving H-NS repression at this promoter, leading to the expression of a downstream cascade of curli genes. In the final chapter, I discuss my work in the greater context of virulence gene regulation and the implications of my findings for the current understanding of transcription mechanisms within both *Salmonella* and other bacteria. In addition, I point to future directions for this work and address remaining unanswered questions.

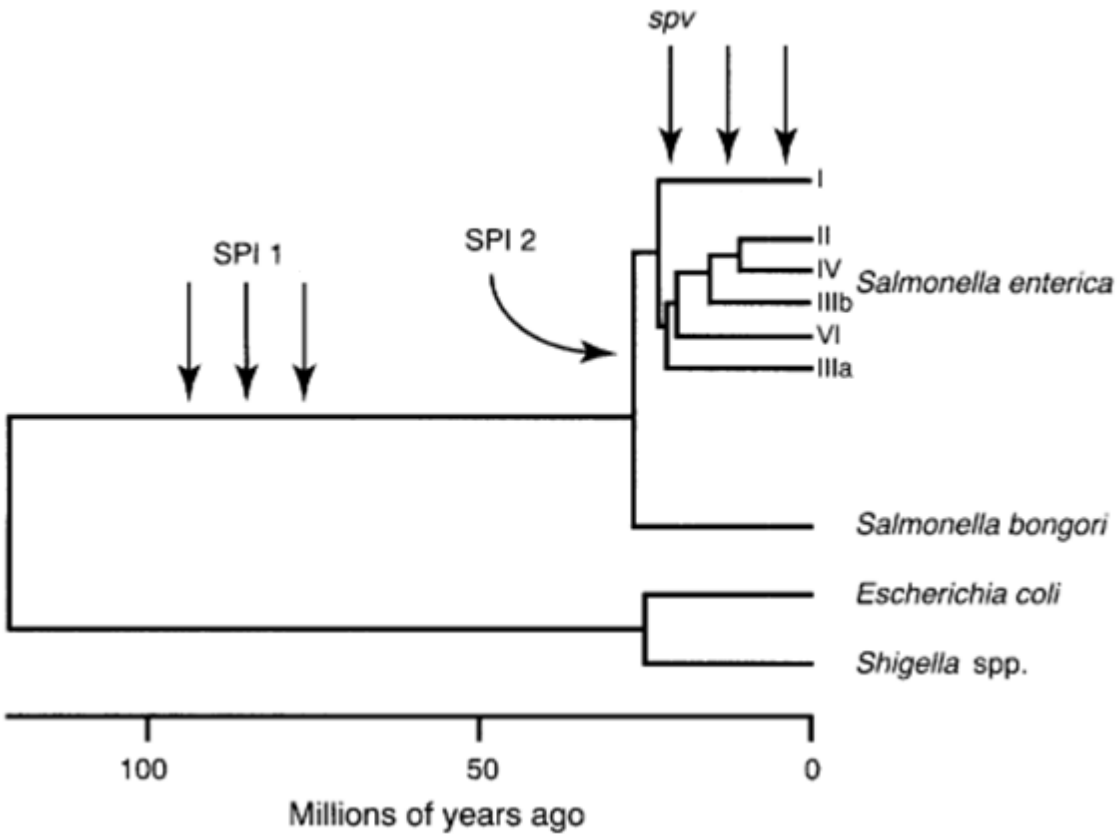


Figure 1.1. Major horizontal gene transfer events in *Salmonella*. Filled arrows represent acquisition points of *Salmonella* virulence genes. Notable events include acquisition of *Salmonella* pathogenicity island 1 (SPI-1), *Salmonella* pathogenicity island 2 (SPI-2) and the *Salmonella* virulence plasmid (*spv).* Adapted from (69).

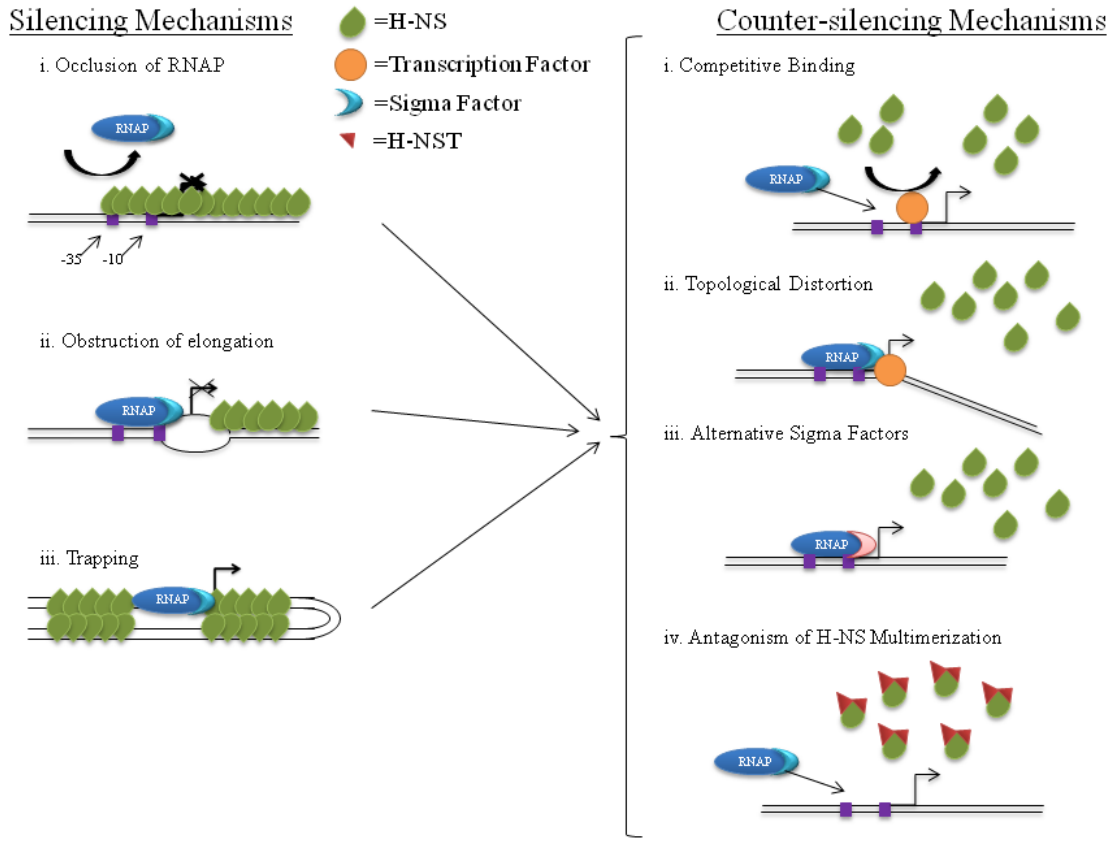


Figure 1.2. H-NS Silencing and Counter-Silencing Mechanisms. Left: Proposed mechanisms of H-NS-mediated silencing include nucleofilament formation to occlude promoter recognition (i), nucleofilament obstruction of elongation (ii), and trapping of RNAP in the bridging conformation (iii). Right: Four different mechanisms of counter-silencing have been proposed, including (i) competition for nucleation sites by promoter-specific transcription factors, (ii) topological distortions induced by promoter-specific transcription factors or environmental stimulation, (iii) activation by alternative sigma factors, and (iv) multimer disruption by protein antagonists. Adapted from (54,55).

Genetic Element	%AT-content
SPI-1	53.0%
SPI-2	55.4%
SPI-3	52.7%
SPI-4	55.2%
SPI-5	56.4%
Plasmid Virulence Genes	54.3%
Salmonella Genome	47.8%

Table 1.1 AT-content of horizontally acquired pathogenicity islands. The *Salmonella* pathogenicity islands and virulence plasmid genes exhibit AT-content substantially above the host genomic average, a hallmark characteristic of horizontally-acquired genes.

Chapter 2: Materials and Methods

Bacterial strains and plasmids

All *Salmonella enterica* serovar Typhimurium strains were constructed in a 14028s genetic background and grown in 50 mM NaCl Luria Bertani (LB) medium, which corresponds to 100 mOsm, unless otherwise noted. This medium has low osmolarity compared to typical LB (396 mOsm). All bacterial strains, plasmids, and primers are described in Table 2.1. Ampicillin and kanamycin were added to media as required at concentrations of 100 μg ml^{-1} and 50 μg ml^{-1}, respectively. Deletions of *csgD, rpoS, crl, ihfA, ihfB,* and *mlrA* were generated using the λ-Red recombinase protocol (70), and antibiotic resistance markers were swapped out using pCP20 (71). Phage P22 HT105/1 *int*-201 was used for transduction of *csgD, rpoS, mlrA*, *crl*, *ihfB,* and *ihfA* mutations into the appropriate strains (72).

Plasmid pSLN15 was constructed by cloning a PCR fragment generated from the primers csgD-OE-F and csgD-OE-R into the *Bam*HI and *Xho*I sites of pET28b (Qiagen). Plasmid pSLN16 was constructed by cloning a PCR fragment generated from the primers EF154 and EF155 with pKD4 as a template into pHR103 (73) and inserted into the *Pvu*I site. Plasmid pSLN17 (pCsgD) was constructed by cloning the PCR product generated from the primers SP20 and SP21 into the *Xba*I and *Eco*RI sites of pSLN16. Plasmid pQE-80L-RpoS was constructed by cloning the PCR product generated from the primers RpoS-OE-F and RpoS-OE-R into the *Sac*I and *Hin*dIII sites of pQE-80L (Qiagen). Plasmid pQE-80L-RpoD was constructed by cloning the PCR product generated from the primers RpoD-OE-F and RpoD-OE-R into the *Sac*I and *Hin*dIII sites of pQE-80L (Qiagen). Plasmid pHN1009 asHNS was constructed by cloning a 128 bp fragment of the H-NS promoter region starting 36 bp upstream of the translational start site and spanning 92 bp into

the gene. The fragment was amplified using primers asRNA HNS F and asRNA HNS R and inserted into the *Nco*I and *Xho*I sites of pHN1009 (74). Plasmid pSLN14 was constructed by cloning a PCR fragment generated from the primers crl-OE-F and crl-OE-R into the *Sal*I and *Xho*I sites of pET28b (Qiagen). Plasmid pSLN31 was constructed by cloning a PCR fragment generated from the primers SP100 and SP103 and moved into the *NdeI* and *HindIII* sites of pET28b (Qiagen). Plasmid pSLN24 (pMlrA) was constructed by cloning the PCR product generated from the primers SP64 and SP65 and inserted into the *Xba*I and *Eco*RI sites of pSLN16.

C-terminal HA-tagged H-NS was created using the λ-Red recombinase protocol (70) with insertion of the *tetRA* locus using a PCR product generated from HNS-tetRA F/R primers into strain SL4482. The *tetRA* element was subsequently replaced with a PCR fragment amplified by HA-HNS fill-in F/R primers, generating strain SLN66. Using SLN66 gDNA as a template, Gibson cloning fragment 1 amplified with primers "HNS Gibson Scramble Fragment 1 F/R," Gibson cloning fragment 2 amplified with primers "HNS Gibson scramble Fragment 2 F/R," and Gibson cloning fragment 3 amplified with primers "H-NS Gibson Scramble Fragment 3 F and R" were PCR-amplified, gel purified and cloned at equimolar ratios into the *Bam*HI site of pRDH10 using Gibson Assembly Master Mix (NEB) and incubated for 90 min at 50°C to construct plasmid pSLN23. pSLN23 was introduced into the *S.* Typhimurium SLN196 recipient strain, containing pSW172, by conjugation using *E. coli* S17-1λpir (FLS306) as the donor strain. Strain selection was performed as previously published (75) to create SLN221 and pSW172 was cured by growth at 37°C.

IVT Vectors. Plasmid pSLN7 was generated by cloning a 3.0 kb fragment of the *csgB* region, amplified using primers *csgA* IVT vector R and *csgA* IVT vector F, and ligated into the *Hin*DIII and *Xba*I sites of pRW20 (Will *et al.*, 2014). Plasmid pSLN25 was generated by cloning a 3kb fragment of the *mlrA* promoter region, amplified using primers *mlrA* IVT vector F and *mlrA* IVT vector R, and ligated into the *Xba*I and *Sal*I sites of pRW20. Plasmid pSLN27 was generated by cloning a 2.7kb fragment of the *ihfB* promoter region, amplified using primers *ihfB* IVT vector F and *ihfB* IVT vector R, and ligated into the *Xba*I and *Sal*I sites of pRW20. Plasmid pSLN13 was generated by cloning a 2.9kb fragment of the *ugtL* promoter region, amplified using primers *ugtL* IVT vector F and *ugtL* IVT vector R, and ligated into the *BamH*I and *Hind*III sites of pRW20. Plasmid pSLN10 was generated by cloning a 2.9kb fragment of the *purE* promoter region, amplified using primers *purE* IVT vector F and *purE* IVT vector R, and ligated into the *Xba*I and *Eco*RI sites of pRW20. Plasmid pRW20-*spvA* was generated by cloning a 2.5kb fragment of the *spvA* promoter region, amplified using primers *spvA* IVT vector F and *spvA* IVT vector R, and ligated into the *Sma*I and *Hind*III sites of pRW20. Plasmid pRW20-*katE* was generated by cloning a 2.9kb fragment of the *katE* promoter region, amplified using primers *katE* IVT vector F and *katE* IVT vector R, and ligated into the *Sma*I and *Hind*III sites of pRW20. Plasmid pRW20-*gmk* was generated by cloning a 2.0kb fragment of the *gmk* promoter region, amplified using primers *gmk* IVT vector F and *gmk* IVT vector R, and ligated into the *Sma*I and *Hind*III sites of pRW20. Plasmid pSLN19 was generated by cloning a 2.9 kb fragment of the *csgD* promoter region, amplified using primers *csgD* IVT vector F and *csgD* IVT vector R, and ligated into the *Pst*I and *Xba*I sites of pRW20. Plasmid DNA for biochemical assays was purified as previously described (10).

RNA isolation and quantitative PCR analysis of *in vivo* gene expression

2 x 10^7 CFUs of overnight cultures grown in LB broth at 37°C were subcultured into 50 mL 100 mOsm LB broth and grown at 30°C. For exponential phase experiments, cells were split into two flasks, and one was induced with 1mM isopropyl β-D-1-thiogalactoside (IPTG) for approximately four hrs before reaching OD_{600}~0.6. For stationary phase experiments, cells were split at OD_{600} ~0.6 into two flasks, and one was induced with 1 mM IPTG. After induction, cells were grown for another four hrs at 30°C. Cells were then pelleted and re-suspended in TRIzol reagent (Life Technologies) and incubated for a minimum of 30 min. After two chloroform extractions, isolated RNA was suspended in isopropanol and stored at -20°C until further processing. RNA was precipitated and washed per manufacturer's instructions. Five U DNase I (Thermo Fisher Scientific) were added to each sample and incubated for one hr at 37°C to remove residual DNA. RNA was extracted with acid-phenol:chloroform (1:1) and precipitated with 95% ethanol. The final RNA pellet was dissolved in RNase-free water. RNA was converted to cDNA using the QuantiTect RT-PCR kit (Qiagen) according to the manufacturer's instructions. cDNA was diluted appropriately (1:20) to bring samples into linear range of detection and transcripts were quantified via qPCR in a CFX90 (BioRad) using SYBR green master mix (76). Oligonucleotide pairs are described in Table 2.1. The relative expression of genes was determined by normalizing expression levels to *gyrB* transcript.

Phenotypic Analyses

Bacterial strains were grown in 100 mOsm NaCl LB overnight at 37°C. The cells were diluted to OD_{600} 1.0, and 5 μL were spot inoculated on LB plates without salt and supplemented with the appropriate antibiotic(s), Congo red (40 μg mL^{-1}), and Coomassie Blue (20 μg mL^{-1}) (77). For

asHNS knock-down experiments, plates were spread with 40 μL of 100 mM IPTG before spotting. Plates were incubated at 28°C or 37°C for three days without inversion.

Western Blotting

The asHNS construct and asVector control were transformed into an *S.* Typhimurium 14028s strain carrying an N-terminal HA-tagged H-NS chromosomal construct. Depletion experiments were conducted as above, but instead of harvesting cells for RNA collection, 0.25 OD_{600} cells were collected at each time point by centrifugation. Pellets were boiled for 5 min at 95˚C in 30μL Laemmli buffer, and total protein was separated using a 4-15% SDS gradient gel. Protein was transferred onto a PVDF membrane and, after blocking with PBS buffer containing 0.05% Tween-20 (PBS-T) and 5% Non-fat dry milk, incubated in blocking solution with 1:2000 anti-HA (Sigma), 1:1000 mouse IgG anti-sigmaS (Biolegend), or 1:80,000 anti-GroEL (Sigma). Membranes were washed in PBS-T and probed with either anti-rabbit IgG or anti-mouse IgG-HRP conjugate secondary antibody (Bio-Rad) diluted 1:10,000 in blocking solution, and visualized with Pierce ECL Western Blotting Substrate (Pierce).

Protein Purification

*Sigma Factors***.** Expression vectors pQE-80L-RpoS and pQE-80L-RpoD were transformed into *E. coli* BL21 (DE3) cells for production and purification of recombinant sigma factors. Cells were grown at 30°C to OD_{600} 0.5 in LB. Expression was induced with 1 mM IPTG for four hrs before cells were pelleted by centrifugation (7,000 × g, 10 min at 4°C) and stored at -80°C. Cells were re-suspended in 20 mL binding buffer (50 mM NaH_2PO_4 , 300 mM NaCl pH 8.0) with 0.5 mg mL^{-1} lysozyme and protease inhibitors (Sigma). The solution was incubated on ice for twenty min and lysed by sonication, and supernatant was clarified with three rounds of centrifugation

(15,000 × g, 30 minutes each). Lysates were then applied to 5 mL HisTrap HP columns (GE Healthcare Life Sciences) equilibrated with binding buffer. Columns were washed first with 5 column volumes (CV) binding buffer followed by 4 CV binding buffer supplemented with 20 mM imidazole. Proteins were then eluted over a 20 CV gradient from 20 mM to 500 mM imidazole in binding buffer. Fractions containing sigma factors were determined by SDS-PAGE and pooled. Eluate was dialyzed overnight at 4°C at a 1:200 ratio to gel filtration buffer (50 mM Tris pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol) and concentrated to 2.5 mL. The sample was injected into a 130 mL Superdex 75 gel filtration column (GE Healthcare Life Sciences) equilibriated with gel filtration buffer. Elution occurred over 180 mL. Pure fractions were identified by SDS-PAGE, dialyzed in storage buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.5 mM DTT, 50% glycerol), and stored at -80°C.

H-NS. H-NS purification was performed as previously using pRW13 (10) except that elution from the His-Trap HP column was performed as follows: 10 CV of linear gradient of buffer containing 20 mM to 250 mM imidazole followed by a 5 CV wash with buffer containing 250 mM imidazole followed by a 5 CV linear gradient of buffer containing 250 to 500 mM imidazole. Purified protein was stored in aliquots at -80°C.

CsgD. pSLN15 was transformed into *E. coli* BL21 (DE3) for production and purification of CsgD. Cells were grown at 37°C to OD_{600}~0.6 in 100 mOsm LB and then induced with 1 mM IPTG for four hrs at 30°C and harvested and stored as above. Cells were re-suspended in 15 mL binding buffer supplemented with 20 mM imidazole, 1 mM PMSF, and protease inhibitors (Sigma) and lysed by sonication. The lysate was clarified by centrifugation (three times at 20,000

× g, 30 minutes each). Lysates were then loaded onto an equilibrated 5 mL His-Trap HP column (GE Healthcare Life Sciences) and washed with 50 mL binding buffer containing 20 mM imidazole. Protein was eluted over a 120 mL linear gradient of imidazole, from 20 to 250 mM, and CsgD-containing fractions were identified through SDS-PAGE. CsgD was concentrated using an Amicon Ultra centrifugal concentrator (Millipore), and the 6xHis-tag was removed using a Thrombin Cleavage Capture Kit (Novagen) with overnight incubation at 4°C, combining 1 mg protein with 1 U Thrombin. Any remaining precipitate was isolated by centrifugation. Residual thrombin was removed with streptavidin agarose per the manufacturer's protocol. Sample was dialyzed against binding buffer overnight at 4°C and then loaded onto 0.5 mL equilibrated Ni-NTA resin (Qiagen). Purified CsgD was collected from the flow-through and verified by SDS-PAGE. Pure cleaved CsgD was dialyzed into CsgD storage buffer (50 mM Tris pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 0.5 mM TCEP, 50% glycerol) and stored at -80°C.

Crl. pSLN14 was transformed into *E. coli* BL21 (DE3) for production and purification of Crl protein. Cells were grown at 37°C to OD_{600} ~0.6 and then induced with 1 mM IPTG for 3.5 hrs at 30˚C before harvesting and storage at -80˚C. Cells were resuspended in 15 mL Crl lysis buffer (50 mM Na_2HPO_4 pH 8.0, 300 mM NaCl), 1 mM PMSF, protease inhibitors (Sigma) and 1 mg/mL lysozyme prior to lysis by sonication. The lysate was clarified by centrifugation (2 times at 12,000 rpm, 30 min each) before the supernatant was combined with 2 mL equilibriated Ni-NTA beads (Qiagen) and incubated for 1 hr at 4˚C. The beads were loaded onto a column and washed with 20 CV Crl lysis buffer containing 20 mM imidazole. Purified 6xHis-tagged Crl was eluted with 10 CV Crl lysis buffer containing 250 mM imidazole. Crl was concentrated using an Amicon Ultra centrifugal concentrator (Millipore), and the 6xHis-tag was removed using a

Thrombin Cleavage Capture Kit (Novagen) with 2 hr incubation at room temperature combining 1 mg protein with 1 U Thrombin. Remaining precipitate was isolated by centrifugation. Residual thrombin was removed with streptavidin agarose per the manufacturer's protocol. Sample was dialyzed against binding buffer overnight at 4°C and then loaded onto 0.5 mL equilibrated Ni-NTA resin (Qiagen). Purified CsgD was collected from the flow-through and verified by SDS-PAGE. Pure cleaved Crl was dialyzed into Crl storage buffer (50 mM Tris pH 7.6, 200 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 50% glycerol) and stored at -80°C.

MlrA. pSLN31 was transformed into *E. coli* BL21 (DE3) for production and purification of MlrA protein. Cells were grown at 37°C to OD_{600} ~0.6 in LB and then induced with 0.1mM IPTG for 3 hrs at 37°C prior to harvesting and storage as above. Cells were resuspended in 20 mL MlrA lysis buffer (50mM Tris pH 8.5, 300mM NaCl, 5mM β-mercaptoethanol) with 1mM PMSF, 1mg/mL lysozyme and protease inhibitors (Sigma) prior to lysis by sonication, and the supernatant was clarified with centrifugation for 30 min at 12,000 rpm. The supernatant was set aside and the resulting pellet resuspended in 20mL Sarcosine Buffer (50mM Tris pH 8.5, 300mM NaCl, 1% Triton X-100, 1.5% N-Lauryl sarcosine, 25mM triethanolamine, 5mM β-mercaptoethanol and 1mM PMSF). The solution was placed over a stir plate in an ice bath and stirred for 30 min before spinning for 10 min at 12,000 rpm, and the resulting supernatant was combined with the supernatant from the lysis procedure and applied to 0.8 mL Ni-NTA beads (Qiagen) equilibriated with MlrA binding buffer (50mM Tris pH 8.5, 300mM NaCl, 20mM imidazole, 10% glycerol, 5mM β-mercaptoethanol and 1 mM PMSF). The supernatant was incubated with the resin for 2 hrs with rotation at 4˚C. Beads were loaded onto a column to allow flow-through and then washed with 10 CV MlrA binding buffer followed by 10 CV MlrA wash

buffer (50mM Tris pH 8.5, 1M NaCl, 20mM imidazole, 10% glycerol, 5mM β-mercaptoethanol and 1mM PMSF). Purified protein was eluted with 10 CV MlrA elution buffer (50mM Tris pH 8.5, 150mM NaCl, 500mM imidazole, 10% glycerol and 1mM PMSF). MlrA was concentrated using an Amicon Ultra centrifugal concentrator (Millipore) and the 6xHis-tag removed using a Thrombin Cleavage Capture Kit (Novagen) with 24 hr incubation at 4°C, combining 2 mg protein with 1 U Thrombin. Remaining precipitate was isolated by centrifugation. Residual thrombin was removed with streptavidin agarose per the manufacturer's protocol. Sample was dialyzed against binding buffer overnight at 4°C and then loaded onto 0.8 mL equilibrated Ni-NTA resin (Qiagen). Purified MlrA was collected from the flow-through and verified by SDS-PAGE. Pure cleaved MlrA was dialyzed into MlrA storage buffer (50mM Tris pH 7.6, 200mM KCl, 10mM $MgCl_2$, 1mM DTT, 0.1mM EDTA and 50% glycerol) and stored at -80°C.

IHF. Purified IHF was received as a gift from Dr. Carlos Catalanos, University of Colorado – Denver and purified using a method described previously (78).

In vitro transcription

All steps of IVT reactions were carried out at 30°C unless otherwise specified. Reactions were assembled in IVT buffer containing 40 mM HEPES, pH 7.3, 1 mM $MgCl_2$, 120 mM potassium glutamate, 0.5 mM $CaCl_2$, 1 mM DTT, 0.05% NP-40, 0.1 mg mL^{-1} BSA and 10% glycerol to a final volume of 20 μL. This concentration of potassium glutamate was used to promote potential sigma-factor specificity (79). To confirm that the elevated potassium glutamate concentration did not impact counter-silencing, counter-silencing IVT reactions were also performed in IVT buffer containing 50 mM potassium glutamate (Supplementary Figure 4.10a). Purified σ^S was incubated with RNAP core (New England Biolabs) at a 10:1 ratio, whereas purified σ^{70} was

incubated with RNAP core at a 2:1 ratio. RNA polymerase holoenzyme was reconstituted in IVT buffer for 30 min at 30°C immediately before use in IVT reactions. Template at a final concentration of 1 nM was combined with 20 U of RiboLock RNase Inhibitor (Thermo Fisher Scientific) and incubated for 10 min in order to equilibrate. For counter-silencing reactions, CsgD was added at 50 nM for 10 min before H-NS was added to a final concentration of 130 nM and incubated for 10 min, although the order of addition was not found to influence counter-silencing (Supplementary Figure 4.10b). Following the addition of regulatory proteins, reconstituted RNA polymerase holoenyzme was incubated with template for 10 min at a final concentration of 10 nM. rNTPs were added to a final concentration of 1 nM and the reaction incubated for an additional 30 min at 30°C. The reaction was stopped by adding 20 μL DNase I buffer containing 10 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 4 U of DNase I (Thermo Fisher Scientific), and incubated for 30 min at 37°C. EDTA was added to final concentration of 5 mM and the reactions incubated for 10 min at 65°C to inactivate DNase I. cDNA synthesis was performed as previously (10) using the sequence-specific reverse oligonucleotide probes as indicated in Table 2.1.

DNase I Footprinting and Fluorescent Primer Extension

Reactions, fluorescent primer extension, and Differential DNA Footprint Analysis (DDFA) were performed as in Will *et al.* (10) using *csgB* FAM sense or *csgB* FAM anti-sense probes as indicated in Table 2.1.

$KMnO_4$ Footprinting

Reactions were assembled as described for IVT reactions, except that DTT was omitted from the IVT buffer. Proteins were sequentially added for 10 min each at 30˚C as for IVT reactions,

except that RNAP was allowed to incubate for 15 min, at which point $KMnO_4$ was added to a final concentration of 10 mM. The reactions were allowed to proceed for 2 min and stopped by the addition of 2 μL of 14 M β-mercaptoethanol, followed by 78 μL of quenching buffer containing 1 M β-mercaptoethanol, 20 mM EDTA, and 385 mM sodium acetate (pH 7.0). Reactions were extracted with phenol:CHCl3:IAA (25:24:1) followed by chloroform and precipitated with 2 μL glycogen and ethanol. Reactions were washed 3 times with 70% ethanol, dried, and re-dissolved in 10 μL H_20. Reactions were detected via fluorescent primer extension as above using the *csgB* FAM sense probe as the primer.

Circular Permutation Assay

A 32-bp region of the *csgB* promoter containing the CsgD-binding site was generated by PCR using primers SP136 and SP137 and cloned into the *Eco*RI and *Sal*I sites of pTrc99a, making plasmid pSLN32. Using this plasmid as a template, circular permutation fragments were generated using PCR with the corresponding primer pairs for each fragment as indicated in Table 2.1 and diagrammed in Supplementary Figure 4.4a. Mobility of CsgD-bound fragments were analyzed using a gel shift assay. 20 ng of fragments (15.4nM) and 25x-molar concentration of purified CsgD (385 nM) were incubated in EMSA buffer (10 mM Tris pH 7.6, 50 mM KCl, 10% glycerol, 100 ng mL^{-1} BSA, 0.05% NP-40, 1 mM $MgCl_2$, and 1 mM DTT) to a total volume of 10 μL. Binding reactions were carried out for 30 min at 30°C. Samples were loaded onto a pre-run 6% 19:1 native acrylamide gel in 0.5x TBE and run at 100V and 4°C. The gel was stained using SybrGold (Thermo Fisher Scientific) in 0.5x TBE at room temperature for 30 min and visualized using a Typhoon 9400 variable mode imager (GE Healthcare, Piscataway, NJ). Bend angle was estimated by determining the ratio of the mobility of the protein-binding site in the

middle of the DNA fragment relative to the mobility of a fragment bound by the DNA at the end (Thompson and Landy, 1988).

In vitro Vitamin B_{12} Transcription Assays

IVT reactions and $E\sigma^S$ holoenzyme were assembled as described above. Vitamin B_{12} (Sigma) dilutions were either covered in foil or pre-treated with 20 min exposure 4 cm from a white light source. One nM supercoiled *csgD* template was incubated with 50 nM or 200 nM MlrA at a 1:1 ratio with vitamin B_{12} for 10 min before a 10 min incubation with either 130 nM H-NS or 20 nM IHF for 10 min followed by H-NS for 10 min. Reconstituted RNA polymerase holoenzyme was added, and the reactions proceeded as above.

Table 2.1. Strains, Plasmids, and Primers used in this Dissertation

Strain, plasmid, or primer	Genotype, relevant characteristics, or sequence	Reference
Strains		
SLN19	14028s pHN1009 asHNS	This Study
SLN36	14028s pHN1009	This Study
SLN66	SL4482 H-NS HA	This Study
SLN86	14028s *rpoS* pHN1009	This Study
SLN87	14028s *rpoS* pHN1009 asHNS	This Study
SLN96	14028s *crl*::FRT::kan::FRT	This Study
SLN101	14028s *rpoS crl*::FRT::kan::FRT	This Study
SLN102	14028s *rpoS hns*	This Study
SLN104	14028s *rpoS hns crl*::FRT::kan::FRT	This Study
SLN112	14028s *csgD*	This Study
SLN114	14028s *csgDrpoS*	This Study
SLN123	14028s *csgD* pHN1009	This Study
SLN124	14028s *csgD* pHN1009 asHNS	This Study
SLN125	14028s *csgDrpoS* pHN1009	This Study
SLN126	14028s *csgDrpoS* pHN1009 asHNS	This Study
SLN134	SLN19 pSLN16	This Study
SLN135	SLN19 pCsgD	This Study
SLN136	SLN36 pSLN16	This Study
SLN137	SLN36 pCsgD	This Study
SLN138	SLN86 pSLN16	This Study
SLN139	SLN86 pCsgD	This Study
SLN140	SLN87 pSLN16	This Study
SLN141	SLN87 pCsgD	This Study
SLN195	14028s *mlrA*::cm	This Study
SLN196	SLN66 pSW172	This Study
SLN198	*E. coli* S17-1λ-pir pSLN23	This Study
SLN199	SLN19 pMlrA	This Study
SLN200	SLN36 pMlrA	This Study
SLN201	SLN86 pMlrA	This Study
SLN202	SLN87 pMlrA	This Study
SLN204	14028s *rpoS mlrA*::cm	This Study
SLN207	14028s *mlrA*::cm pHN1009	This Study
SLN208	14028s *mlrA*::cm pHN1009 asHNS	This Study
SLN209	14028s *rpoS mlrA*::cm pHN1009	This Study
SLN210	14028s *rpoS mlrA*::cm pHN1009 asHNS	This Study

SLN211	14028s *rpoS* pSLN16	This Study
SLN212	14028s *rpoS* pMlrA	This Study
SLN221	SLN66 derivative with scrambled H-NS promoter	This Study
SLN222	SLN221 pHN1009	This Study
SLN223	SLN221 pHN1009 asHNS	This Study
SLN225	14028s *ihfA*::cm	This Study
SLN242	14028s *ihfB*::tetRA	This Study
SLN 272	14028s *ihfA*::cm pHN1009	This Study
SLN273	14028s *ihfA*::cm pHN1009 asHNS	This Study
SLN274	14028s *ihfB*::tetRA pHN1009	This Study
SLN275	14028s *ihfB*::tetRA pHN1009 asHNS	This Study
SLN276	14028s *ihfA*::cm *ihfB*::tetRA	This Study
SLN277	14028s *ihfA*::cm *ihfB*::tetRA pHN1009	This Study
SLN278	14028s *ihfA*::cm *ihfB*::tetRA pHN1009 asHNS	This Study
BL21 (DE3)	*fhuA2* [*lon*] *ompT gal* (λDE3) [*dcm*] Δ*hsdS* λDE3 = λs*BamHI*o Δ*EcoRI*-B int::(*lacI*::P*lacUV5*::T7 gene1) i21 Δ*nin5*	New England Biolabs
SL4482	14028s *rpoS**	(56)
DH10B	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* ***λ***- *rpsL nupG*	Invitrogen
FLS305	*E. coli* DH5a sup E44, Δ*lacU169* (Φ*lacZ*ΔM15), *recA1*, *endA1*, *hsdR17*, *thi-1*, *gyrA96*, *relA1*, λ*pir* phage lysogen	(80)
FLS306	S17-1 λ*pir* TpR SmR *recA, thi, pro, hsdR*-M+RP4: 2-Tc:Mu: Km Tn7 λpir	(80)
Plasmids		
pHN1009	Paired termini hairpin knock-down vector	(74)
pHN1009 asHNS	H-NS promoter fragment cloned into pHN1009 for induced knock-down	This Study
pHR103	*bla ΔlacIQ* P_{trc} oricolE1	(73)
pET28b	pBR322 *lacI*	Qiagen
pQE-80L	pBR322 oricolE1	Qiagen
pRW13	14028s *hns* cloned into *NcoI* and *XbaI* sites of pTrc99a for H-NS overexpression	(10)
pRW20	Transcriptional terminators cloned into pWKS30. Used for *in vitro* vector construction	(10)
pKD3	*bla* FRT *cm* FRT PS1 PS2 ori6K	(70)

pKD4	*bla* FRT *kan* FRT PS1 PS2 ori6K	(70)
pKD46	*bla araC*-P_{araB} γβ*exo* oriR101 repA101(Ts)	(70)
pCP20	*bla cat* cI857 lPr *flp* PSC101 *oriTS*	(70)
pCsgD	14028s *csgD* moved into the *XbaI* and *EcoRI* sites of pSLN16 for constitutive expression	This Study
pRW20-*gmk*	14028s *gmk* moved into pRW20 for *in vitro* transcription reactions	This Study
pRW20-*katE*	14028s *katE* moved into pRW20 for *in vitro* transcription reactions	This Study
pRW20-*spvA*	14028s *spvA* moved into pRW20 for *in vitro* transcription reactions	This Study
pQE-80L-RpoD	14028s *rpoD* cloned into pQE-80L for protein overexpression	This Study
pQE-80L-RpoS	14028s *rpoS* cloned into pQE-80L for protein overexpression	This Study
pRDH10	*SacRB ori6K mobRP4* suicide plasmid	(80)
pSW172	OriR101 *repA*101ts Carb	(81)
pSLN7	14028s *csgB* region cloned into pRW20 for *in vitro* transcription reactions	This Study
pSLN10	14028s *purE* moved into pRW20 for *in vitro* transcription reactions	This Study
pSLN13	14028s *ugtL* moved into pRW20 for *in vitro* transcription reactions	This Study
pSLN14	14028s *crl* cloned into pET28b for protein overexpression	This Study
pSLN15	14028s *csgD* moved into the *BamHI* and *XhoI* sites of pET28b for overexpression	This Study
pSLN16	Kanamycin cassette moved into pHR103	This Study
pSLN19	14028s *csgD* moved into pRW20 for *in vitro* transcription reactions	This Study
pSLN23	Gibson assembly of scrambled H-NS promoter into pRDH10	This Study
pSLN24 (pMlrA)	14028s *mlrA* cloned into pSLN16 for constitutive expression	This Study
pSLN25	14028s *mlrA* moved into pRW20 for *in vitro* transcription reactions	This Study
pSLN27	14028s *ihfB* moved into pRW20 for *in vitro* transcription reactions	This Study
pSLN31	14028s *mlrA* cloned into pET28b for protein overexpression	This Study

pSLN 32	*csgB* CsgD binding site moved into pTrc99a for circular permutation template	This Study
Primers		
asRNA HNS F	GCCTGCTGCTCTCGAGAACAAACCACCCCA ATATAAGTTTGAG	This Study
asRNA HNS R	AGCAGTTGACACCATGGTTCCAGCATTTCT TCAAGCGTTTCCAG	This Study
csgA IVT vector R	GGATCAAGCTTGCCAGCAGGCGTGA	This Study
csgA IVT vector F	CGTGTTCTAGAGCAGTTTTCCGGTTATGG	This Study
CsgD-OE-F	GCAGTGGATCCGTTTAATGAAGTCCATAGT AGTC	This Study
CsgD-OE-R	GAGTCCTCGAGTTACCGCCTGAGATTATCG	This Study
CsgD constitutive Plasmid insertion F	CTG TAG AAT TCA TGT TTA ATG AAG TCC ATA GTA GTC	This Study
CsgD constitutive Plasmid insertion R	CTGTATCTAGATTACCGCCTGAGATTATCG TTTGC	This Study
EF154	CACCGATCGGTCATTTCGAACCCCAGAGTC CCGC	(73)
EF155	CACGATCGGATCCCCTCACGCTGCCGCAAG CACTC	(73)
RpoS-OE-F	GAGCTCATGAGTCAGAATACGCTGAAAGTT C	This Study
RpoS-OE-R	AAGCTTTTACTCGCGGAACAGCGCTTC	This Study
RpoD -OE-F	GAGCTCATGCCGCATATCGATCGGGAAG	This Study
RpoD-OE-R	AAGCTTTTAATCGTCGAGGAAGCTGCGCAG	This Study
csgD IVT vector F	CGTGTCTAGAGGCATTGTTTCTGAAACCAT TCTG	This Study
csgD IVT vector R	CGTGCTGCAGCGGTATTAATATTGGTCAAC AAGC	This Study
pagC qPCR F	GGGTCTGTTGAGCCTGAAGG	This Study
pagC qPCR R	GCCATCCTGAGTGGAATGTTC	This Study
csgB qPCR F	CACAAGAAGGAGGAAATAATCG	This Study
csgB qPCR R	TATTGGCCTTATTTCCAGAACC	This Study
csgB FAM anti-sense probe	5'- /56-FAM/CGCACCCAGTATTGTCAACATC	This Study
csgB FAM sense probe	5’-/56-FAM/ATACACTATCTTCTGTGGCC	This Study

katE qPCR F	GTATTCCACGCAGTTATCG	This Study
katE qPCR R	TGTGACTCATCCCATACC	This Study
purE qPCR F	GTCTTCCCGCAATAATCC	This Study
purE qPCR R	GGAAACCACTTCTACATGG	This Study
gyrB qPCR F	GATGGGTTTTCCAGCAGGTATTC	This Study
gyrB qPCR R	AGGTCTGATTGCGGTGGTTTC	This Study
csgD qPCR F	ATCTCTGCAAGCTACGG	This Study
csgD qPCR R	TCCGCTTCCATCATATCC	This Study
mlrA qPCR F	AGTAGCGAACAACCTAACG	This Study
mlrA qPCR R	ATGTTTGGGCAGGATAATCC	This Study
ihfA qPCR F	TCTGTTTGATAAGCTTGGGC	This Study
ihfA qPCR R	CAAAACCAGAGAGTTTCACC	This Study
ihfB qPCR F	GGTAAAAGAGATGCTGGAGC	This Study
ihfB qPCR R	TTACCCGGTTTAAAGTGAGG	This Study
gmk qPCR F	TCAGGTTTCCGTTTCACATACC	This Study
gmk qPCR R	GTAGTAATTGCCAAACACTTCCG	This Study
spvA qPCR F	ACAATGGCACGAACCTGAG	This Study
spvA qPCR R	TGGAAAATGATACGGCAGC	This Study
ugtL qPCR F	TACCGTTTCGTTATCGGATGGGCT	This Study
ugtL qPCR R	AACCCTGCGATGATACCGTAATGC	This Study
hns qPCR F	ATGAGCGTCGTGAAGAAGAAAGCG	This Study
hns qPCR R	GCAGCCATGCTATTCAGCAGTTCA	This Study
ompR qPCR F	CGCCTGCGTAGTCAAAGTAA	This Study
ompR qPCR R	GATTTCCAGCCCGACGATAC	This Study
ssrB qPCR F	CATACGAGCCTGACATACTTATCC	This Study
ssrB qPCR R	CTTGTTGGTATGCTGTGTAAACC	This Study
delta*rpoS* F	TTGCTAGTTCCGTCAAGGGATCACGGGTAG GAGCCACCTTGTGTAGGCTGGAGCTGCTTC	(82)
delta*rpoS* R	AAGGCCAGTCGACAGACTGGCCTTTTTTTG ACAAGGGTACATGGGAATTAGCCATGGTCC	(82)
delta*csgD* F	CAGCTGTCAGATGTGCGATTAAAAAAAGTG GAGTTTCATCGTGTAGGCTGGAGCTGCTTC	This Study
delta*csgD* R	CTCTGCTGCTACAATCCAGGTCAGATAGCG TTTCATGGCCCATATGAATATCCTCCTTAG	This Study
HA-HNS F	CCATACGATGTTCCAGATTACG	This Study
HA-HNS R	GCGTAATCTGGAACATCGTATGG	This Study

HNS Gibson Scramble Fragment 1 F	GCGACCACACCCGTCCTGTGCAAAGTACTG GAATATCTAATTTGTC	This Study
HNS Gibson Scramble Fragment 1 R	GACTGATCAGTTTGTTGAGCTAATAATAGA GC	This Study
HNS Gibson Scramble Fragment 2 F	GCTCAACAAACTGATCAGTCCGTACTTTGA GATTGTATCTATGAGTGAGGCTCTTAAGAT CCTGAATAATATCCGCACACTGCGTGCTC	This Study
HNS Gibson Scramble Fragment 2 R	ACGACGCTCATTAACGACAACTTCTAATTT TTCAAGCATTTCTTCCAGAGTCTCAAGTGT GCACTCCCTAGCTTGAGCACGCAGTGTGCG	This Study
HNS Gibson Scramble Fragment 3 F	CGCTCTATTATTAGCTCAACAAACTGATCA GTCCGTACTTTGAGATTGTATCTATGAGTG AGGCTCTTAAGATCCTGAATAATATCCGCA CACTGCGTGCTCAAGCTAGGGAGTGCACAC TTGAGACTCTGGAAGAAATGCTTGAAAAAT TAGAAGTTGTCGTTAATGAGCGTCG	This Study
HNS Gibson Scramble Fragment 3 R	ACGATGCGTCCGGCGTAGAGCGGTCGCGG GATTGGGAA	This Study
H-NS tetRA F	AGAACAAGGTAAGCAACTGGAAGATTTCC TGATCAAGGAATTAAGACCCACTTTCACAT T	This Study
H-NS tetRA R	CCGCCAGCGGCGGGATTTTAAGCATCCAGG AAGTAAATTACTAAGCACTTGTCTCCTG	This Study
H-NS HA-tag fill-in F	CCGCCAGCGGCGGGATTTTAAGC	This Study
H-NS HA-tag fill-in R	AGAACAAGGTAAGCAACTGGAAGATTTCC TGATCAAGGAAGGAGCCGGAGCCTACCCA TACGATGTTCCAGATTACGCTTAATTTACTT CCTGGATGCTTAAAATCCCGCCGCTGGCGG	This Study
Circ. Perm. F_1 F	CGCTCAAGGCGCACTCCC	This Study
Circ. Perm. F_1 R	GACGGAAACATTTTTAAT	This Study
Circ. Perm. F_2 F	GGATAATGTTTTTTGCGC	This Study
Circ. Perm. F_2 R	CCAAGCTTGCATGCCTGC	This Study
Circ. Perm. F_3 F	TCATAACGGTTCTGGCAA	This Study
Circ. Perm. F_3 R	TCTTCTCTCATCCGCCAA	This Study
Circ. Perm. F_4 F	CTGAAATGAGCTGTTGAC	This Study
Circ. Perm. F_4 R	TTTAATCTGTATCAGGCT	This Study

Circ. Perm. F_5 F	ATCATCCGGCTCGTATAA	This Study
Circ. Perm. F_5 R	ATCAGACCGCTTCTGCGT	This Study
Circ. Perm. F_6 F	GGAATTGTGAGCGGATAA	This Study
Circ. Perm. F_6 R	TGCCGCCAGGCAAATTCT	This Study
Circ. Perm. F_7 F	TCACACAGGAAACAGACC	This Study
Circ. Perm. F_7 R	GTCAGGTGGGACCACCGC	This Study
Circ. Perm. F_8 F	ATTCCCACGCGTGGGTGA	This Study
Circ. Perm. F_8 R	TTCACTTCTGAGTTCGGC	This Study
mlrA IVT vector F	CGTGTCTAGACGCCACGCAGCG	This Study
mlrA IVT vector R	CGTGGTCGACGCCACGTTATCCAACAGGGATCG	This Study
ihfB IVT vector F	CGTGTCTAGAGCGTTGACGGCCTGCTGC	This Study
ihfB IVT vector R	CGTGGTCGACGCCAAAGACTTTGTGATATAACCG	This Study
katE IVT vector F	CTGCGATGTACCCGGGGCGCACTCTCCATCAGTAACGGC	This Study
katE IVT vector R	CGTGTAGCTTAAGCTTCCTGTCTGAAGGGTTAACGG	This Study
spvA IVT vector F	CTGCGATGTACCCGGGAATCGCTTGTCTGCCGGAC	This Study
spvA IVT vector R	CGTGTAGCTTAAGCTTGAGGTCCACATTGTCACCG	This Study
gmk IVT vector F	CTGCGATGTACCCGGGCGCCTTGTAGTCCGTTACCC	This Study
gmk IVT vector R	CGTGTAGCTTAAGCTTGCTTAATGAGGATGACGCG	This Study
purE IVT vector F	GGTGTTCTAGAATGGTTACTTTCCACACTAATCACG	This Study
purE IVT vector R	CGTGTGAATTCTTATTTAAGCTTACTTTGCGC	This Study
ugtL IVT vector F	GGTGTGGATCCTACACCTGCCTGGGCGATGCC	This Study
*ugtL*IVT vector R	CGTGTAAGCTTTCAGGGTCTCTGCTTAACGG	This Study
crl-OE-F	GCAGTGTCGACACGTTACCGAGTGGACACCCG	This Study
crl-OE-R	GAGTCCTCGAGTTATGCCGACAGTTTTACCGGC	This Study
SP64	CTGTAGAATTCATGGCGCTTTACACAATTGG	This Study

SP65	CTGTATCTAGATTAAACGCCAAGGGGATGAATGTCG	This Study
SP100	CGTGAAGCTTTTAAACGCCAAGGGGATGAATGTCG	This Study
SP103	GGAATTCCATATGGCGCTTTACACAATTGGTG	This Study
SP136	GCTGGAATTCCCACGCGTGGGTGAGTTATTAA	This Study
SP137	CAGCGTCGACGGAAACATTTTTAATAACTCAC	This Study
delta-*mlrA*-F	TCTAAAGTTAAACCGGGACCTCGCGAGCAAGGGTGAAACGGTGTAGGCTGGAGCTGCTTC	This Study
delta-*mlrA*-R	TAAAAGGAGTATACATTAAAGCGAATTTGTTAGCTTCTGTCATATGAATATCCTCCTTAG	This Study
delta-*ihfA*- F	TTAAAAGAGCGATTCCAGGCATCATTGAGGGATTGAACCTGTGTAGGCTGGAGCTGCTTC	This Study
delta-*ihfA*-R	TGCCGCAATACACCCTGATGGATGTTATGCCTGGATCTGACATATGAATATCCTCCTTAG	This Study
delta-*ihfB*-F	ACGGCTGCAGCCAATTTGCCTTTAAGGAACCGGAGGAATCGTGTAGGCTGGAGCTGCTTC	This Study
delta-*ihfB*-R	CTGACGGTGCTTTTTTCGGGTTCAAGTTTTGCGTTAAAACCATATGAATATCCTCCTTAG	This Study
delta-*crl*-F	TTGATTTGGTAAAACAGTTGCTTCATTAAAGGAGATCGCAGTGTAGGCTGGAGCTGCTTC	This Study
delta-*crl*-R	TGTGCCTGATGGCGCTGCGCCATCAGGCATGGCAGCAGGGCATATGAATATCCTCCTTAG	This Study

Chapter 3: Role of σ^S as a Transcriptional Regulator and Counter-silencer

Summary

The bacterial sigma factor σ^S is responsible for the selective transcription of a subset of genes involved in adaptation to the stationary phase of growth and stress resistance. However, close relatedness to the housekeeping sigma factor σ^{70} and similar binding site sequences have raised questions regarding the basis of sigma factor selectivity at specific promoters. Some H-NS-silenced genes are transcribed by RNA polymerase (RNAP) associated with σ^S but not with σ^{70}, suggesting that σ^S might serve as a counter-silencer that allows RNAP to overcome H-NS-mediated silencing. If true, this might account for σ^S selectivity at certain promoters co-regulated by H-NS. To determine whether σ^S can directly counter-silence H-NS and whether co-regulation by H-NS accounts for selective σ^S activation of certain promoters, I examined the *spvAB* and *csgBA* promoters, which are known to be regulated by both σ^S and H-NS. Using genetic and *in vitro* transcription assays, I found that σ^S alone is unable to counter H-NS-mediated silencing at these genes. Rather, additional regulatory proteins are required to disrupt H-NS silencing of these promoters.

Introduction

Bacterial transcription in *Salmonella* is initiated by core RNAP (RNA polymerase) coupled with one of five sigma factors: RpoD (σ^{70}), RpoN (σ^{54}, σ^N), RpoH (σ^{32}, σ^H), RpoS (σ^{38}, σ^S) and RpoE (σ^{24}, σ^E). Each sigma factor directs RNAP to a different subset of promoters to alter patterns of gene expression. Formation of holoenzymes with different sigma factors is triggered by environmental cues or facilitated by expression of other cellular factors. Two of the

most prominent sigma factors are RpoD and RpoS. RpoD (σ^{70}) is the housekeeping sigma factor, responsible for transcribing the majority of *Salmonella* genes. RpoS (σ^{S}) is most important during times of environmental stress. This alternative sigma factor allows cellular adaptation to environmental factors such as pH, temperature, oxidative stress and nutrient deprivation (83,84). The latter response is commonly associated with activation of the RpoS regulon, because RpoS levels rise during stationary phase as cells experience starvation conditions. At that time, RpoS directly or indirectly promotes the expression of more than 500 genes in stationary phase, comprising just over 10% of the *Salmonella* genome (85). An RpoS response triggered by any of these stressors provides broad resistance to multiple stress conditions (86).

The mechanism of promoter selectivity for specific sigma factors has been intensively studied. RpoS itself is regulated at multiple levels, including transcription, translation, degradation and activity (for review see (85). RpoS protein is essentially undetectable during exponential phase but levels increase during stationary phase and peak at about 1,600 copies per cell. This is still lower than stationary-phase levels of RpoD (7,000 copies per cell) or RNAP core (typically 2,500 copies per cell) (87). Moreover the affinity of RpoS for core RNAP is five-fold less than that of RpoD (88), raising the question of how $E\sigma^{S}$ effectively competes with $E\sigma^{70}$ at RpoS-regulated promoters.

Currently, there are three general theories for the basis of sigma factor selectivity at regulated promoters: (a) differences in promoter elements (89), (b) changes in promoter topology (90), and (c) participation by other inputs or global regulators to facilitate the formation or promoter interactions of specific RNAP holoenzymes (91) and thereby confer selectivity (92,93). Because RpoD and RpoS share considerable structural homology, promoter recognition sequences for these two sigma factors are very similar. Transcription from the promoters for

many genes are able to be initiated by either sigma factor *in vitro* (90), and *de novo* promoter sequences optimized for *in vitro* expression by $E\sigma^S$ retain the same core promoter elements as those for $E\sigma^{70}$ (94). Subtle structural changes in promoter elements, such as a conserved cytosine at -13 (94) or a more degenerate -35 site (95) can sometimes, but not always, drive a shift toward σ^S selectivity. Topologically, σ^S may have more flexibility regarding the geometric alignments of the -10 and -35 sites (96), creating more tolerance for supercoiling and non-optimal spacer lengths. However, exceptions to this observation occur as well (97).

Nucleoid-associated proteins can promote σ^S-selectivity through a variety of mechanisms. In some instances, activators, such as cAMP-CRP at the *csiD* promoter, are essential and only able to interact with $E\sigma^S$ rather than $E\sigma^{70}$ (98). In other instances, $E\sigma^S$ appears to be more efficient at disrupting transcriptional inhibitors, such as Crp, Lrp and IHF at the *osmY* promoter (92). Many σ^S-dependent genes have been shown to be transcribed independently of σ^S in an *hns* mutant strain, and by either sigma factor *in vitro*. These include many virulence genes such as *csgBA* (99) and *hdeAB* in *E.coli*, (66) and *spv* (68) and *csiD* (98) in *S.* Typhimurium. Based on these observations, it has been suggested that H-NS is a determinant of sigma factor selectivity, owing to a unique ability of σ^S to overcome H-NS mediated silencing (100).

Studies looking to examine the relationship between H-NS and RNAP have suggested multiple possible mechanisms by which H-NS represses transcription. Some observations have indicated that H-NS multimers occlude the promoter and prevent RNAP access (10,101), while others have correlated RNAP occupancy with H-NS binding at more than 65% of H-NS bound promoters (102), suggesting a model in which RNAP may be trapped by H-NS (52,66,103). However, such interactions implicate H-NS binding in a "bridging" mode (104), which occurs under conditions of high magnesium concentrations (51,52). Increasing evidence, however,

suggests that the linear filament or "stiffening" mode of H-NS binding is correlated with transcriptional silencing (53,54).

As the RpoS response is activated under conditions of environmental stress, as during *Salmonella* infection of a host, it seems plausible that RpoS could serve as a counter-silencer to promote the expression of virulence genes silenced by H-NS. Some have even suggested that RpoS promoter selectivity is a specific function of H-NS (85). However, while a number of studies have investigated a possible counter-silencing role for RpoS (66,67,92,98), other researchers have posited that RpoS indirectly stimulates the expression of H-NS-repressed genes by acting upstream in complex regulatory cascades (105).

In this chapter, I describe genetic and biochemical approaches to definitively characterize the role of σ^S-associated RNAP in alleviating H-NS mediated silencing at the *Salmonella spvAB* and *csgBA* genetic loci. Although my observations are inconsistent with the original hypothesis that RpoS directly counters H-NS silencing, I provide experimental support for the concept that RpoS antagonizes H-NS silencing of specific promoters by acting upstream to stimulate the expression of additional regulatory proteins that are responsible for H-NS counter-silencing.

Results

H-NS depletion restores *spvB* expression in *rpoS* mutant *S.* Typhimurium

My initial studies to investigate the potential role of RpoS as a counter-silencer utilized the *spv* operon. The *spv* genes are found in specific non-typhoidal *Salmonella* strains and are arranged in two operons, with the gene encoding a positive transcriptional regulator (*spvR*) transcribed upstream of the *spvABCD* structural genes. The *spv* genes are typically carried on the pSLT virulence plasmid (106), but in some cases, integration into the chromosome has been

noted (107). Expression is required for maximal virulence of *Salmonella* in mice and is induced upon intracellular localization. Both the transcriptional regulator SpvR and RpoS are required for expression (108), and H-NS has been shown to *silence spv* expression (68).

To better understand the co-regulatory relationship between H-NS and σ^S, we performed an *in vivo* genetic analysis of the *spvABCD* operon. Previous studies have found that *S.* Typhimurium *hns* mutants exhibit profound growth defects and rapidly acquire compensatory mutations in *rpoS,* resulting in reduced σ^S activity (56,109). To avoid adventitious compensatory mutations in *rpoS,* we used an IPTG-inducible antisense RNA construct to conditionally deplete *hns* mRNA *in vivo* (74). The paired-termini anti-sense *hns* (asHNS) transcript hybridizes to sequences flanking the ribosome-binding site and start codon of the *hns* promoter, occluding these regions and inhibiting translation (Figure 3.1), allowing inducible control of H-NS protein levels. Although σ^S protein levels also decrease during H-NS depletion, this is likely a result of H-NS acting indirectly as a positive regulator of *rpoS,* a phenomenon that has been observed previously (110,111). To confirm that the asHNS construct was not also directly inhibiting *rpoS* translation, the upstream portion of the *hns* transcript recognized by asHNS was mutated to disrupt asRNA binding but maintain a functional *hns* coding sequence (Supplementary Figure 3.1). H-NS and σ^S protein levels, as determined by immunoblot, were unchanged in strains encoding the mutant *hns* transcript, indicating that the decrease in σ^S levels observed in the wild-type strain is due to indirect regulation via H-NS and not due to a non-specific interaction between the *rpoS* transcript and asHNS.

Levels of the *spvA* transcript were measured during stationary phase in strains containing either the asHNS construct or an asRNA vector control. Experssion of *spvA* is maximal during stationary phase (112). However, depletion of H-NS with asHNS resulted in an additional log-

fold increase in expression (Figure 3.2a), suggesting that under these conditions, H-NS is still able to silence *spv* expression to some extent despite the presence of RpoS. In an *rpoS* mutant, *spvA* expression was decreased by over one-log (Figure 3.2a), confirming that RpoS is required for *spv* transcription. Depletion of H-NS increased transcript levels by 2-fold, but levels of *spvA* transcription remained significantly lower than in wild-type (Figure 3.2a), indicating that σ^S and H-NS have independent roles in the regulation of *spvA* expression. Quantitation of control genes specifically dependent on either RpoS (*katE*) (Figure 3.2b) or H-NS (*pagC*)(Figure 3.2c) by qRT-PCR yielded the expected results.

$E\sigma^S$ is unable to counter-silence *spvAB in vitro*

To understand the direct roles of σ^S and H-NS in the regulation of *spvAB*, the *spvAB* transcriptional circuit was reconstituted *in vitro* from individual purified components on large supercoiled targets, which are likely to be more physiologically and structurally indicative of *spvAB* regulation *in vivo* than the small linear targets typically used in such studies. *In vitro* transcription assays using purified proteins show some target specificity, as H-NS exhibits increased repression for target genes with lower GC content (Figure 3.3a). $E\sigma^{70}$ stimulated higher levels of *gmk* transcription than $E\sigma^S$, whereas the reverse was observed for *katE* transcription (Figure 3.3b), indicating that these assays could detect σ factor promoter selectivity. However, the *spv* promoter did not exhibit a preference for either sigma factor, as levels of *spvA* transcription were similar for $E\sigma^{70}$ and $E\sigma^S$ (Figure 3.3b). To determine whether either RNAP holoenzyme was able to overcome H-NS-mediated silencing at the *spvAB* promoter, IVT assays were performed with either $E\sigma^{70}$ or $E\sigma^S$ on *spvAB* template incubated with increasing concentrations of H-NS. The two σ-factors exhibited nearly identical susceptibility to H-NS

silencing at this promoter (Figure 3.4a), suggesting that σ^S alone does not function as a counter-silencer of H-NS at the *spvAB* promoter.

Based on these observations, we investigated whether σ^S might promote counter-silencing in concert with additional regulatory proteins. As a precedent, the regulatory proteins SlyA and PhoP act in combination to relieve H-NS-mediated repression of the *Salmonella pagC* promoter (10). cAMP-CRP (98) and YncC (113) are also believed to be required for σ^S-mediated counter-silencing at their respective promoters, either by initiating sigma-specific recruitment or by altering the structure of the nucleoprotein complex at the promoter. In the case of *spvAB*, the LysR-family regulator SpvR is a good candidate as it has previously been shown to directly bind the *spvA* promoter region to up-regulate transcription (114) and has also been implicated in σ^S-mediated counter-silencing (68). However, numerous efforts to purify SpvR were unsuccessful, as the native protein was found to be insoluble, so I was unable to confirm this hypothesis *in vitro*. Nevertheless, the *in vitro* analysis of *spvAB* suggested that σ^S by itself is unable to counter-silence the actions of H-NS at this locus.

Eσ^S is unable to counter-silence *csgB in vitro*

I next focused my attention on the *csgBA* promoter. *In vitro* assays again confirmed that σ^S alone was not sufficient to promote counter-silencing (Figure 3.4b). However, at the *csgBA* locus, there are two other regulators in addition to RpoS and H-NS that have a possible regulatory influence, one being Crl (115). Crl was an attractive candidate for participation in sigma-mediated counter-silencing because of its ability to specifically interact with σ^S (116) and promote σ^S-dependent transcription initiation (115). Crl was first identified as a regulator of the *csg* genes that is important for curli fiber production (117) and the rdar morphotype (115). Crl

production is highest at lower temperatures (28˚C), suggesting a potential role as a thermosensor (118) that links expression of the *csgBA* operon to temperature. Although *crl* mutations may decrease the expression of many RpoS-regulated genes (91), the strength of the direct interaction between the two proteins is considered to be relatively low (119), and the impact of Crl on gene expression is variable for different promoters (120,121).

In contrast to *Salmonella* and *E. coli*, many bacterial species that contain *rpoS* do not possess the *crl* gene (122), suggesting that it may have been horizontally acquired and supporting the idea that it may play a particularly important role in the regulation of horizontally-acquired genes such as the *csg* locus. Proposed mechanisms of Crl action include the modification of $E\sigma^S$ positioning to alter DNA-RNAP complex architecture or enhancement of DNA melting by $E\sigma^S$ (116). Either of these activities could effectively serve to disrupt H-NS silencing to allow transcription.

To test this hypothesis, a *crl* mutation was created to determine whether *crl* is necessary for *csgB* gene expression in the presence or absence of H-NS. Expression of *csgB* in wild-type cells occurs during stationary phase (123), but a *crl* mutation was not found to affect *csgB* expression (Figure 3.5a), suggesting that Crl is not required for *csgB* transcription in stationary phase. As *rpoS* mutants are defective for *csgB* transcription in stationary phase, expression was unaffected by a *crl* mutation in this background as expected. An increase in *csgB* transcript levels in stationary phase compared to exponential phase in an *rpoS* mutant, even with H-NS present, suggested that additional factors such as DNA supercoiling may affect H-NS silencing during stationary pahse. However, the introduction of an *hns* mutation made *csgB* expression independent of both σ^S and Crl, enhancing transcription in exponential phase close to 10,000-fold, to a level comparable to that of stationary phase wild-type cells (Figure 3.5a). This suggests

that σ^S and Crl are not required for *csgB* transcription in the absence of H-NS. Furthermore, Crl does not appear to be responsible for counter-silencing of H-NS at the *csgB* promoter.

To further confirm this hypothesis, I incubated purified Crl for *in vitro* reactions with the $E\sigma^{70}$ and $E\sigma^S$ holoenzymes. This experiment verified that Crl does not enhance sigma factor activity or selectivity *in vitro* (Figure 3.5b).

Discussion

During the past two decades, several investigators have suggested that the ability of RpoS to overcome H-NS silencing may represent a mechanism of sigma factor selectivity. However, little direct *in vitro* evidence to support this hypothesis has been presented. My analysis of two different *Salmonella* genetic loci that exhibit regulation by both H-NS and σ^S suggests that RpoS is not in fact a direct counter-silencer of H-NS. My observations have shown that neither sigma factor is capable of disrupting H-NS under silencing conditions (Figure 3.4), and although RpoS is required for *spv* and *csg* gene expression in intact cells (Figures 3.2a and 3.5a), some relief of H-NS-mediated silencing can be observed during stationary phase that is independent of RpoS (Figure 3.5a), and that over 90% of H-NS-regulated genes are independent of σ^S (Supplementary Figure 3.2). This suggests that the involvement of σ^S is most likely independent of H-NS.

Some reports have suggested that only $E\sigma^S$ alone is able to form an open-complex in the presence of H-NS (98), using linear templates that are exclusively σ^S-regulated. Despite evidence that these same promoters in supercoiled templates exhibited equal levels of activation by either sigma factor, the effect of H-NS under these conditions was not studied. Other studies that have utilized supercoiled templates to conclude σ^S-specific counter-silencing have been based on the idea that H-NS can form bridges that can trap $E\sigma^{70}$ but not $E\sigma^S$ (66). However, the

bridging model relies on magnesium concentrations (10 mM) that are well above physiological concentrations (124). Since bridging in this model is the basis for selectivity, it would be curious to see if the same results occur under low magnesium concentrations when H-NS is in a stiffening mode. My study using supercoiled templates at biologically relevant magnesium concentrations (1 mM) has revealed that transcription levels are equivalent with $E\sigma^{70}$ and $E\sigma^{S}$ holoenzymes under these conditions and that H-NS is equally able to silence expression driven by either form of RNAP (Figure 3.4a).

To investigate the possibility of σ^{S} serving as a counter-silencer at other H-NS regulated promoters, and whether this activity could account for the apparent σ^{S}-selectivity of some promoters, i.e., whether $E\sigma^{S}$ is better able than $E\sigma^{70}$ to overcome H-NS-mediated silencing, we looked to the literature to identify genes within the σ^{S}-H-NS co-regulon. Although no studies have specifically compared H-NS, σ^{S}, and σ^{70} binding profiles in log and stationary phase, an analysis of previously published datasets suggests that significant overlap between the H-NS and RNAP-σ^{S} regulons is unlikely. Previous ChIP experiments revealed that H-NS binds approximately 35% of all σ^{S} -dependent genes during exponential phase (125), including the *csg* operon (101). However, many datasets describing the σ^{S} regulon rely on transcriptomic data, which is a poor indicator of actual σ^{S} binding, as they fail to account for cascading downstream effects that reflect indirect actions of σ^{S} (126). ChIP datasets from *E. coli* further reveal that as many as 90% of σ^{S}–regulated genes, including *csgD*, are also bound by σ^{70} to some extent, even during stationary phase (127). With these caveats in mind, we performed a comparative analysis of existing ChIP-chip (56) and transcriptomic datasets (128) from *S.* Typhimurium. Our analysis found that only 1% of all genes exhibit regulation by both H-NS and σ^{S} (Supplementary Figure 3.2). In view of this limited overlap, it appears unlikely that H-NS counter-silencing is a general

characteristic of the σ^S-regulatory network. Further investigation of these genes revealed that nearly all relied on additional transcription factors for transcriptional activation and possible disruption of H-NS (Supplementary Table 3.1). This observation by itself suggests that it is unlikely that H-NS is a major determinant of sigma factor selectivity at most σ^S -regulated promoters. Additional regulatory factors are likely to preferentially promote σ^S-binding (89,92) and to mediate counter-silencing.

Returning to the literature that served as the basis for this investigation, there are also indications that other proteins are involved in H-NS counter-silencing of RpoS-regulated genes. For example, regulatory proteins that bind to the *hdeAB* promoter region, such as GadW/X, are able to overcome H-NS silencing (129,130) of this RpoS-dependent gene (131), suggesting that RpoS acts at an upstream level. Another well-studied LysR regulator, LeuO, has been demonstrated to relieve H-NS repression (132,133), setting a precedent for SpvR-mediated counter-silencing.

The organizations of the *spv* and *csg* operons are similar in terms of having an auto-regulated transcription factor that is responsible for the downstream activation of structural genes. As will be discussed in Chapter 4, the transcriptional regulator of the *csg* structural genes, CsgD, is in fact a counter-silencer of H-NS. It is attractive to speculate that SpvR operates in a similar manner, disrupting H-NS silencing of the structural *spv* genes. In this scenario, RpoS would act upstream at the level of *spvR* transcription, which could account for my observation that H-NS can silence gene expression promoted by either sigma factor in the absence of SpvR. Further *in vitro* studies with purified SpvR protein would elucidate its potential role as a counter-silencer, but this will await technical advances that allow the purification of soluble protein. The basis for σ^S-regulation of the *spvR* promoter will also need to be explored. Although NAPs can

enhance σ^S binding (92), the interactions between RpoS and NAPs are likely to be much more complex, involving upstream actions and additional regulatory factors. Although a more comprehensive analysis of additional promoters will need to be done before H-NS is definitively excluded as a mechanism of σ^S-selectivity, the *in vitro* and transcriptomic analyses presented in this study suggest that this is improbable.

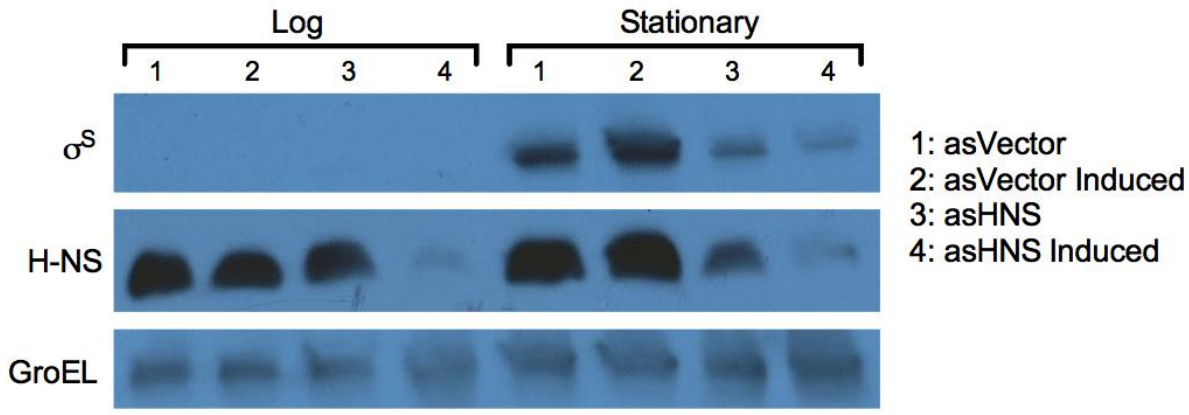


Figure 3.1. Depletion of H-NS using an inducible asHNS construct. H-NS was quantified from exponential and stationary phase cultures of SLN90 containing pHN1009 (asVector) and pHN1009 asHNS, which encodes an asRNA complimentary to the region flanking the ribosome binding site of *hns*. asRNA expression was induced with IPTG for 4 hrs, cells were harvested, and H-NS levels visualized via immunoblot analysis using an anti-HA antibody. σ^S is absent in log phase but increases in abundance during stationary phase. GroEL was detected as a loading control.

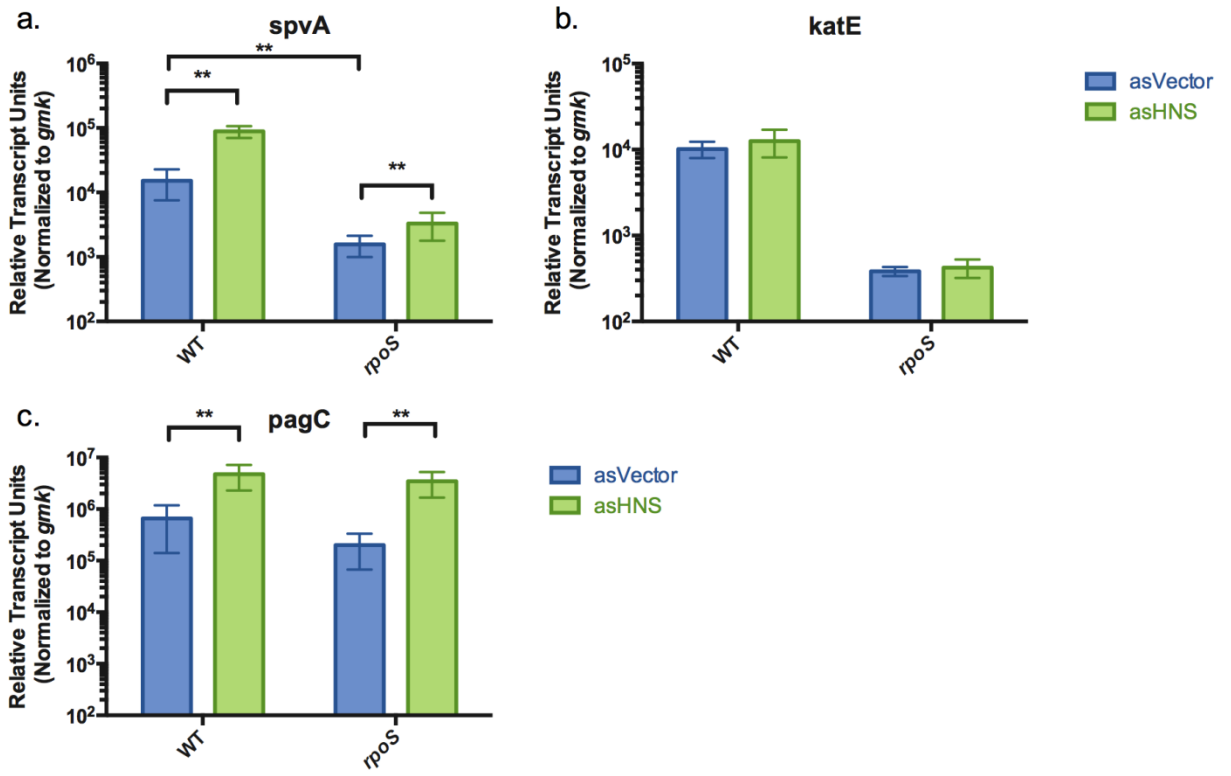
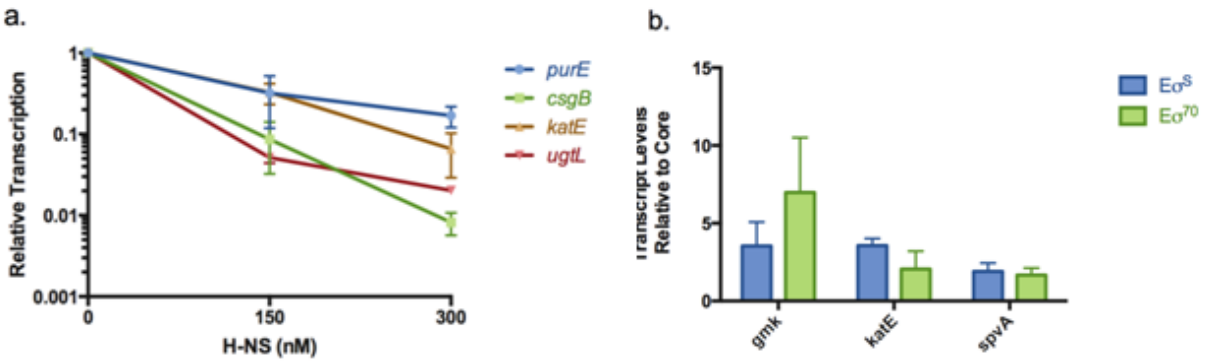


Figure 3.2. H-NS and σ^S play distinct and independent regulatory roles in *spvA* expression *in vivo*. Transcript levels of *spvA* (a), *katE* (b) and *pagC* (c) were quantified using qRT-PCR in wild-type and *rpoS S.* Typhimurium strains containing asVector or asHNS with IPTG added to deplete H-NS. Data are presented as the mean +/-SD of three replicates normalized to *gmk*. ** indicates $p<0.01$ by ratio-paired t-test.



Gene Name	GC-content of Gene	%GC 500bp Upstream	%GC 200bp Upstream
purE	60.2	62.4	62
katE	49.6	53.9	57
ugtL	40.4	35.6	34
csgB	44.3	37.4	41.5

Figure 3.3. *In vitro* transcription assays with different target promoters exhibit regulatory factor specificity. a) Four supercoiled-template targets with various GC-contents were incubated with commercial holoenzyme and tested for H-NS silencing by adding increasing concentrations of H-NS. Genes with known high AT-content that are regulated by H-NS (*ugtL* and *csgB*) exhibit more silencing by H-NS than genes with higher GC-content (*purE* and *katE*). Data are presented as the mean +/-SD of three replicates and normalized to an RNAP-only control.

b) Reconstituted holoenzymes $E\sigma^S$ and $E\sigma^{70}$ both show transcriptional activity *in vitro*. $E\sigma^{70}$ showed higher levels of transcription of the housekeeping gene *gmk*, but $E\sigma^S$ had 2-fold higher transcription of the RpoS-regulated *katE* gene. The RpoS-regulated *spvA* gene exhibited no sigma factor selectivity *in vitro*. Data are presented as the mean +/-SD of six replicates and normalized to RNAP core without a sigma factor. ** indicates $p<0.01$ by ratio-paired t-test.

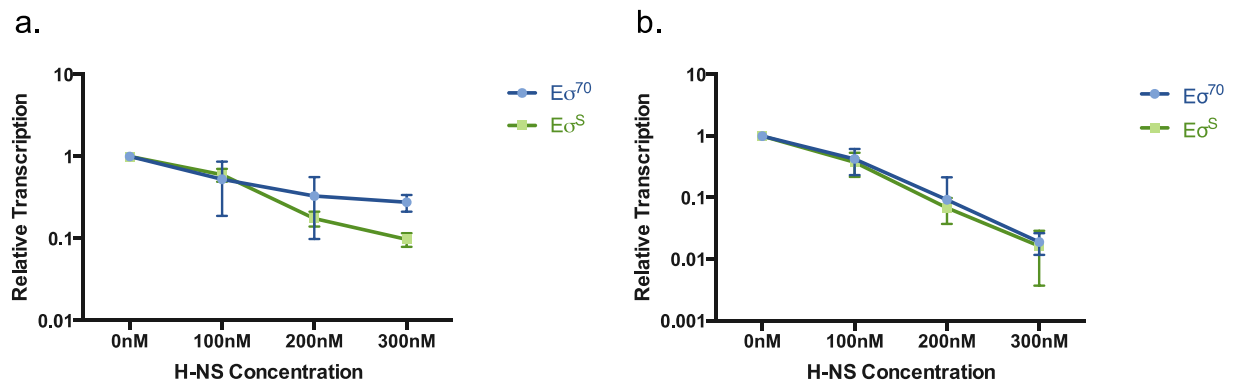


Figure 3.4. Neither Eσ^{S} nor Eσ^{70} show resistance to H-NS silencing *in vitro*. *In vitro* transcription analysis of *spvA* (a) or *csgB* (b) shows that promoters transcribed by Eσ^{70} or Eσ^{S} exhibit concentration-dependent inhibition by H-NS at similar levels. Data are presented as the mean +/-SD of three replicates and normalized to an RNAP-only control.

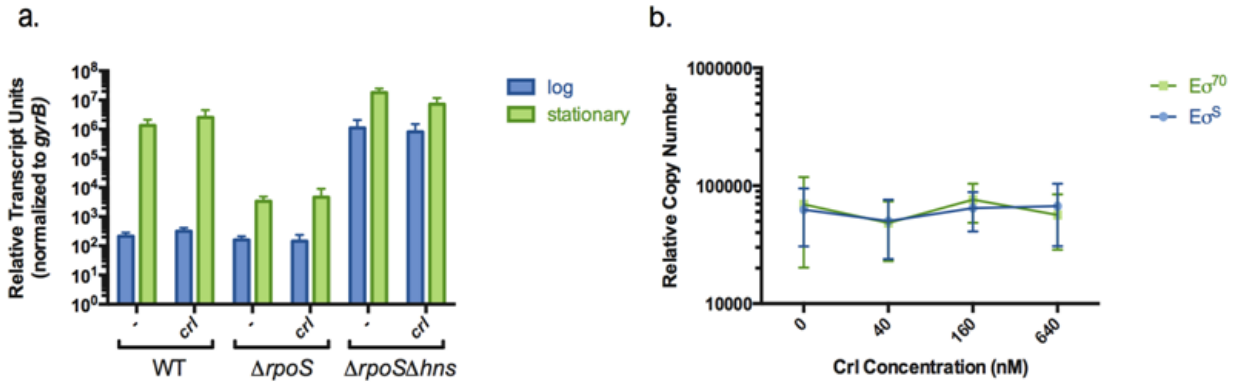
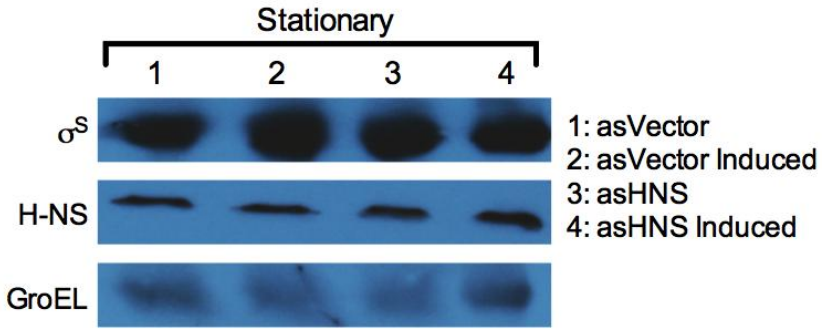
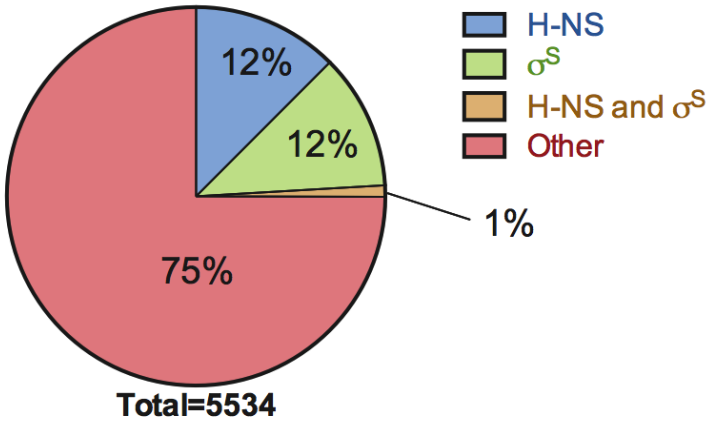


Figure 3.5. Crl does not mediate sigma-specific counter-silencing nor promoter selectivity at *csgB*. a) The *csgB* gene exhibits stationary phase regulation *in vivo* that is reduced in the absence of *rpoS* but unaffected by a *crl* mutation. Introduction of an *hns* mutation results in a 4-log increase in expression during exponential phase that is also independent of *rpoS* and *crl*. b) The addition of purified Crl with either $E\sigma^S$ or $E\sigma^{70}$ *in vitro* does not stimulate *csgB* transcription. Data are presented as the mean +/-SD of three replicates normalized to *gyrB* expression (a) or expressed as relative copy number (b).



Supplementary Figure 3.1. The asHNS transcript does not directly deplete σ^S. To establish that pHN1009 asHNS does not directly target σ^S, the *hns* transcript sequence targeted by asHNS was mutated to disrupt recognition while maintaining *hns* expression. pHN1009 asHNS was induced for 4 hrs and σ^S and H-NS levels visualized via immunoblot. GroEL was used as a loading control.



	# of Regulated Genes
H-NS	691
σ^S	641
H-NS and σ^S	54
Other	4148

Supplementary Figure 3.2. H-NS and σ^S exhibit little regulatory overlap in *Salmonella* Typhimurium. Previous transcriptomic studies of the σ^S (Lévi-Meyrueis, Monteil *et al.* 2014) and H-NS (Navarre *et al.*, 2006) regulons were compared to identify co-regulated genes. σ^S and H-NS each regulate approximately 12% of the genome, whereas only approximately 1% of the genome is co-regulated by both σ^S and H-NS.

Supplementary Table 3.1. The H-NS-σ^S co-regulon in *S*. Typhimurium. Accessory proteins are those found to bind or have binding sites near the promoter based on data from *Salmonella* spp. or *Escherichia coli*. Blank entries indicate genes for which no accessory proteins have been identified or no data exists.

	Accessory Proteins	Function	Supplemental Reference
bcfA	GcvA; Hfq	fimbrial subunit	1, 2
STM14_0341		putative RHS-like protein	
STM14_0421		putative cytoplasmid protein	
yahO		putative periplasmic protein	
ybaJ	CpxR; PdhR (in *E coli*); BasR	modulates Hha toxicity in *E coli*	3,4,5
ybeF	AgrA	Lys-R family transcriptional regulator	1
kdpE		DNA binding transcriptional activator	
STM14_0886, *fumC?*	SoxS, MarA, Crp (*E. coli*), ArcA, Fnr	fumarate hydratase	6,7,8,9,10
ycaM		putative APC transporter	
focA	Crp, IHF, FnR, NarL, ArcA, wrcA (*E. coli*)	formate transporter	11,12,13,14
yccD	Fis	chaperone-modulator protein CbpM	15
STM14_1454	Zur	periplasmic lysozyme inhibitor; possible phage protein	
STM14_1486		putative cytoplasmic protein	
pagC	SlyA, PhoP	virulence membrane protein	20
STM14_1523		putative protein	
STM14_1526		putative response regulator	
STM14_1529		putative cytoplasmic protein	
spy	CpxR, BaeR	Periplasmic stress response chaperone	42
celD	Crp,NagC, ChbR,Cap	DNA-binding transcriptional regulator ChbR	14,43
ydiV	TyrR,SdiA,FliZ	anti-FlhDC factor	1,44,45
STM14_1829		putative cytoplasmic protein	
ydeJ	Fnr (*E. coli*)	competence damage-inducible protein A	16
ydeI	Fnr, RcdA (*E. coli*), Rcs, PhoPQ, PmrAB	important for systemic infection	16,17,18,19
yddX	RcsB (E. *coli*), Fnr	biofilm dependent modulation protein	1,20,21
yncC	YgiV (*E. coli*)	DNA binding transcriptional regulator	22
tyrR	TyrR (*E. coli)*	DNA binding transcriptional regulator	23
osmB	RcsB (*E. coli*), FabR?	osmotically and stress inducible	24,25

		lipoprotein	
yciG	YncC	hypothetical protein	26
yciF	YncC	hypothetical protein	26
yciE	YncC	hypothetical protein	26
STM14_2094	TyrR, CyaR small rna (*E. coli*)	putative catalase	1,27
ftnB	CpxR (*E. coli*)	ferritin B	3
STM14_3108	TyrR, Crp	putative cytoplasmic protein	1
STM14_3193		hypothetical protein	
hin		DNA invertase	
ygaU	CpxR (*E. coli*)	uncharacterized protein containing LysM domain	28
yqaE	CpxR, CyaR (*E. coli)*	Membrane transporter	27,29
nrdF	Fur, NrdR, IscR (*E. coli)*	ribonucleoside-diphosphate reductase 2, beta subunit,	30,31
luxS	GcvA (*Salmonella*), CyaR small RNA (*E. coli*)	S-ribosylhomocysteine lyase/auto-inducer production	1,27
mscL		large conductance mechanosensitive channel protein	
yhjY		lipase	
yiaG	Crp	Transcriptional regulator	1
yibL		conserved cytoplasmid protein	
fidL		Putative Inner Membrane Protein	
marT		Transcriptional Regulator of the ToxR family	
lsrR	LsrR, Crp	Transcriptional Regulator of the SorC family	32
lsrD	LsrR, Crp	Autoinducer-2 ABC transporter - membrane subunit	32
lsrA	LsrR, Crp	AI-2 ABC transporter - ATP binding subunit	32
lsrB	LsrR, Crp	Autoinducer-2 ABC transporter - periplasmic binding protein	32
metJ	Fur, MetJ	Transcriptional Repressor	33,34
STM14_5188		AraC-type DNA binding protein	
spvA	SpvR	Plasmid Virulence Gene	35
slrP	RstA, PhoP, SsrB, HilD	E3 ubiquitin-protein ligase	36,37,38
dps	MntR, Fis, OxyR, IHF, LysR	DNA starvation/stationary phase protection protein	1,39,40,41

Chapter 4: CsgD Serves as a Transcriptional Regulator and Counter-Silencer at the *csgB* locus

Summary

The *csg* locus required for curli fimbrial production in *Salmonella* and *E. coli* is stringently regulated under conditions of bacterial stress. With a primary role in environmental survival and host colonization, the transcriptional activation of this locus depends on σ^S and CsgD, whereas repression is mediated by the nucleoid-associated protein H-NS. In the previous chapter, I have shown that σ^S is not responsible for direct H-NS antagonism. In this chapter, I describe my investigations of the role of the LuxR-family transcriptional activator CsgD in disrupting H-NS. Using genetic and *in vitro* biochemical analyses, I have found that σ^S promotes *csgBA* expression via an indirect mechanism upstream of CsgD regulation. I further show that CsgD directly counter-silences the *csgBA* promoter by altering the structure of the nucleoprotein complex to disrupt H-NS-mediated silencing, in addition to directing the binding and stabilization of the RNAP open complex.

Introduction

The *csg* locus in *Salmonella* is a horizontally-acquired region (80) that encodes the production of curli fimbriae, which are important for both virulence in mice and bacterial survival in the environment (134). Curli are produced by many enteric pathogens, including many *Escherichia* and *Salmonella* spp., and promote community behavior and host colonization. In the environment, curli are important for adhesion to surfaces, cell aggregation, and biofilm formation, and are induced under conditions of low temperature and osmolarity, as well as in stationary phase (67). Organized as two divergently transcribed operons, the *csg* region consists

of *csgBA*, which encodes the major structural subunits of the fibrous polymers, and *csgDEFG*, which encodes the major biofilm transcriptional regulator (CsgD) and the protein components important for secretion and assembly of the curli structures (CsgEFG). While regulation of this operon has been studied extensively in *E. coli*, the binding pattern of CsgD has been noted to be very different between *E. coli* and *Salmonella* (135), despite high conservation of the immediate upstream regulatory sequences. An intergenic alignment between *E. coli* and *S.* Typhimurium shows that only 70% of the residues are conserved, with much of the divergence located further upstream into possible H-NS nucleation sites (Supplementary Figure 4.1), warranting additional study to compare genetic regulatory mechanisms between the two bacterial species.

The *csg* genes are regulated by the alternative sigma factor σ^S (Olsen *et al.*, 1993) but are also regulated by the LuxR-family transcriptional regulator CsgD and the nucleoid-associated silencing protein H-NS. Given that neither σ^S nor Crl has been implicated in H-NS counter-silencing at *csgB* (Chapter 3), it seems possible that CsgD plays a role in regulating *csgB* expression in the presence of H-NS. Disruptive counter-silencing by transcriptional regulators involves the remodeling of the H-NS-DNA complex to allow RNAP binding or transcript initiation and elongation (136). While H-NS binds non-specifically to many promoters, sequence-specific DNA-binding proteins such as CsgD have in some cases been shown to competitively antagonize H-NS. Such regulators typically exhibit consensus recognition sites that are relatively AT-rich (137,138) to coincide with AT-rich H-NS nucleation sites.

Mechanisms of counter-silencing by transcriptional activators can be grouped into three categories. The first involves competitive binding to recognition motifs by more specific regulatory proteins, leading to H-NS displacement from the DNA. This is observed with the H-NS homolog Ler. The binding affinity of Ler for the Lee5p promoter is 40-fold greater than that

of H-NS, so Ler is able to displace H-NS directly (139). Other proteins, such as ToxT, may indirectly displace H-NS by binding to high-affinity upstream sites and oligomerizing to lower affinity downstream sites to disrupt H-NS-DNA complexes (140). A second mechanism involves the structural alteration of a promoter to disrupt H-NS filament formation. For instance, VirB in *Shigella flexneri* binds and bends DNA around itself, disrupting H-NS nucleoprotein filaments (141). LeuO binding creates a physical barrier to H-NS oligomerization, indirectly inhibiting the ability of H-NS to silence transcription of the *ompS1* gene (142,143). A third mechanism by which transcriptional regulators can mediate counter-silencing is through stabilization of the RNAP complex. PhoP and SlyA, for instance, work cooperatively to distort the DNA in a way that stabilizes the RNAP open complex (10). Transcriptional elongation by RNAP may also be required to disrupt downstream H-NS filaments or bridging interactions (104,144). Transcriptional regulators may exhibit dual activities, as is observed with ToxT, which both counters H-NS silencing by competing for binding and stimulates RNAP recruitment to activate gene expression (140).

In this study, we used genetic and biochemical approaches to characterize the mechanism of H-NS-mediated silencing at the *csgBA* promoter and investigate the role of CsgD and σ^S-associated RNAP in alleviating this silencing. Contrary to some previous studies, we found that counter-silencing at this promoter is σ^S-independent, as the formation of an H-NS filament occludes RNAP binding, regardless of the associated sigma factor. The requirement for σ^S in curli expression is more likely to be at a level upstream of the *csgBA* operon, as previously suggested (105). Rather, it is the curli transcriptional regulator CsgD that disrupts H-NS-mediated silencing through the formation of a sharp bend in the promoter DNA, altering the structure of the H-NS-DNA filament and allowing directed binding of RNAP. This finding

highlights a novel counter-silencing protein, supports DNA bending as a fundamental mechanism of counter-silencing, and adds to a growing body of evidence suggesting that counter-silencing is a predominant mechanism of transcriptional regulation in bacteria.

Results

H-NS depletion restores *csgB* expression in *rpoS* and *csgD* mutant *S.* Typhimurium

The *csgBA* operon encoding curli fimbrial production is regulated by both H-NS and σ^S. To better understand the co-regulatory relationship between H-NS and σ^S, we performed an *in vivo* genetic analysis of the *csgBA* operon using an IPTG-inducible antisense RNA construct to deplete H-NS as above. The *csgB* transcript levels were then measured in strains containing either the asHNS construct or an asRNA vector control to examine the relationship between H-NS, σ^S, and CsgD. During exponential growth, *csgB* expression is low and unaffected by the mutation of either *rpoS* or *csgD* (Figure 4.1a), neither of which are normally expressed under these conditions (Figure 3.1) (145). However, in stationary phase, both *rpoS* and *csgD* are up-regulated (145), resulting in over a 350-fold increase in *csgB* expression relative to exponentially growing cells. Both σ^S and CsgD appear to be required for this growth phase-dependent expression, as mutation of either *rpoS* or *csgD* resulted in decreased *csgB* transcript levels similar to those observed in exponential phase (Figure 4.1b). However, the depletion of H-NS with asHNS resulted in a 4- to 6-fold increase of *csgB* transcript levels during exponential phase and a 10- to 21-fold increase during stationary phase in *rpoS* and *csgD* mutant strains, respectively, suggesting that these proteins oppose H-NS-mediated regulation of *csgB*. In wild-type cells during stationary phase, depletion of H-NS had no effect because both σ^S and CsgD were present and silencing was disrupted. Although a moderate decrease in *csgB* expression was

observed under these conditions, this is likely due to decreased concentrations of σ^S resulting from H-NS depletion (Figure 3.1). The regulatory phenotypes of the *hns*, *rpoS*, and *csgD* mutant strains were confirmed by quantifying the expression of genes regulated by either H-NS alone (*pagC*) or σ^S alone (*katE*) by quantitative RT-PCR (Supplementary Figure 4.2).

The comparable levels of *csgB* expression in *rpoS* and *csgD* mutants and the restoration of *csgB* transcription to similar levels by asHNS suggests epistatic regulation of *csgB* by σ^S and CsgD. Although σ^S regulates *csgD* (Figure 4.2a)(145), it is unknown whether σ^S-mediated regulation of *csgB* occurs directly at the *csgB* promoter or indirectly via *csgD*. To experimentally uncouple *csgD* and *rpoS*, and to determine the regulatory role of CsgD independent of *rpoS*, the plasmid pCsgD, expressing *csgD* from a constitutive promoter was transformed into wild-type and *rpoS* mutant strains. Wild-type cultures containing pCsgD exhibited a 30-fold increase in *csgB* transcription during stationary phase (Figure 4.2b) as a result of increased *csgD* expression from the multicopy plasmid (Figure 4.2a)(146). In an *rpoS* mutant, *csgB* transcription decreased but was restored to wild-type levels in strains containing pCsgD, suggesting that σ^S is required for CsgD synthesis but not directly required for *csgB* transcription. H-NS depletion in an *rpoS* mutant did not result in any further increase in *csgB* expression in strains containing pCsgD (Figure 4.2b), suggesting that CsgD is able to completely alleviate H-NS-mediated silencing.

H-NS depletion restores RDAR phenotypes in an *rpoS* mutant

Expression of curli, along with cellulose and other polysaccharides, results in the formation of RDAR (red, dry, and rough) colonies in many *Salmonella* strains under conditions of low temperature (30°C) and osmolarity, as part of a multicellular stress response (147). The RDAR phenotype is dependent on the expression of a functional *csg* region. Wild-type cultures

are typically unable to form RDAR colonies at 37°C, but cultures expressing the asHNS construct at 37°C exhibit a partial RDAR phenotype (Figure 4.3a), suggesting that the temperature-sensitivity is a product of H-NS-mediated regulation. Although the colony structure of the asHNS strain is distinct, this may reflect a decrease in overall fitness due to the prolonged absence of H-NS, altering multicellular behavior, rather than a specific function of H-NS (56). *rpoS* mutant cells are unable to form RDAR colonies (Figure 4.3a). However, depletion of H-NS resulted in a rough phenotype in an *rpoS* mutant after three days at 30˚C, and a partial restoration of the RDAR phenotype at 37˚C.

Cultures expressing *csgD* constitutively from pCsgD were not temperature-sensitive, forming RDAR colonies at both 30°C and 37°C, as previously described (Kader *et al.*, 2006), and pCsgD partially rescued the RDAR phenotype in *rpoS* strains, even in the presence of H-NS (Figure 4.3b), suggesting that CsgD is a critical determinant of RDAR colony formation. However, none of the strains tested were capable of fully complementing an *rpoS* mutation and forming wild-type RDAR colonies, indicating that additional σ^S-dependent factors contribute to RDAR morphology.

CsgD alone can counter-silence *csgB in vitro*

To understand the direct roles of σ^S, H-NS, and CsgD in the regulation of *csgB*, the *csgB* transcriptional circuit was reconstituted *in vitro* from individual purified components on large supercoiled targets, which are likely to be more physiologically and structurally indicative of *csgB* regulation *in vivo* than the small linear targets typically used in such studies. *In vitro* transcription assays with both $E\sigma^{70}$ and $E\sigma^S$ indicated that the *csgB* promoter does not exhibit any basal σ-factor selectivity, as both holoenzymes transcribed *csgB* at similar levels (Figure

4.4), an observation that has been reported for other σ^S-dependent promoters (148,149). They both exhibit nearly identical susceptibility to H-NS silencing (Figures 4.4 and 3.4b). However, when purified CsgD was incubated with the H-NS silenced template, transcription increased 4-fold with $E\sigma^S$ and 8-fold with $E\sigma^{70}$ (Figure 4.4), suggesting that counter-silencing at *csgB* is dependent on CsgD rather than σ^S.

Biochemical analysis of CsgD-mediated counter-silencing

To understand the structure of the nucleoprotein complexes involved in silencing and counter-silencing of *csgB*, Differential DNA Footprint Analysis (DDFA), a highly sensitive quantitative method for analyzing DNA-protein interactions and DNA structure in footprinting experiments, was performed (10). This approach utilizes large targets and signal normalization to facilitate the analysis of DNA interactions with proteins such as H-NS that bind DNA in a relatively non-specific manner, which is not possible with conventional footprinting methods. Supercoiled *csgB* target was incubated with H-NS, as in the IVT studies described above, and the resulting complexes were analysed by DNase I footprinting. Analysis of the anti-sense strand revealed that although H-NS binds extensively along the *csgB* promoter region, two regions of concentrated binding are evident. The first is located upstream of the promoter, ranging from position -93 to -76 relative to the transcriptional start site (TSS), while a second region is detectable near the RNAP binding site from -37 to -5 (Figure 4.5a, Supplementary Figure 4.3b). These locations may represent sites of H-NS nucleation, and indeed, exhibit AT-rich sequences with some similarity to a previously defined high-affinity binding site (150). The absence of hypersensitive sites on either strand (Figure 4.5a, Supplementary Figure 4.5a), indicated by large upward peaks on DDFA plots and representative of DNA bends or distortions, suggests that H-

NS binds the *csgB* promoter in a "stiffening" mode to form a nucleoprotein filament, corroborating evidence that H-NS forms filaments at low magnesium concentrations (54).

Observed protected sites at -20 to -25 and -86 on the anti-sense strand (Figure 4.5b) and at -40 and -60 on the sense strand (Supplementary Figure 4.5b) suggest the locations of the CsgD footprint, which align more closely to previous findings in *E. coli* (135) than had been predicted in *Salmonella* (151). Binding closer to the promoter region introduced a hypersensitive site at position -33 (Figure 4.5b, Supplementary Figure 4.3c), suggesting that CsgD bends the DNA upon binding upstream of the *csgB* promoter. This is significant, as recent studies of SlyA-PhoP-mediated counter-silencing suggested that DNA-bending disrupts the H-NS-DNA filament to alleviate silencing (Will, *et al.,* 2014). The induction of a bend was confirmed by electrophoretic mobility shift assays (EMSAs) with circularly permutated DNA fragments (152). The differential gel mobility of identically sized CsgD-bound framents with a binding site situated in the middle or at the ends demonstrated a bend, as a central bend results in increased distortion and slower migration compared to fragments with a peripheral bend. The ratio of the mobility shift exhibited by the fragment with a central site (F_4=μ_M) compared to the fragment with a peripheral site (F_8=μ_E) was 0.58 (Supplementary Figure 4.4), corresponding to a bend angle of approximately 108° (153).

CsgD and H-NS are able to concomitantly bind the *csgB* promoter, which can be discerned from the fact that the combined footprint of an H-NS + CsgD complex is distinct from that of each individual protein (Figure 4.5). Binding of CsgD to H-NS-bound DNA produces the same hypersensitive site as is observed with CsgD alone (Figure 4.5e). CsgD binding between -20 and -25, and near -86 (Figure 4.5b), overlaps both potential H-NS nucleation sites, suggesting that CsgD may disrupt filament formation through direct competition

for binding at H-NS nucleation sites. Although H-NS binding was also perturbed at other positions, such as -75 and -7, these positions exhibited significant variability, suggesting that the H-NS-CsgD-DNA complexes at these locations are unstable, perhaps as a result of a disrupted nucleation site. As we have observed at other promoters (10), most H-NS was not displaced despite profound changes in the architectural structure of the promoter introduced by CsgD, particularly upstream of the transcriptional start site at -5 and adjacent to the RNA polymerase binding site, at -25 (Figure 4.5e), indicating that the structure of the H-NS-DNA complex, rather than simple binding, is a critical determinant of H-NS activity. This is not entirely unexpected, as other studies have recognized that H-NS may remain bound to DNA during counter-silencing conditions, and the force imparted by RNAP elongation is sufficient to overcome bridging interactions (104,144,154).

Differential DNA Footprinting Analysis of the sense strand demonstrated CsgD binding at previously described recognition motifs near -60 (Supplementary Figures 4.5b and 4.6c), again in a pattern more similar to that observed in *E. coli (135)* than what had been predicted in *Salmonella* (151). H-NS is able to protect this strand at the -10 and -35 regions (Supplementary Figures 4.5a and 4.6d) but is displaced in the presence of CsgD, as reflected by the positive change at these sites when CsgD and H-NS are both present as compared to H-NS alone (Supplementary Figure 4.5c). However, the most striking distortion still appears to take place on the anti-sense strand, as noted above.

To investigate the mechanism of counter-silencing, $KMnO_4$ footprinting was performed, which identifies sites of open-complex formation through reaction with single-stranded DNA exposed by RNAP binding (155). H-NS silencing of the *csgB* promoter via an occlusion mechanism would be expected to result in the disappearance of an open complex upon H-NS

addition. However, silencing by a trapping mechanism would be expected to still show an open complex following the addition of H-NS, and silencing would result from abortive transcription (104). The addition of RNAP holoenzyme alone to the *csgB* promoter produced a stable open complex (Figure 4.6a, Supplementary Figure 4.7b), indicating that this promoter is transcriptionally competent. However, in the absense of other proteins, the open complex formed approximately 50 bp upstream of the usual transcription start site (99). This open complex was abrogated by the addition of H-NS (Figure 4.6b, Supplementary Figure 4.7e), suggesting that H-NS silences the *csgB* promoter by occlusion rather than by bridging and trapping (52). DNase I footprinting showed that RNAP is unable to bind in the presence of H-NS, particularly in the -20 region (Supplementary Figure 4.8b), eliminating the possibility that H-NS is able to bind but unable to form an open complex. The addition of CsgD restored the ability of RNAP to form an open complex in the presence of H-NS (Figure 4.6b, Supplementary Figure 4.7f) at a location corresponding to the usual transcription start site. RNAP also forms an open complex at this location with CsgD in the absence of H-NS (Figure 4.6a, Supplementary Figure 4.7d), suggesting that CsgD recruits RNAP to this site. These observations support the hypothesis that CsgD alters the H-NS nucleoprotein complex to allow RNAP binding and generate a transcriptionally competent promoter.

Discussion

Previous studies have shown that regulation of the *E. coli csgBA* curli fimbrial operon involves the interplay of several transcription factors, including H-NS, CsgD, and σ^S, with σ^S required for expression *in vivo* (67). In this study, we demonstrate that CsgD acts as a counter-silencer of H-NS in *S.* Typhimurium, remodeling the H-NS-DNA complex at the *csgB* promoter

to restore RNAP binding, whether associated with σ^S or σ^{70}, and that the specific requirement for σ^S is indirect and upstream of *csgD* expression. In addition, as is typical for a classical activator, CsgD directs RNAP binding to facilitate open complex formation at the *csgBA* promoter. These findings provide further evidence that counter-silencing is a major mechanism of transcriptional regulation in bacteria.

In agreement with previous studies (99), we found that *csgBA* expression is dependent on σ^S *in vivo*, but becomes σ^S-independent following depletion of H-NS by asHNS (Figure 4.1). Similarly, *csgBA* transcription is reduced in a *csgD* mutant but is restored by depletion of H-NS. Furthermore, transcription from the *csgBA* promoter becomes σ^S-independent in the absence of H-NS, as well as when *csgD* is constitutively expressed (Figure 4.2). Although previous studies suggested that H-NS is unable to silence transcription in the presence of σ^S (66), our data indicate that H-NS is able to repress σ^S-bound promoters both *in vivo* and *in vitro*. This suggests a direct requirement for CsgD, rather than σ^S, to mediate counter-silencing and drive transcription of the *csgBA* promoter. σ^S activates a feed-forward regulatory loop at the *csg* operon, in which σ^S either directly or indirectly stimulates expression of *csgD* at the onset of stationary phase (145), and in turn CsgD enhances expression of σ^S indirectly through transcriptional activation of *iraP*, which stabilizes σ^S (Gualdi *et al.* 2007). The apparent σ^S-dependence of *csgB* may be attributable to the σ^S-CsgD feed-forward loop. Correspondingly, the lack of σ^S is analogous to the absence of CsgD, abolishing transcription of *csgBA*.

Our DNase I footprinting DDFA studies (Figure 4.5) are consistent with H-NS nucleation at high affinity "TpA" steps and oligomerization along lower affinity regions flanking the *csgBA* promoter (54,156), beginning upstream of the RNAP binding site and extending into the coding region, likely resulting in the occlusion of RNAP binding (95). DDFA plots of CsgD binding at

csgBA revealed that CsgD binds immediately upstream of the TSS, competing with H-NS nucleation sites and inducing a sharp bend in the H-NS-bound DNA at the promoter, a structural change that is likely to disrupt H-NS-DNA filament formation (10,136). Previous studies have suggested that CsgD functions as a classical activator due to its binding site overlapping the -35 box of the RNAP binding site (157), and our observation of directed open complex formation in the presence of CsgD supports that hypothesis. However our observations also suggest that CsgD-mediated counter-silencing is essential for *csgB* expression.

There appear to be additional uncharacterized requirements for CsgD-mediated regulation at some promoters, as CsgD is reported to regulate other genes, including its own promoter (145), but is unable to counter-silence or activate its own promoter *in vitro* (Supplementary Figure 4.9). A likely explanation is that an additional factor acts as a co-counter-silencer with CsgD. However, the identity of potential co-counter-silencers at *csgD* is presently unknown.

CsgD is a member of the LuxR-family of transcriptional regulators, which have been shown to be involved in biological processes including quorum sensing, virulence, motility, plasmid transfer, and biofilm formation in a variety of bacterial species (158). Previous studies have suggested that LuxR-family members can serve as either direct activators or repressors of transcription (159,160), but there is also a precedent for LuxR and its homologs to serve as counter-silencers. In *Vibrio* species, LuxR acts in conjunction with σ^S at the *lux* bioluminescence genes to relieve H-NS silencing, as neither LuxR nor σ^S are necessary for *lux* expression in an *hns* mutant (161,162). In *Pseudomonas aeruginosa*, the LuxR regulators RhlR and LasR promote stationary-phase expression of *lecA*, which is silenced by the H-NS functional homologue, MvaT (163), and in other enteric bacteria, LuxR regulators such as BglJ (found in *Salmonella*, *E. coli*,

and *Shigella*) (164) and EcpR (found in both enteropathogenic and enterohemorrhagic *Escherichia coli)* (165) have also been found to disrupt H-NS silencing. The recognition sequences of LuxR-family proteins tend to be degenerate in nature, allowing them to recognize a variety of foreign promoters (166,167). Additional studies have suggested that, like CsgD, LuxR bends DNA upon binding (Stevens, Dolan *et al.*, 1994), which might allow it to disrupt the H-NS-DNA filament and function as a counter-silencer. In view of these observations, the LuxR-family transcriptional regulators may function primarily as counter-silencers rather than classical activators. As this family of transcriptional regulators can be found in many bacterial species, counter-silencing by these proteins may be a conserved mechanism to incorporate horizontally acquired genes into host genomes.

In summary, we have demonstrated for the first time that CsgD induces distinct structural changes at the *csgBA* promoter and promotes transcription by disrupting H-NS silencing and directing RNAP binding and open complex formation. As σ^S does not act as a counter-silencer of *csgBA* expression, the basis for the σ^S-dependence of the curli operon is presently unclear. A role of σ^S in *csgD* regulation has not been excluded. However, given the number of additional regulators at the *csgD* promoter (145), the explanation is likely to be more complex and awaits further investigation, as will be discussed in Chapter 5.

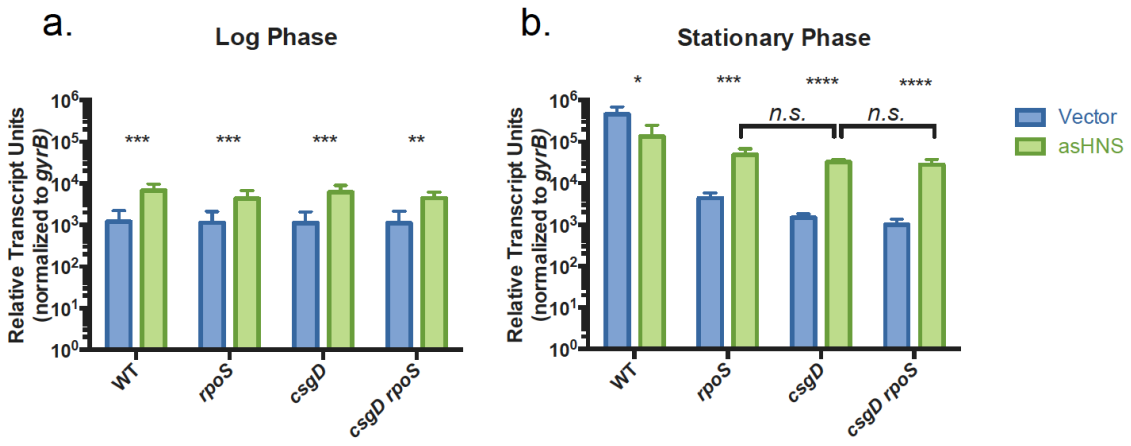


Figure 4.1. CsgD and σ^S up-regulate *csgB* in stationary phase. *csgB* transcript levels were quantified by qRT-PCR during a) exponential or b) stationary phase in wild-type, *rpoS*, *csgD*, and *rpoS csgD S.* Typhimurium strains containing pHN1009 or pHN1009asHNS, with IPTG added to deplete H-NS. Data are presented as the mean +/- SD of three replicates normalized to *gyrB*; * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, and **** indicates $p<0.0001$ by ratio-paired t-test.

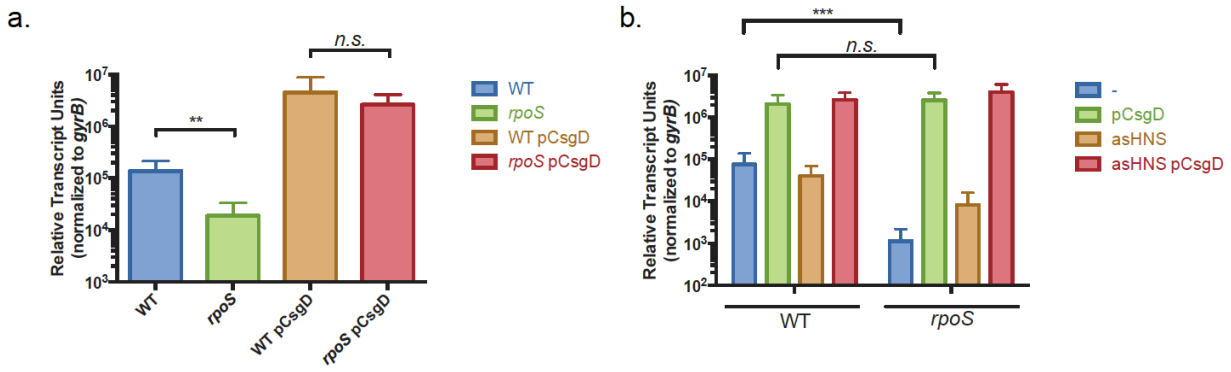


Figure 4.2. Constitutive *csgD* expression *in trans* stimulates σ^S-independent *csgB* transcription. a) *csgD* transcript levels were quantified using qRT-PCR in wild-type and *rpoS S.* Typhimurium containing pCsgD, which contains *csgD* downstream of a constitutive promoter. b) *csgB* transcript levels were quantified in response to *csgD* constitutive expression in strains containing both pCsgD and pHN1009asHNS. Data are presented as the mean +/- SD of three replicates normalized to *gyrB* expression; ** indicates $p<0.01$, and *** indicates $p<0.001$ by ratio-paired t-test.

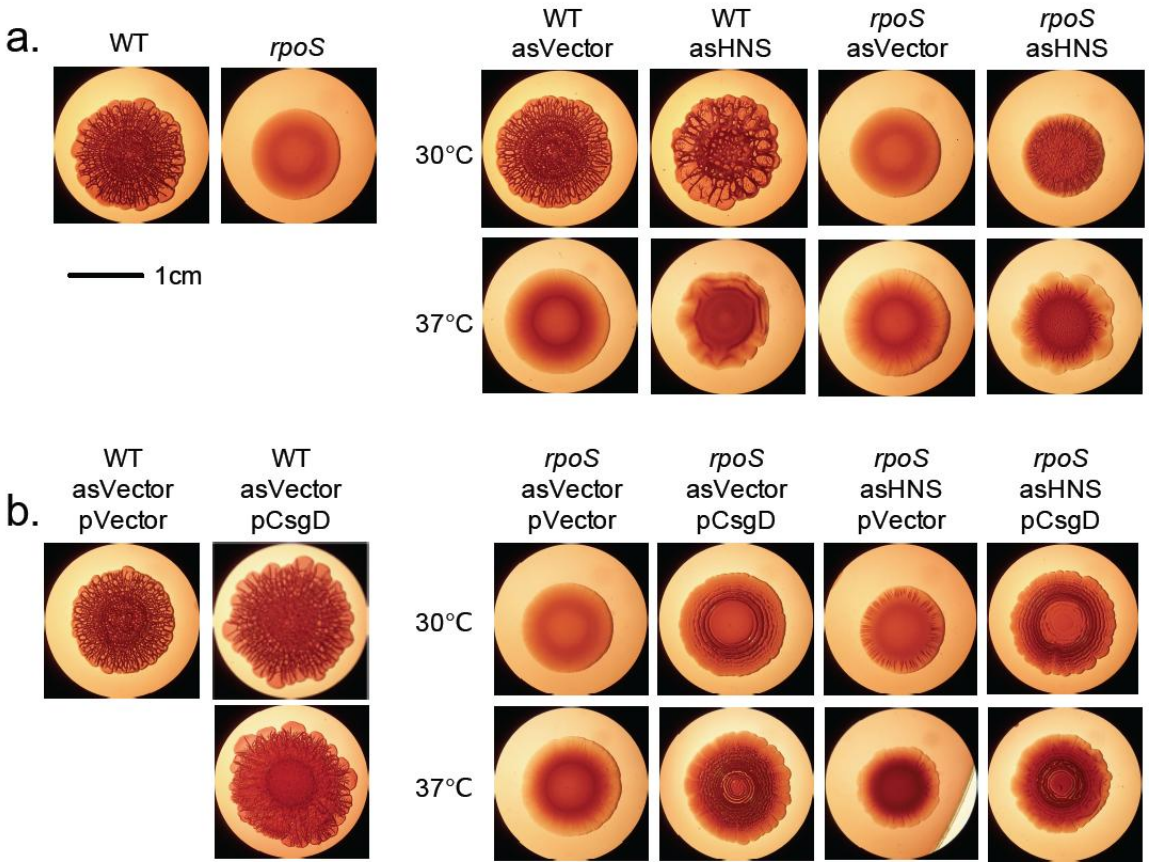


Figure 4.3. Depletion of H-NS restores biofilm formation in σ^S-deficient *S.* Typhimurium.

a)Wild-type and *rpoS* strains of *S.* Typhimurium were spotted on Congo Red plates and incubated at 30 or 37° C to determine their RDAR phenotypes. b) *rpoS* strains containing pCsgD exhibited enhanced RDAR morphology in an *rpoS* mutant when compared to an *rpoS* strain containing the control vector.

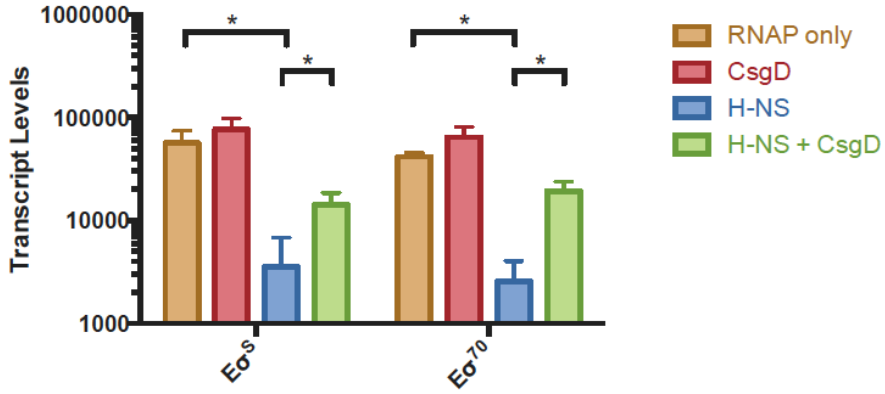


Figure 4.4. CsgD counter-silences the *csgB* promoter *in vitro*. The *csgB* transcriptional circuit was reconstituted *in vitro* using purified, supercoiled DNA template containing a 3kb-region of DNA surrounding the *csgB* promoter and purified protein components. Either $E\sigma^S$ or $E\sigma^{70}$ can initiate transcription of the *csgB* promoter *in vitro*. CsgD-mediated counter-silencing was assayed by incubating the *csgB* template in the presence of 50 nM CsgD before adding 130 nM H-NS. Transcript levels are expressed as relative copy number. Data are presented as the mean +/- SD of three replicates; * indicates $p<0.05$ by ratio-paired t-test.

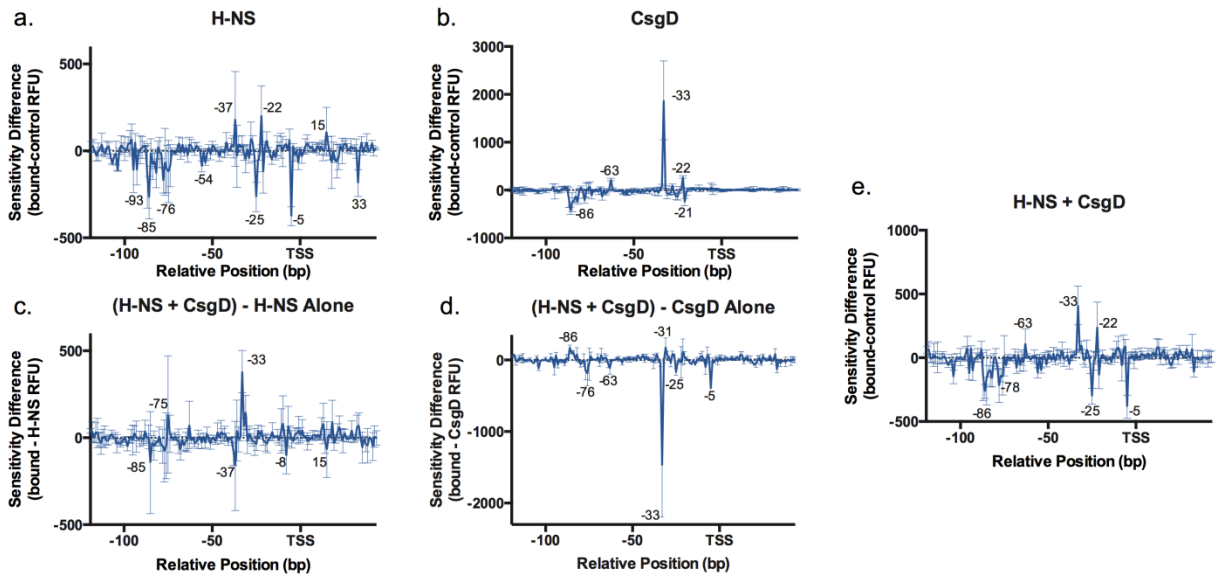


Figure 4.5. Anti-sense strand DNaseI Differential DNA Footprint Analysis (DDFA) of the *csgB* promoter region. *In vitro* DNase I footprinting at the *csgB* promoter was performed with 130 nM H-NS and 50 nM CsgD, as indicated. Peaks are regions of hypersensitivity, which are suggestive of distorted or bent DNA, whereas valleys indicate sites of protection. Base positions are indicated relative to the TSS. a) H-NS, b) CsgD, or e) CsgD and H-NS were added to the *csgB* promoter and the fluorescent peak height following DNase I cleavage determined relative to the protein-free control in relative fluorescent units (RFU). The differences compared to the H-NS control (c) and the CsgD control (d) were also analyzed. Data are presented as the mean +/- SD of three replicates.

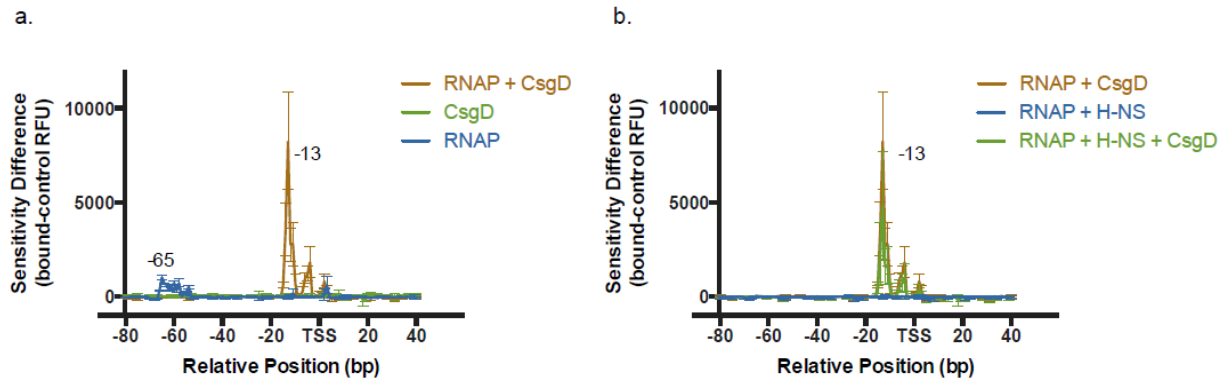
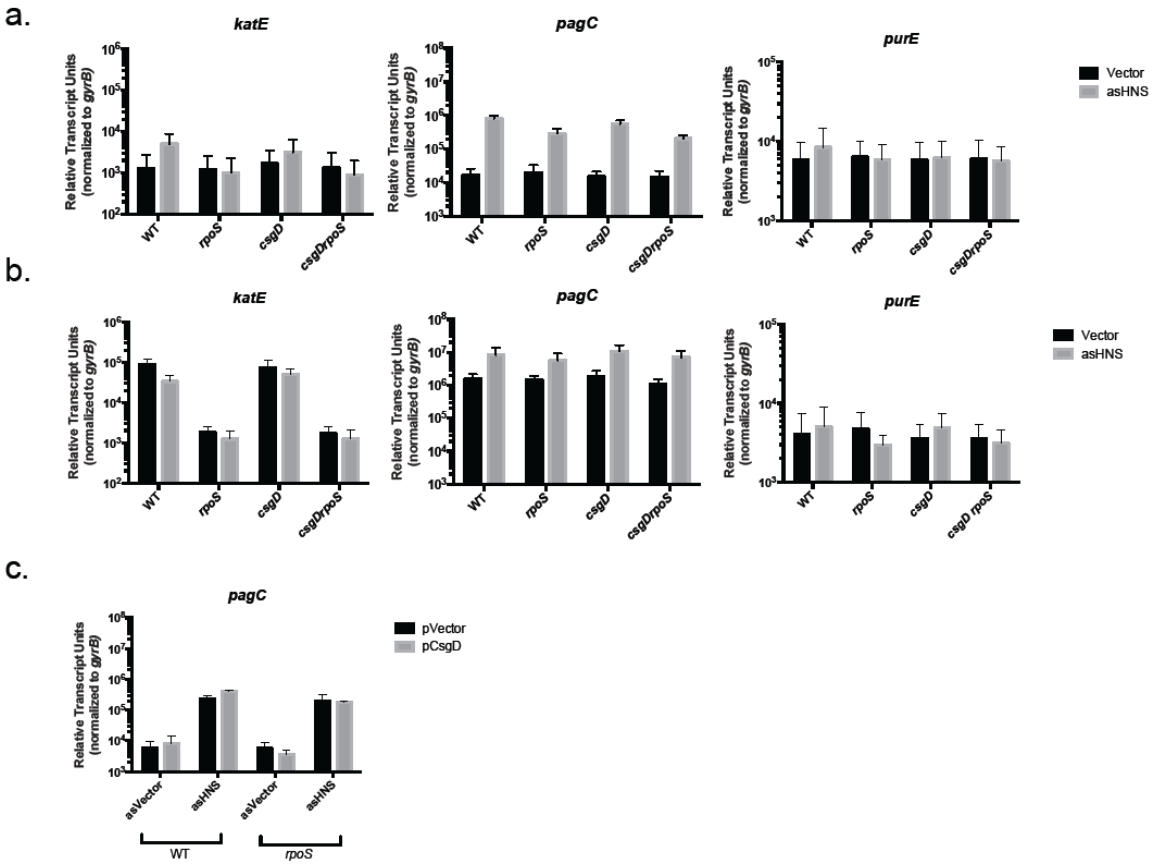
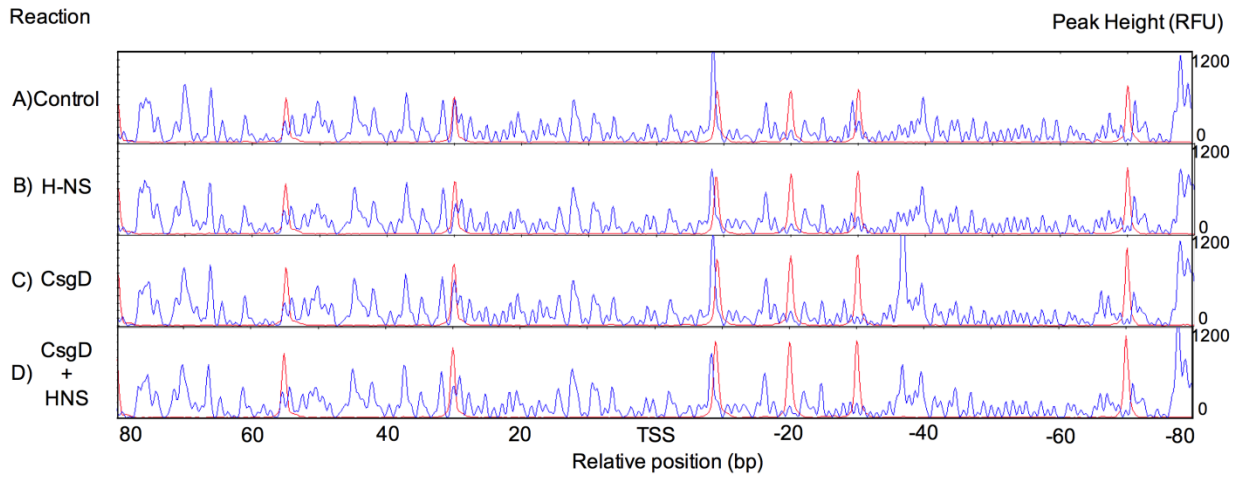


Figure 4.6. $KMnO_4$ Differential DNA Footprint Analysis (DDFA) at the *csgB* promoter region. $KMnO_4$ footprinting reactions, which detect regions of single-stranded DNA generated during open-complex formation, were performed on the *csgB* promoter region with RNAP, CsgD, and H-NS, as indicated. Peaks, in relative fluorescent units (RFUs), indicate regions of single stranded DNA present in the experimental samples that are not present in control (no RNAP) reactions. Base positions are indicated relative to the TSS. a) $KMnO_4$ footprinting analysis indicates that the location of RNAP open complex formation on the naked DNA promoter is dependent on the presence or absence of CsgD. RNAP alone is capable of forming a weak open complex at an upstream site, but the addition of CsgD directs RNAP to the previously identified transcriptional start site. b) RNAP is unable to form an open complex in the presence of H-NS, but the addition of CsgD restores open complex formation at the *csgB* promoter. Data are presented as the mean +/- SD of three replicates.

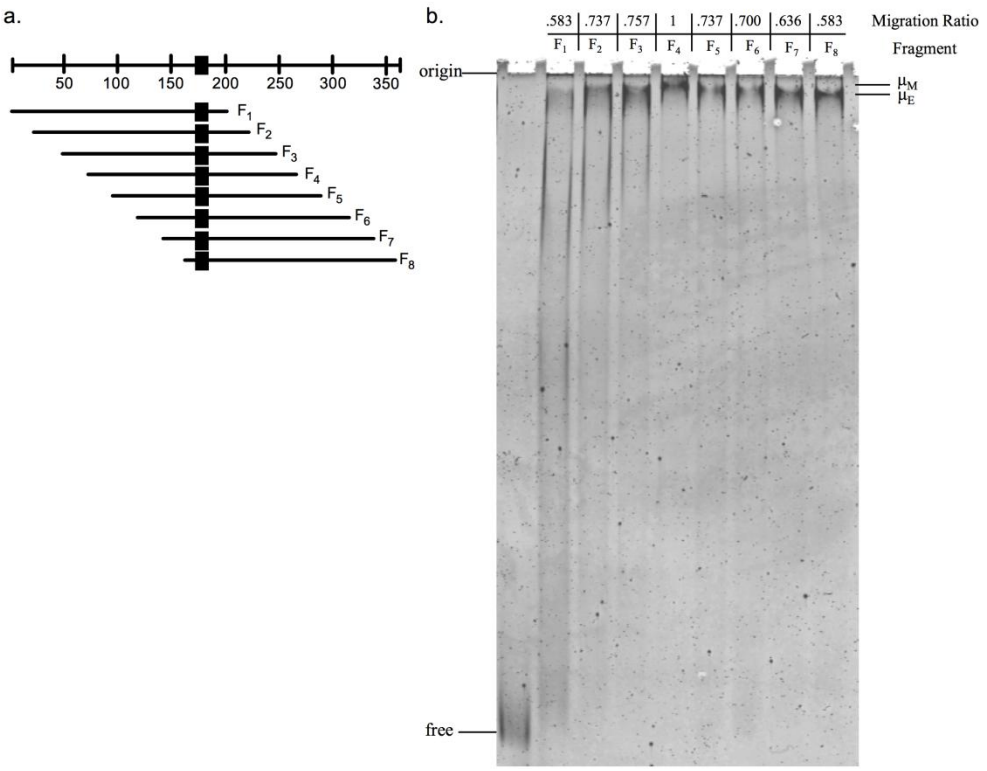
Supplementary Figure 4.1. Alignment between *S. enterica* 14028s and *E. coli* K12 *csg* intergeneic region. Muscle multiple sequence alignment using MacVector software identified 526 conserved identities between the *S. enterica* 14028s and *E. coli* K12 *csg* intergenic regions, corresponding to 70% conservation.



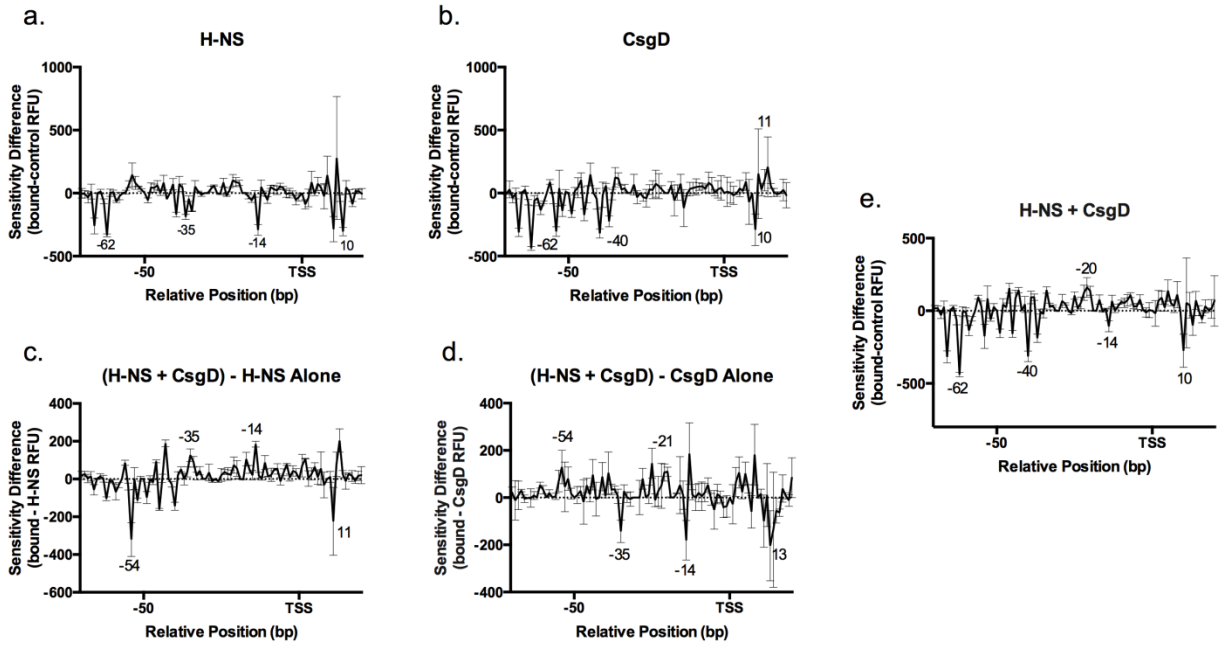
Supplementary Figure 4.2. H-NS depletion and mutation of *rpoS* or *csgD* have distinctive regulatory effects. To confirm the specific regulatory effects of H-NS depletion, or the inactivation of the *rpoS* and *csgD* genes, transcript levels of well-characterised σ^S- and H-NS- regulated genes (*katE* and *pagC*, respectively), along with an unregulated control gene (*purE*), were quantified by qRT-PCR during a) exponential phase, b) stationary phase, and c) in strains containing pCsgD. Data are normalized to *gyrB* and presented as the mean +/- SD of three replicates.



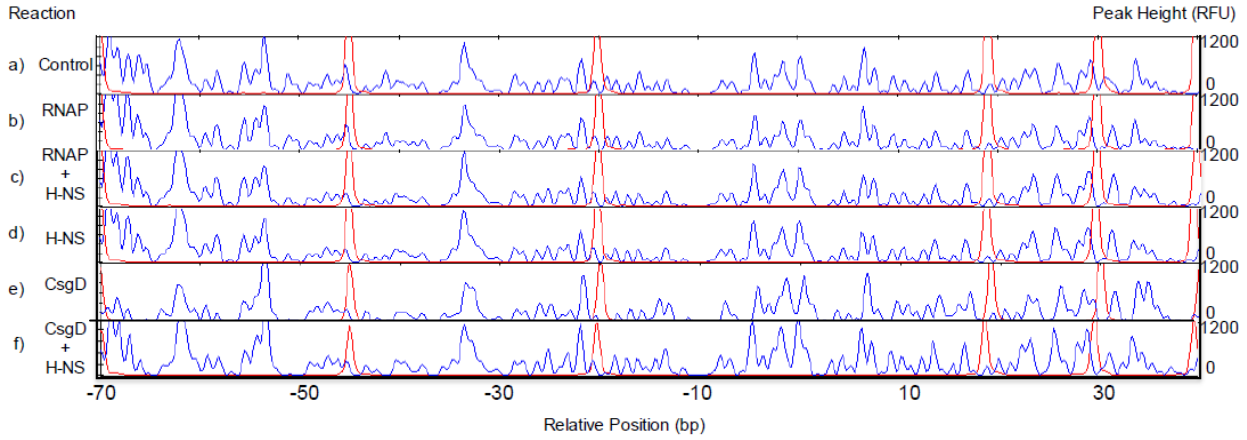
Supplementary Figure 4.3. Anti-sense strand DNase I footprinting at the *csgB* promoter. Representative chromatograms are shown for DNase I footprinting reactions presented in Figure 4.5. Purified *csgB* template was incubated with 130 nM H-NS (b), 50 nM CsgD (c) or both (d) as indicated adjacent to each reaction. The fluorescence of each reaction is normalized to the total signal for all analyzed samples. Base positions are indicated relative to the TSS. The red trace denotes the GeneScan 500 LIZ size standard.



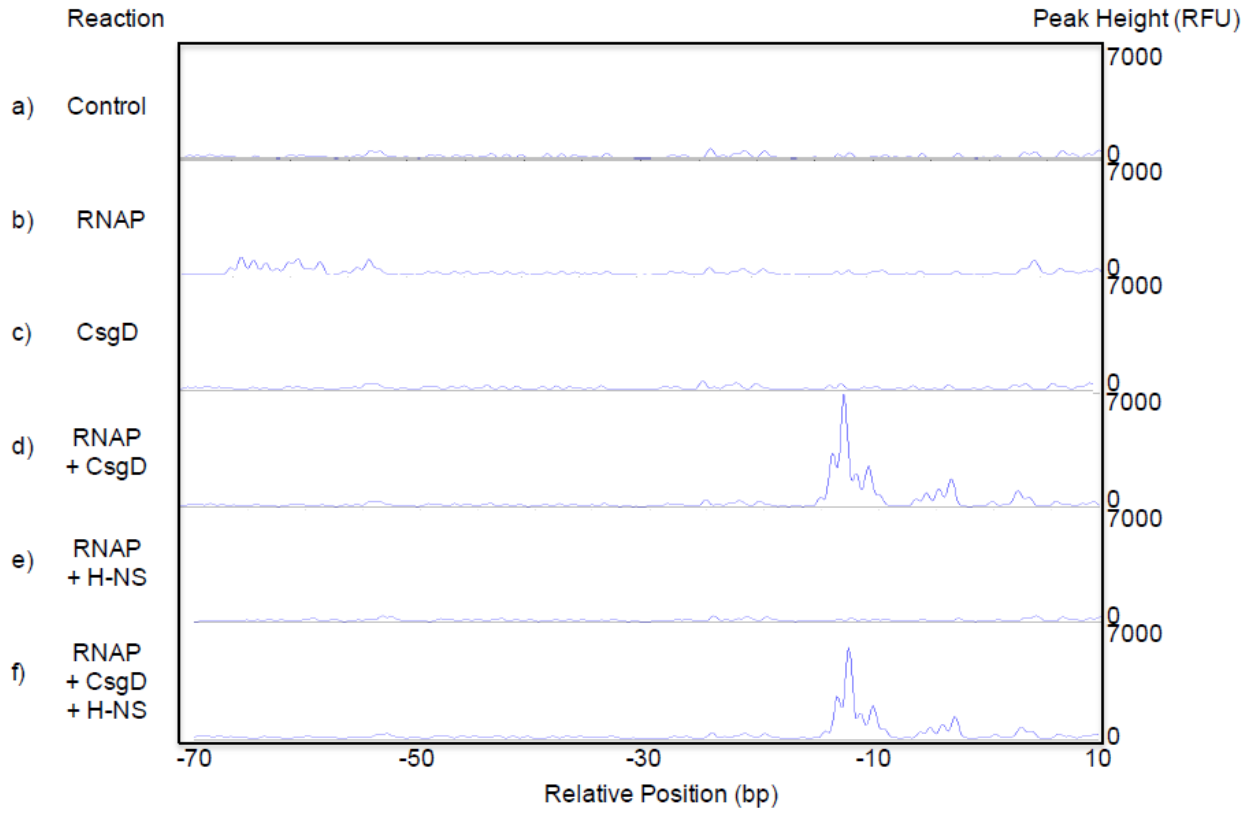
Supplementary Figure 4.4. Circular Permutation Analysis of the DNA bending induced by CsgD binding. a) Structure of the DNA fragment used to generate circularly permutated sequences. The filled box indicates the site of the CsgD binding site within the sequence. PCR primers were used to amplify 200-bp fragments containing the CsgD binding site at various positions, as indicated by the filled box. The resulting fragments are denoted as F_1 – F_8. b) Electrophoretic mobility shift assay of CsgD binding to circularly permutated fragments. 20ng of DNA were loaded along with a 25x molar concentration of CsgD and run on a native 6% polyacrylamide gel. The position of unbound DNA is indicated as free, and the origin of the gel is also noted. μ_M indicates the position of mobility of the slowest fragment, wherein the CsgD binding site is in the middle of the fragment, while μ_E points to the position of the fastest fragment, in which CsgD binds near the end. The relative migration ratio of CsgD-bound complexes to μ_M is noted above each fragment.



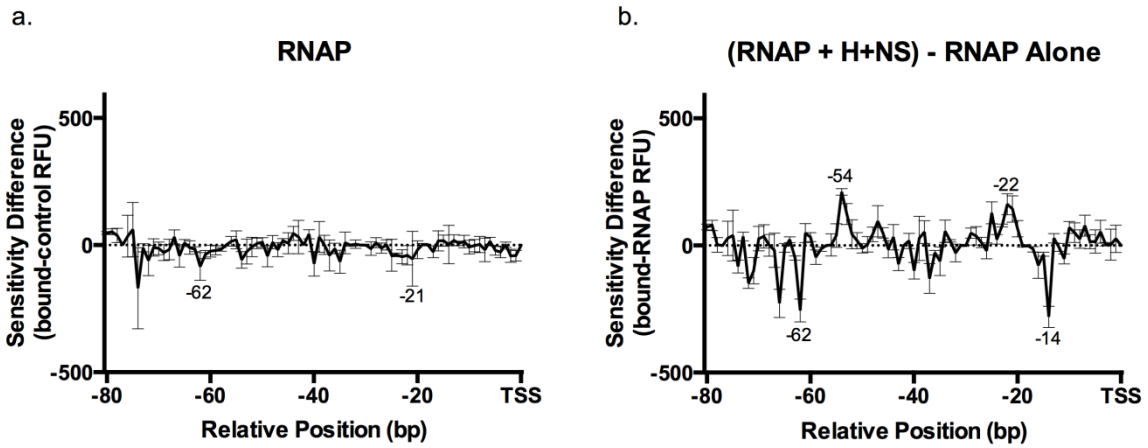
Supplementary Figure 4.5. Sense-strand DNase I Differential DNA Footprint Analysis (DDFA) of the *csgB* promoter region. *In vitro* DNase I footprinting at the *csgB* promoter was performed with 130 nM H-NS and 50 nM CsgD, as indicated and as in Figure 4.5, except an alternative probe was used to detect sensitivity on the sense-strand of the DNA. Peaks are regions of hypersensitivity, which are suggestive of distorted or bent DNA, whereas valleys indicate sites of protection. Base positions are indicated relative to the TSS. a) H-NS, b) CsgD, or e) CsgD and H-NS were added to the *csgB* promoter and the fluorescent peak height following DNase I cleavage determined relative to the protein-free control in relative fluorescent units (RFU). The differences compared to the H-NS control (c) and the CsgD control (d) were also analyzed. Data are presented as the mean +/- SD of three replicates.



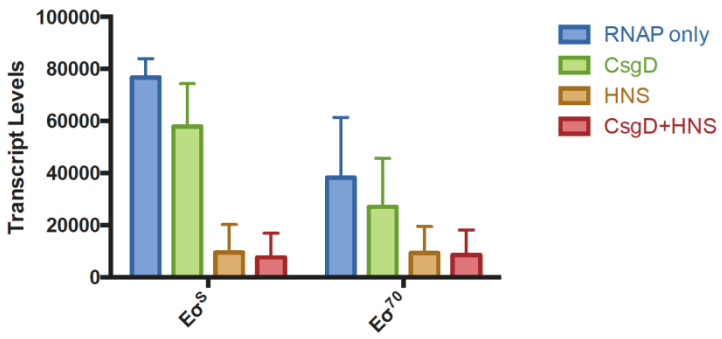
Supplementary Figure 4.6. Sense-strand DNase I footprinting at the *csgB* promoter. Representative chromatograms are shown for sense-strand DNase I footprinting reactions presented in Supplementary Figure 4.5. Purified *csgB* template used in IVT reactions was incubated with 10 nM commercial holoenzyme (a), 130 nM H-NS (d), RNAP + H-NS (c), 50 nM CsgD (e), or CsgD + H-NS (f). The fluorescence of each reaction is normalized to the total signal for all analyzed samples. Base positions are indicated relative to the TSS. The red trace denotes the GeneScan 500 LIZ size standard.



Supplementary Figure 4.7. $KMnO_4$ footprinting analysis of the *csgB* promoter region. Representative chromatograms are shown for $KMnO_4$ footprinting reactions performed with 50 nM CsgD, 130 nM H-NS, and 10 nM commercial RNAP as indicated next to each reaction. Base positions are indicated relative to the TSS, while relative fluorescence is indicated on the vertical axis.

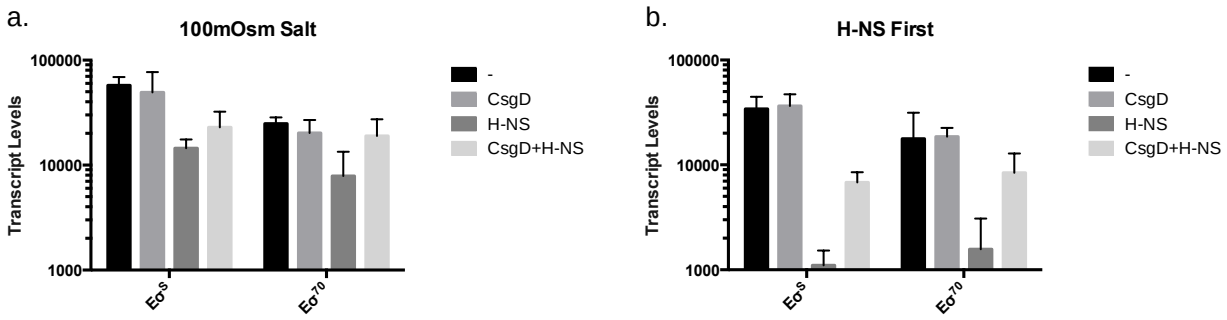


Supplementary Figure 4.8. Sense-strand DNase I DDFA of RNAP at the *csgB* promoter. *In vitro* DNase I footprinting at the *csgB* promoter was performed with 130 nM H-NS and 10 nM commercial RNAP, as indicated. Base positions are indicated relative to the TSS. In a) RNAP was added to the *csgB* promoter and the fluorescent peak height following DNase I cleavage determined relative to a protein-free control in relative fluorescent units (RFU). Addition of H-NS to RNAP bound DNA (b) and normalized to RNAP-only bound sample reveals changes in RNAP-binding as a result of H-NS. Data are presented as the mean +/- SD of three replicates.



Supplementary Figure 4.9. CsgD does not counter-silence its own promoter *in vitro*.

Purified, supercoiled *csgD* template was incubated in the presence of 50 nM CsgD and 130 nM H-NS as indicated, along with $E\sigma^S$ or $E\sigma^{70}$, and transcriptional output assayed. Data are presented as the mean +/- SD of three replicates.



Supplementary Figure 4.10. CsgD counter-silences the *csgB* promoter *in vitro* under varying conditions. *In vitro* transcription assays were carried out as in Figure 4.4, except that potassium glutamate levels were reduced to 50 mM (a) or 130 nM H-NS was added prior to 50 nM CsgD (b). Transcript levels are expressed as relative copy number. Data are presented as the mean +/- SD of three replicates.

Chapter 5: MlrA Antagonizes H-NS Silencing at the *csgD* promoter

Summary

Transcriptional regulation of the *csgD* promoter is complex, involving many transcription factors that mediate activation or repression. These factors confer responsiveness to different environmental conditions and regulate biofilm formation in response to stress. The mechanisms of several of these proteins have been well-characterized, but the role of the MerR-family regulatory protein, MlrA, has been largely undefined. In this study, I have demonstrated the role of MlrA as an H-NS counter-silencer at the *csgD* promoter *in vitro* and further suggest that DNA binding by IHF (integration host factor) can facilitate MlrA-mediated counter-silencing. *In vivo* analyses indicate that both of these proteins are highly σ^S-dependent, pointing toward an explanation for sigma factor selectivity in biofilm expression. In addition, I have sought to identify environmental modulators of *csg* gene expression and suggest a possible novel role of vitamin B_{12} as an allosteric regulator of MlrA.

Introduction

CsgD is widely viewed as the master regulator of curli expression and biofilm formation in *Salmonella* and *E. coli*, serving as both a transcriptional activator and counter-silencer (Chapter 4). Biofilms are resistant to a variety of stress conditions (168) and antibiotics (169,170), thus regulatory networks that respond to these factors are critically important to ensure the expression of curli genes under appropriate conditions. Curli genes are selectively expressed during stationary phase, but the mechanisms responsible for the stationary phase expression of *csg* genes are incompletely defined. The stationary phase sigma factor σ^S is known to be required for curli gene expression during stationary phase, but in Chapter 4, I showed that

σ^S does not directly regulate the *csgBA* promoter. Here I examine the regulation of *csgD*, which encodes the transcriptional activator and counter-silencer of the *csgBA* promoter. Regulation of the *csgD* promoter is complex, with binding demonstrated by ten different transcriptional regulators and five small RNAs. However, expression of *csgD* under particular conditions involves only a subset of these regulators, as each factor responds to different environmental conditions. For instance, RstA and OmpR serve as activators in response to acid stress (145,171), while CpxR monitors membrane stress (172). Small RNAs regulate mRNA levels in response to osmolarity, carbon-limitation, and cell envelope stress (see (173) for review). While studies have implicated roles for OmpR (174) and SsrB (175) in countering CpxR or H-NS activity, respectively, at *csgD*, neither of these regulators provides an explanation for σ^S selectivity (Supplementary Figure 5.1), so I examined the potential role of other candidate regulatory proteins.

MlrA is a member of the MerR-family of transcriptional regulators. MerR family members are thought to function as allosteric regulators, with an N-terminus containing a helix-turn-helix DNA-binding domain and a C-terminus, which varies between family members, interacting with a sensor molecule (176). Interaction with an inducer can lead to positive regulation resulting from transcriptional activation (177) or promotion of oligomerization (178), but in other cases binding can cause the regulator to release DNA, resulting in derepresson (178). Binding sites of MerR-family members are typically situated between the -10 and -35 regions of a promoter, with preferential binding in regions with spacer lengths of 17+/-1 bp, non-optimal for σ^{70} recognition. Allosteric changes cause these proteins to underwind the DNA, changing the superhelicity and modulating the distance between the -10 and -35 to make it more accessible to RNAP.

Given the properties of the MerR-family members, I hypothesized that MlrA might account for sigma-factor selectivity at the *csgD* promoter. As mentioned previously (Chapter 3), σ^S-associated RNAP is better able to recognize promoters with non-optimal spacer regions, and previous studies have suggested a strong correlation between sigma-factor selectivity and DNA supercoiling (90), with σ^S-coupled RNAP better able to transcribe DNA in a relaxed state than RNAP containing σ^{70} (179). Additional studies have suggested that MerR is able to directly interact with the sigma-domain of RNAP (180), raising the question as to whether this property imparts sigma-factor selectivity to MlrA. Earlier studies have suggested that MlrA binds close to the -35 region of *csgD*, as single base pair mutations at -44 result in MlrA-independent regulation (181). However, more recently, the MlrA-binding region in *E. coli* has been identified to occur from -113 to -146, far upstream of the RNAP-binding site (182). In view of these discrepant observations, I also considered how MlrA might be able to facilitate H-NS counter-silencing by binding far upstream of the promoter.

Another possible contributor to stationary phase *csgD* regulation is integration host factor (IHF). IHF is a nucleoid-associated protein that has been demonstrated to play an important role in gene regulation (183). It is composed of two subunits, IHFα and IHFβ, that interact to form a functionally active heterodimer that binds to the minor groove of DNA (184). Most notably, IHF is capable of bending DNA up to 160˚ (185), which creates a promoter architecture critical for regulation of some genes. Previous studies have shown that IHF mutants exhibit reduced expression of *csgD*, (171) and that IHF levels increase up to 3-fold upon entry into stationary phase (145). IHF has a higher binding affinity for DNA than H-NS, so in some cases, it may outcompete H-NS (145), suggesting a potential role of IHF as a counter-silencer (111,165,186).

However, when multiple IHF binding sites are present, IHF may simultaneously bind with H-NS as well (145).

In a recent study in *Vibrio harveyi*, IHF was found to act synergistically with the LuxR transcriptional activator, serving as a co-activator of gene expression. Sites critical for LuxR regulation are positioned far upstream of the transcriptional start site of *luxC*, preventing productive interactions with RNAP (187). IHF bends the DNA to reorganize the promoter architecture, bringing the LuxR binding site into closer proximity to the -35 promoter region (188,189). In view of its established binding site at the *csgD* promoter between the transcriptional start and MlrA-binding sites (171), I posited that IHF might facilitate MlrA-directed activation of *csgD* transcription by remodeling promoter architecture as well as counter-silencing H-NS.

The studies described in this chapter were performed to characterize the role of MlrA at the *Salmonella csgD* promoter. In view of the conflicting previous reports regarding the location of MlrA binding, *in vitro* DNaseI footprinting was performed to establish the site of MlrA binding. In addition, I investigated potential cooperative interactions between IHF and MlrA in counter-silencing of H-NS at the *csgD* promoter. Lastly, I sought to identify a small molecular ligand that could interact with MlrA to mediate allosteric regulation, and speculate regarding its possible role in facilitating counter-silencing and biofilm formation.

Results

H-NS depletion restores *csgD* expression in *rpoS* and *mlrA* mutant *S.* Typhimurium

In vivo genetic analyses with inducible antisense RNA to deplete H-NS demonstrated that, as seen previously with *csgB*, expression of *csgD* is regulated by H-NS during stationary

phase (Figure 5.1). Analysis of *mlrA* and *rpoS* mutants revealed that MlrA and σ^S are required for expression in intact cells, as mutations in either locus resulted in a 6- to 7-fold decrease in *csgD* expression. However, depletion of H-NS under these conditions resulted in a 2.3-to-3.3-fold increase in *csgD* expression in *rpoS* and *mlrA* mutant strains, respectively, suggesting that these proteins are required to oppose H-NS silencing of *csgD*. In contrast to *csgB* expression, wherein a *rpoS csgD* double-mutant phenocopies the single mutants (Chapter 4, Figure 4.1), there appears to be an additive effect of of *mlrA* and *rpoS* mutations compared to either individually (Figure 5.1), suggesting that the contributions of these proteins are at least in part independent. The *mlrA* gene itself does exhibit σ^S regulation (Figure 5.2a), so the plasmid pMlrA expressing *mlrA* from a constitutive σ^S-independent promoter was used to determine if σ^S plays a direct role in counter-silencing at *csgD*. Wild-type cultures containing pMlrA exhibited a 56-fold increase in *csgD* transcription during stationary phase (Figure 5.2b) as a result of increased *csgD* expression from the multicopy plasmid (146). In an *rpoS* mutant containing pMlrA, *csgD* expression could be restored to wild-type levels, suggesting that σ^S is not directly required for H-NS counter-silencing. H-NS depletion did not result in increased *csgD* expression in *rpoS*-null strains containing pMlrA, suggesting that MlrA is sufficient to alleviate silencing (Figure 5.2b).

Investigation of the σ^S dependence of other loci implicated in the regulation of the *csgD* promoter revealed that *ihfB* is also highly σ^S-dependent (Figure 5.3a). While not regulated by MlrA or H-NS, expression of *ihfB* drops more than three-logs in an *rpoS* mutant during stationary phase, corresponding to previous observations that IHF levels increase during stationary phase (190). This may account for the additive effect of *mlrA* and *rpoS* mutations. Current studies are underway to examine levels of *csgD*, *rpoS*, and *mlrA* transcripts in *ihfB* mutants during stationary phase. Preliminary experiments indicate that H-NS is effectively able

to silence *csgD* in stationary phase in an *ihf* mutant and that depletion of H-NS through antisense induction restores *csgD* expression to wild-type levels, suggesting that IHF is only required in the presence of H-NS (Figure 5.3b).

Given the previously established MlrA-binding site, it is possible that IHF function is necessary for MlrA activity. If that is the case, then we would expect the *ihfB* and *mlrA* mutants to exhibit a similar phenotype. It is important to note that in the absence of *rpoS*, constitutive expression of MlrA leads to levels similar to WT (Figure 5.2b), suggesting that *ihfB* is not necessary for MlrA activity in the presence of H-NS. However, *ihfA* levels are unaffected by an *rpoS* mutation (Figure 5.3a). In theory, since IHFα and IHFβ function as a heterodimer, IHF protein levels should also be reduced, but the possibility of the formation of a functional homodimeric protein cannot be excluded (191) and requires further investigation. The regulatory phenotypes of the various mutants were confirmed by individual quantification of the expression of genes regulated by MlrA and H-NS (Supplementary Figure 5.2).

MlrA is able to serve as a counter-silencer to H-NS independently of IHF *in vitro*

To better understand the interplay of H-NS, IHF, MlrA and σ^S in the regulation of *csgD*, the *csgD* transcriptional circuit was reconstituted *in vitro* using purified proteins and large supercoiled templates. *In vitro* transcription assays with both $E\sigma^{70}$ and $E\sigma^S$ revealed that neither *csgD, mlrA,* nor *ihfB* exhibit basal σ-factor selectivity, as both holoenzymes transcribed these promoters at similar levels (Figures S4.9 and 5.4a). At *csgD,* purified MlrA did not demonstrate any properties as an activator when paired with $E\sigma^S$, but when incubated with H-NS-silenced template, transcription increased 5.3-fold (Figure 5.4b), suggesting that MlrA is able to drive counter-silencing at *csgD* independent of other factors. Current studies are underway to examine

if a similar level of counter-silencing occurs when MlrA is coupled with $E\sigma^{70}$ at the *csgD* promoter and whether the addition of purified IHF enhances MlrA-counter-silencing activity *in vitro*.

MlrA shows sequence similarity to other MerR-family members sensitive to vitamin B_{12}

As previously noted, MerR-family members typically regulate transcription through allosteric changes driven by the interactions between the C-terminus and a specific ligand (176). To date, no ligand has specifically been identified for MlrA. Since interaction with a binding molecule is important for protein function of these family members, identifying the compound(s) with which MlrA interacts is of considerable interest. Using the on-line COACH server (192) complemented with Phyre2 (193), structural predictions and comparisons to binding-specific substructure were made to identify homologous proteins and predict ligand-binding sites. The two analyses revealed several proteins with structural and sequence similarity to MlrA (Supplementary Tables 5.1 and 5.2). Notably, half of these proteins showed high sequence and structural similarity at the C-terminus and frequently had been shown to bind vitamin B_{12}.

Vitamin B_{12} is recognized as an essential vitamin in humans and has been shown to be an important cofactor for enzymes involved in many cellular processes in both eukaryotes and bacteria. The making and breaking of carbon and cobalt bonds drives B_{12}-dependent enzymatic reactions important in methylation, DNA synthesis, fatty acid production and amino acid metabolism (194,195). Vitamin B_{12} has also played a critical role as a ligand in different riboswitches (196) and as a cofactor for light-sensing regulatory proteins (178). Also known as cobalamin, vitamin B_{12}, exists in two forms *in vivo*: adenosylcobalamin ($AdoB_{12}$) and methylcobalamin (MeB_{12}). Upon photo- or thermo-excitation, both $AdoB_{12}$ and MeB_{12} undergo

homolytic cleavage of the carbon-cobalt bond, creating the photoproduct hydroxocobalamin (OHB_{12}).

In many organisms, vitamin B_{12} plays a vital role in facilitating protein function, typically leading to a conformational change upon binding. For instance, in *Bacillus* and *Salmonella*, the transcriptional regulators LitR (197) and MetR (198) cause transcriptional repression upon interaction with cobalamin. MlrA shows the highest degree of homology with the protein CarH in *Thermus thermophilus* (Figure 5.5). B_{12} binding to CarH in the dark causes protein oligomerization and repression of carotenoid genes, but photolysis of $AdoB_{12}$ to OHB_{12} destabilizes this complex, leading to gene activation (178).

Although not designated as a homologous hit by COACH, AerR in *Rhodobacter capsulatus* binds B_{12} in an alternative regulatory mechanism. While AerR does not have an identifiable DNA-binding domain, it is thought to control expression of genes by modulating the activity of the transcriptional repressor CrtJ (199,200). Only AerR bound with B_{12} can interact with CrtJ, which inhibits CrtJ's ability to bind DNA, thereby causing derepression (201). In view of the roles of B_{12}-modulated proteins in the direct and indirect activation of gene transcription, we have considered whether this ligand might modulate the transcriptional regulatory activity of MlrA at *csgD in vitro*.

Light Exposure of the vitamin B_{12}-MlrA complex leads to high levels of transcriptional activation and counter-silencing *in vitro*

Studies are presently underway with our collaborators to determine whether direct binding of MlrA by cobalamin can occur (202). In parallel, we are investigating whether the incubation of MlrA, with B_{12}, alters its regulatory properties. Preliminary studies suggest that B_{12}

can stimulate MlrA activity by nearly four-fold, and this activity is exclusively light-dependent (Figure 5.6a). In addition, *in vitro* experiments suggest that intact B_{12} in the absence of photolysis may inhibit the ability of MlrA to counter H-NS activity (Figure 5.6b), although confirmation of these findings is pending.

Discussion

Regulation of the *Salmonella csgD* promoter is complex, with input from more than ten different regulatory proteins and five small RNAs acting under various environmental conditions. In this study, we explored the role of the MerR-family regulator MlrA as both an activator and a counter-silencer at the *csgD* promoter, examined conditions under which *csgD* is induced, and investigated whether IHF is required for MlrA regulation. Some studies are still ongoing, but it appears that MlrA may serve as a novel vitamin B_{12}-sensor, and B_{12} may modulate MlrA counter-silencing of H-NS and biofilm formation.

My *in vivo* studies have confirmed the role of σ^S, MlrA, and IHF as important regulatory inputs into *csgD* expression and demonstrated that they are no longer required if H-NS is depleted (Figures 5.1-5.3). However, the mechanisms by which these transcription factors interact to relieve H-NS silencing is unclear. The importance of σ^S may stem from direct transcriptional dependency of both *mlrA* and *ihfB* (Figures 5.2 and 5.3), but whether or not IHF is required for the counter-silencing actions of MlrA *in vivo* still needs to be investigated. My *in vitro* experiments have suggested that this is not the case, as MlrA is able to relieve H-NS-mediated silencing at *csgD* independent of IHF (Figure 5.4). However, studies are underway to better characterize the relationship of these factors in the presence or absence of different forms of B_{12} and to determine whether the counter-silencing by MlrA is sigma-factor specific.

Nearly all *Salmonella* isolates are able to synthesize cobalamin *de novo* (203), with close to 1% of the *S.* Typhimurium genome devoted to the synthesis and uptake of cobalamin, and another 1% involved in metabolic pathways that require it (204). However, vitamin B_{12} has not been previously implicated in *Salmonella* biofilm formation. In *Pseudomonas*, as in *Salmonella*, vitamin B_{12} production is closely linked to the availability of oxygen. During anaerobic conditions, B_{12} production is impaired and exogenous exposure to B_{12} helps to alleviate stress and enhance growth (205). While some groups have suggested that cobalamin production in *Salmonella* occurs only under conditions of anaerobic growth (206), others suggest that oxygen is needed at some level to facilitate B_{12} synthesis (207). Previous studies have suggested that CsgD expression is maximal under aerobic or microaerophilic conditions (208), correlating with observations that biofilm formation is greatest under microaerophilic and CO_2-rich conditions (209) and inhibited under anaerobic conditions (210,211). In this context, it is possible that the environmental cues that influence curli gene expression are conditions of low oxygen or exposure to exogenous B_{12}, a scenario thought to exist during infection, as members of the intestinal microbiota are prominent producers of the molecule (212). In our *in vivo* genetic experiments, the cultures are heavily aerated, suggesting that the bacteria are not primed to produce their own vitamin B_{12}. Nevertheless, we still observe MlrA-dependent *csgD* expression (Figure 5.1). Luria Bertani medium, as used in this study, contains yeast extract, an important source of vitamin B_{12} in microbial growth media, and likely provides an exogenous source that cues this transcriptional response. Studies under minimal media conditions would need to be carried out to verify that MlrA-dependent counter-silencing *in vivo* is mediated by environmental cues from interaction with vitamin B_{12}.

An additional signal worth considering is light. As previously mentioned, vitamin B_{12} in itself is extremely light sensitive and often requires exposure to light for cleavage into an active form. Preliminary *in vitro* data suggest that photolysis of B_{12} may be necessary for MlrA activity (Figure 5.6). As biofilms are a known defense mechanism against UV exposure (213), one possibility is that B_{12} confers light-responsiveness to biofilm formation by inhibiting MlrA counter-silencing in the dark and promoting curli formation following cleavage by UV light. Previous studies in *E. coli* have examined the influence of blue light on the MlrA paralogue, BluR, suggesting that exposure to blue light leads to derepression of genes important for biofilm formation (214). These authors suggested that light-regulation in this case was inadvertent and likely a result of evolutionary descent from an ancestral light-responsive protein.

I hypothesize a model in which MlrA promotes a conformational change in DNA that disrupts H-NS silencing, leading to *csgD* expression and biofilm formation (Figure 5.7). Vitamin B_{12}, either produced endogenously under microaerobic conditions or provided exogenously by the gut microbiota, modulates this response. Dark-state cyanocobalamin may sequester and modify MlrA to inhibit DNA binding, while photo-activated cobalamin restores MlrA DNA-binding activity. Understanding the mechanisms by which MlrA, IHF, and σ^S act coordinately to relieve H-NS-mediated silencing remains a work in progress. It is possible that MlrA and IHF act together to relieve counter-silencing, but *in vivo* and *in vitro* transcriptional assays suggest that MlrA is independently capable of this activity as well. Planned DNase I footprinting studies will confirm the binding site of MlrA, as it is possible that another binding site more proximal to the -35 region also exists (181), which may obviate the need for IHF. These studies, coupled with $KMnO_4$ and UV laser footprinting assays, will provide insight into the mechanism by which MlrA mediates counter-silencing. Further exploration of the possible role of vitamin B_{12} as an

allosteric regulator modulating MlrA counter-silencing may identify a novel environmental context in which curli gene expression and biofilm formation is initiated.

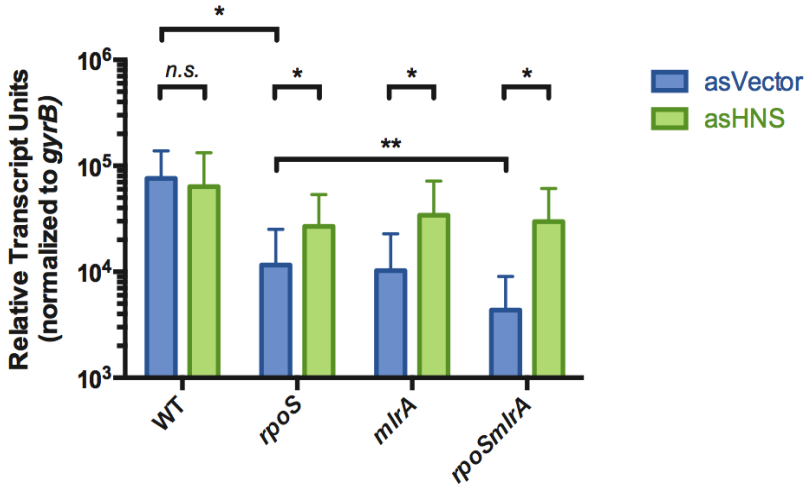


Figure 5.1. MlrA and σ^S up-regulate *csgD* in stationary phase. *csgD* transcript levels during stationary phase were quantified by qRT-PCR in wild-type, *rpoS*, *mlrA*, and *rpoS mlrA S.* Typhimurium strains containing pHN1009 asVector or pHN1009 asHNS with IPTG added to deplete H-NS. Data are presented as the mean +/- SD of 3 replicates normalized to *gyrB*; * indicates $p<0.05$, and ** indicates $p<0.01$ by ratio-paired t-test.

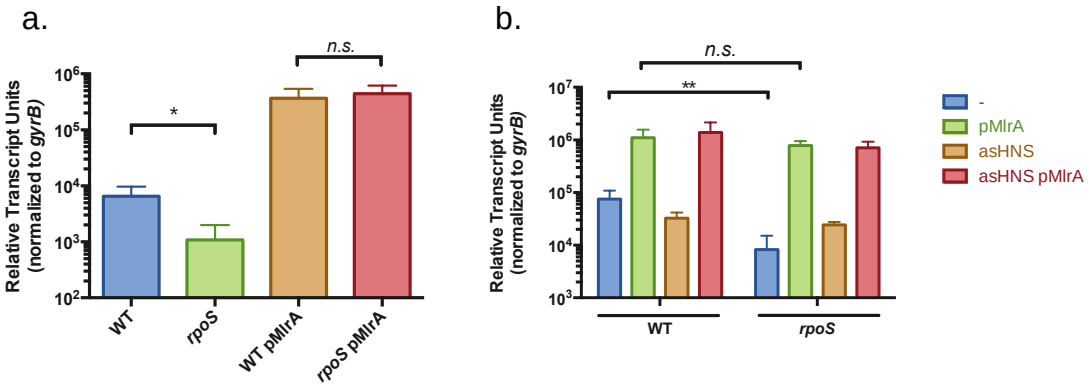


Figure 5.2. Constitutive *mlrA* expression *in trans* stimulates σ^S-independent *csgD* transcription. a) *mlrA* transcript levels were quantified using qRT-PCR in wild-type and *rpoS* mutant *S.* Typhimurium containing pMlrA, which contains *mlrA* downstream of a constitutive promoter. b) *csgD* transcript levels were quantified in response to *mlrA* constitutive expression in strains containing both pMlrA and asHNS. Data are presented as a mean +/- SD of 3 replicates normalized to *gyrB* expression; * indicates $p<0.05$, and ** indicates $p<0.01$ by ratio-paired t-test.

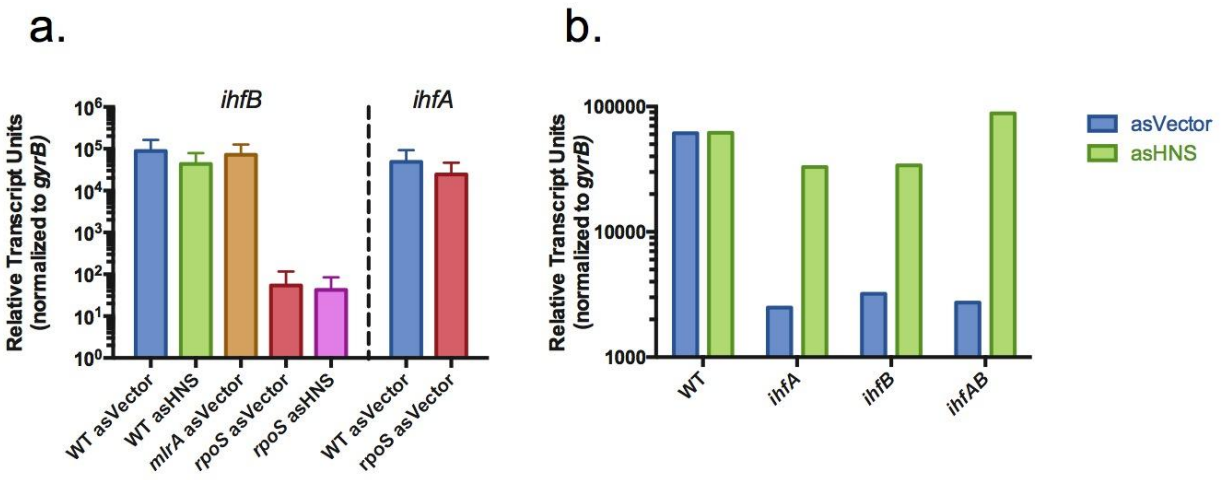


Figure 5.3. *ihfB* expression is dependent on σ^S and required for *csgD* transcription in the presence of H-NS. a) *ihfB* or *ihfA* transcript levels during stationary phase were quantified using qRT-PCR in wild-type, *mlrA*, and *rpoS S*. Typhimurium strains containing pHN1009 asVector or pHN1009 asHNS, with IPTG added to deplete H-NS. Data are presented as the mean +/- standard deviation of 3 replicates normalized to *gyrB* expression. b) *csgD* expression during stationary phase was measured using qRT-PCR in *ihf* mutants of *S.* Typhimurium containing asVector or asHNS. Results shown are from one experiment.

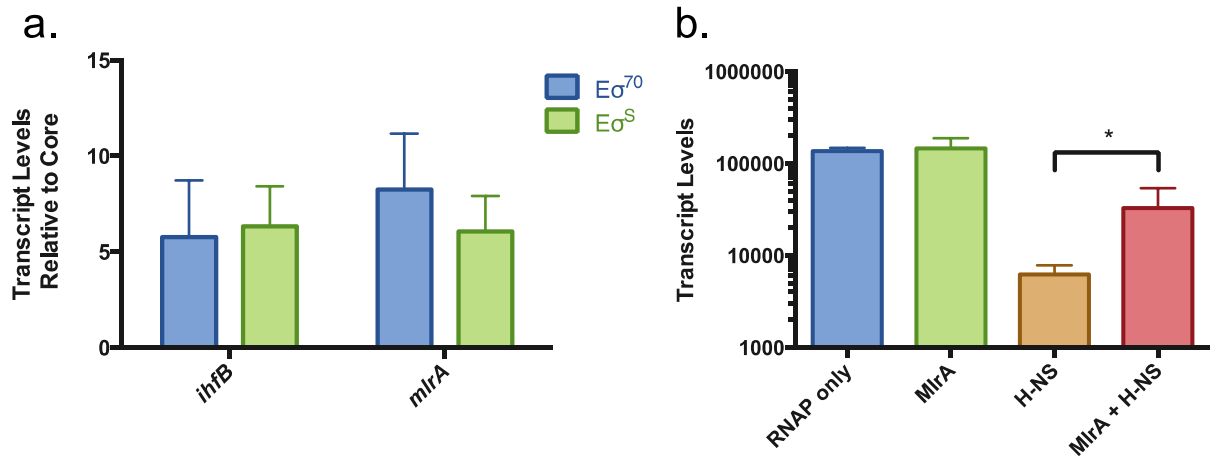


Figure 5.4. MlrA counter-silences the *csgD* promoter *in vitro*. a) Reconstituted holoenzymes $E\sigma^S$ and $E\sigma^{70}$ showed equal transcriptional activity *in vitro* on supercoiled templates containing the *ihfB* or *mlrA* promoter regions, suggesting no sigma-factor selectivity. Data are presented as the mean +/- SD of 3 replicates and are normalized to RNAP core without a sigma-factor. b) $E\sigma^S$ alone can initiate transcription of the *csgD* promoter *in vitro.* MlrA-mediated counter-silencing was assayed by incubating *csgD* template in the presence of 100nM MlrA for 20 min before adding 130nM H-NS. Transcript levels are expressed as relative copy number. Data are presented as the mean +/- SD of 4 replicates; * indicates $p<0.05$ by ratio-paired t-test.

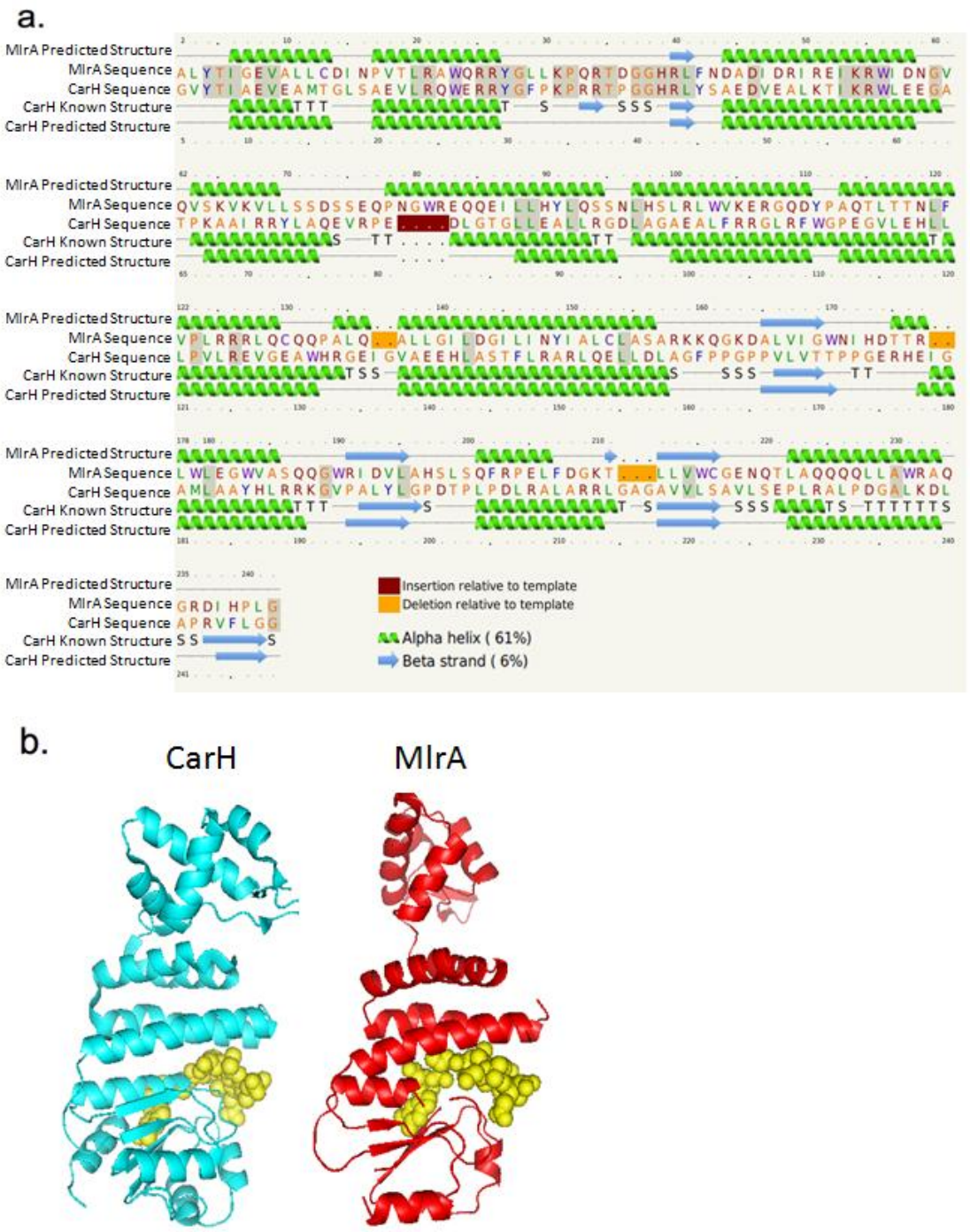


Figure 5.5. MlrA protein structure is similar to CarH. a) Utilization of the PHYRE server suggested similar protein structures based on amino acid sequence for *T. thermophilus* CarH and *S.* Typhimurium MlrA. b) Cartoon diagrams of the conformation of CarH (blue) and MlrA (red) proteins in complex with vitamin B_{12} (yellow spheres). The N-terminal DNA binding domain is located at the top and the C-terminal sensor domain is at the bottom.

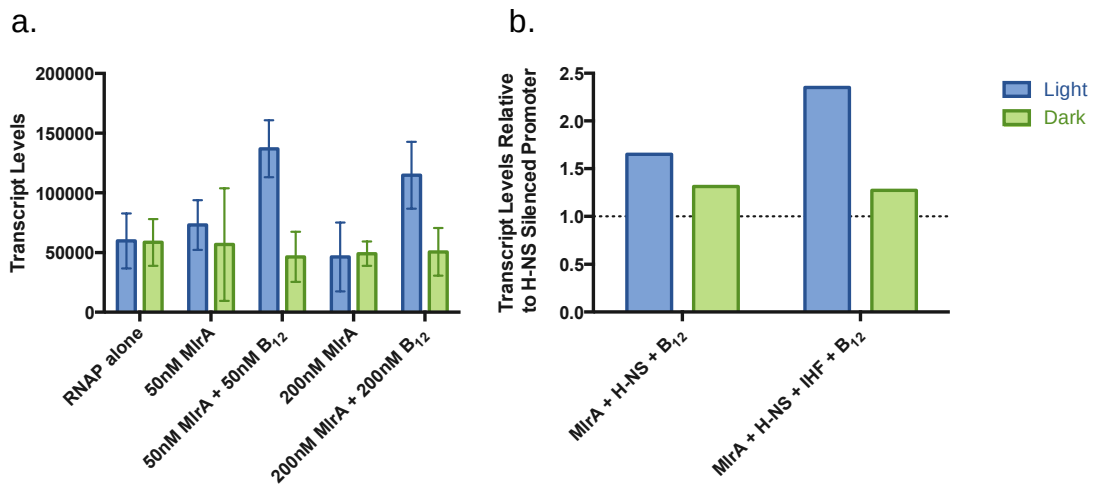


Figure 5.6. Modulation of MlrA activity by vitamin B_{12} is light-dependent. a) Vitamin B_{12} was incubated with varying concentrations of MlrA at a 1:1 ratio, added to *csgD* supercoiled template along with $E\sigma^S$, and incubated at 30˚C under white light or covered with foil. Data shown are the results of two experiments. b) 100nM MlrA was incubated with vitamin B_{12} at a 1:1 molar ratio for 10 min at the *csgD* supercoiled template, followed by 20nM IHF, 130nM H-NS, and 10nM $E\sigma^S$ each for 10 min at 30˚C. Reactions were placed either under white light or covered with foil. Transcript levels are normalized to levels of promoter silenced by H-NS alone under light or dark conditions. Data shown are from one experiment.

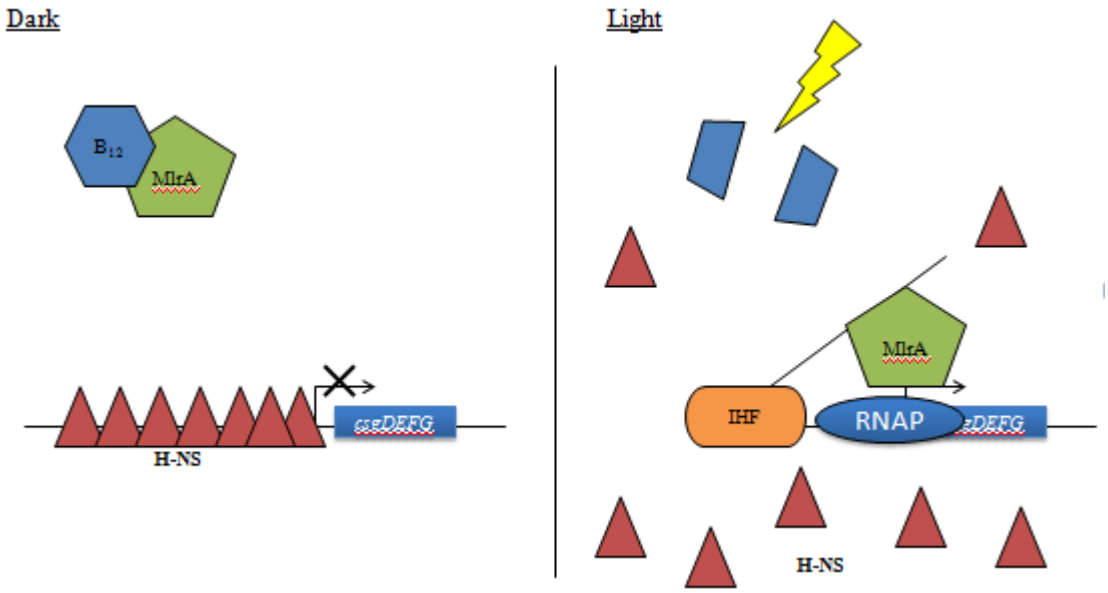
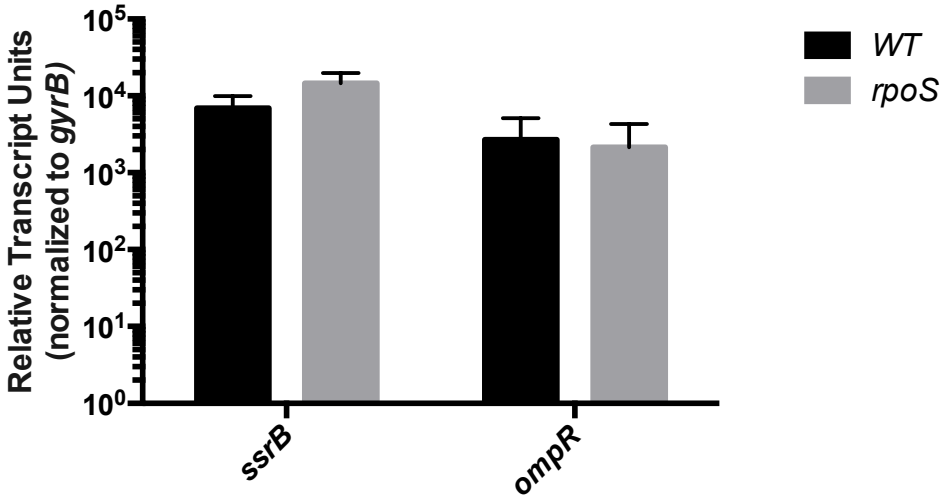
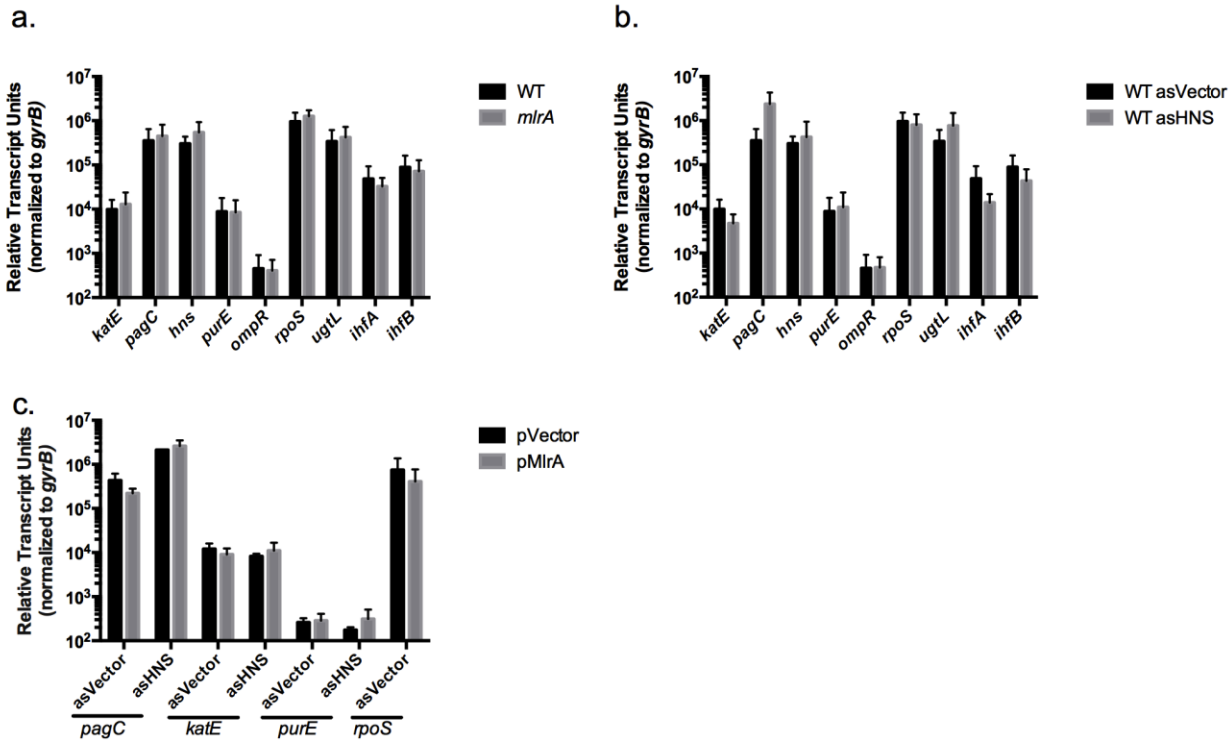


Figure 5.7. Model of B_{12}-modulated regulation of *csgD* expression by MlrA and IHF. Under dark conditions, vitamin B_{12} binds to MlrA, inhibiting its ability to interact with DNA and allowing H-NS to bind and silence expression. Following photolysis, B_{12} is no longer able to bind and sequester MlrA. IHF expression in stationary phase bends DNA to facilitate localization of upstream-bound MlrA more proximally to the promoter, thereby disrupting the H-NS complex and allowing transcription.



Supplementary Figure 5.1. σ^S-dependent curli expression does not result from SsrAB or OmpR regulation. qRT-PCR expression measured transcriptional activity of the *ssrA* or *ompR* promoters in stationary phase wild-type or isogenic *rpoS* mutant *S.* Typhimurium. Data are presented as the mean +/- SD of 3 replicates and normalized to *gyrB.*



Supplementary Figure 5.2. MlrA and H-NS do not influence the expression of other *csgD* regulators. Quantitative RT-PCR was used to measure gene expression during stationary phase in wild-type or *mlrA* mutant *S.* Typhimurium (a), after H-NS depletion by anti-sense expression (b), or during constitutive MlrA expression (c). Data are presented as the mean +/- SD of 3 replicates and normalized to *gyrB.*

Supplementary Table 5.1. Molecules most structurally-related to MlrA retrieved by the PHYRE server.

PDB identity	Confidence	Identity %	Protein/Function	Species	MerR-Type Regulator	Binds B_{12}
5c8e	100	18	CarH: Light-Dependent Transcriptional Regulator	*Thermus thermophilus*	Yes	Yes
1r8d	99.8	23	MtaN: Transcriptional Regulator	*Bacillus subtilis*	Yes	No
3hh0	99.8	18	Transcriptional Regulator	*Bacillus cereus*	Yes	No
3gpv	99.8	17	Transcriptional Regulator	*Bacillus thuringiensis*	Yes	No
3qao	99.8	16	Transcriptional Regulator	*Listeria monocytogenes*	Yes	No
2zhh	99.8	19	SoxR: oxidative-stress transcriptional regulator	*Escherichia coli*	Yes	No

Supplementary Table 5.2. Molecules most structurally-related to MlrA retrieved by COACH. COACH is a meta-server approach to protein-ligand binding site prediction that combines the results of methods such as COFACTOR, FINDSITE, and ConCavity to increase the strength of a prediction. Confidence scores range from 0-1, where a higher score indicates a more reliable prediction.

PDB identity	Confidence Score	Protein/Function	Species	MerR-Type Regulator	Binds B_{12}
5c8eG	.19	CarH Nucleic acid bindindgdomain	*T. thermophilus*	Yes	Yes
5c8aB	.12	CarH B_{12} binding domain	*T. thermophilus*	Yes	Yes
3IV9A	.06	B_{12}-dependent methionine synthase (MetH)	*E. coli*	Yes	Yes
4REQA	.04	Methylmalonyl-coA mutase	*Propionibacterium shermanii*	No	Yes
1R8EA	0.02	BmrR	*Bacillus subtilis*	Yes	No

Chapter 6: Significance and Future Directions

Curli gene expression has been extensively studied in both *E. coli* and *Salmonella* during the past two decades. My studies have identified new insights into the mechanisms by which curli expression is regulated at the level of *csgB* and *csgD* transcription. I have found that counter-silencing plays a central role in the regulation at both genes, and that modulation of the expression of counter-silencing proteins by the stationary phase sigma factor σ^S may be responsible for growth phase-dependent curli expression.

Data presented in Chapter 3 explored direct actions of σ^S at the *csgBA* and *spvAB* promoters during stationary phase. These genes, also regulated by H-NS, could not be transcribed by σ^S alone *in vitro* when H-NS was present (Figure 3.4), indicating that σ^S is unable to overcome H-NS silencing of these promoters and rather influences *csgBA* expression indirectly. Many of the genes co-regulated by H-NS and σ^S (Supplementary Table 3.1) are controlled by additional regulators, suggesting that the counter-silencing of these genes is complex. Future studies including comparative ChIP-seq analyses of H-NS and σ^S under different growth conditions would provide a more detailed picture of the degree of overlap between the H-NS and σ^S regulons. Although I cannot generalize from the data presented herein regarding two promoters to all σ^S-H-NS regulated promoters, my observations suggest that σ^S may not serve as a direct counter-silencer but may nevertheless modulate counter-silencing by indirect actions.

While previous studies have suggested that CsgD serves as a transcriptional activator (157), the experiments described in Chapter 4 show that CsgD is also responsible for relieving H-NS silencing at the *csgB* promoter. *In vitro* analyses have revealed that H-NS inhibits transcription by occluding RNAP (Figure 4.6), but CsgD distorts the DNA to disrupt the H-NS

filament and restore RNAP-DNA contacts (Figure 4.5). As discussed in this chapter, CsgD is a member of the LuxR-family of transcriptional regulators. Therefore, it would be interesting to conduct future studies to identify if this mechanism of counter-silencing is common to other LuxR-family members. Studies by other groups have suggested this is a possibility (161-165), and our *in vitro* system could provide a more detailed view of how these proteins function in the presence of H-NS. This would lend additional insight into the regulation of genes involved in virulence and quorum sensing across bacterial species.

Chapter 5 describes my efforts to identify the source of stationary phase regulation of the curli genes. The *csgD* promoter itself is not σ^S-specific *in vitro* (Supplementary Figure 4.9), but two upstream regulators of *csgD*, MlrA and IHFβ, are σ^S-dependent *in vivo* (Figures 5.2 and 5.3). While I have been able to show that MlrA alone is able to relieve H-NS silencing, future studies will be required to determine whether IHF also plays an integral role *in vivo* and *in vitro* in *csgD* counter-silencing. DNaseI footprinting could either confirm the predicted MlrA-binding site (182) or suggest that it might bind further downstream (181), which would help to explain how MlrA is able to serve as a counter-silencer independent of other factors. That neither *mlrA* nor *ihfB* show sigma-factor selectivity *in vitro* (Figure 5.4), underscores that more work will be needed to understand the direct basis of σ^S-mediated curli regulation. Further characterizion of the environmental cues responsible for MlrA-mediated counter-silencing is also of considerable interest. *In silico* modeling has suggested that MlrA is able to interact with vitamin B_{12}. Other studies have suggested that vitamin B_{12} can serve as an allosteric regulator (178,197,198), so it will be of interest to determine whether vitamin B_{12} can modulate MlrA-activity. *In vivo* studies using the H-NS knock-down system in minimal medium lacking or supplemented with vitamin

B_{12} could suggest whether vitamin B_{12} modulates curli gene expression and suggest the environmental conditions in which expression is triggered.

In conclusion, the analysis of counter-silencing mechanisms in *Salmonella enterica* can provide important insights into mechanisms of horizontal gene transfer and the accommodation of new genetic sequences into pre-existing regulatory networks. Many of the proteins described in this dissertation are conserved across different bacterial species, so it is likely that the mechanisms by which they act are similar in diverse bacteria. The molecular mechanisms by which bacterial virulence genes are regulated is also critical for an understanding of bacterial pathogenesis. As multi-drug resistant strains become more prevalent, understanding these processes could aid in targeting future drug development. Finally, an understanding of how horizontally acquired genes become integrated into host regulatory networks can provide insight into how transcriptional mechanisms have facilitated the evolution of *Salmonella* over time.

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Vita

Shannon Newman is originally from St. Charles, Illinois. She graduated *summa cum laude* in course and *summa cum laude* in independent research at Lawrence University in Appleton, Wisconsin, where she earned a Bachelor of Arts in Biochemistry with a minor in German in 2011 and was inducted into the prestigious Phi Beta Kappa honor society. Shannon joined the Molecular and Cellular Biology Interdisciplinary Graduate Program at the University of Washington in the Fall of 2011. In Ferric Fang's lab, she was a two-time honorable mention of the National Science Foundation Graduate Research Fellowship and the recipient of the Cellular and Molecular Biology Training Grant. She defended her dissertation regarding the molecular and biochemical mechanisms of gene regulation in *Salmonella enterica* on June 23rd, 2017. Passionate about education and fostering meaningful learning experiences, Shannon will be beginning a position after graduation as Associate Professor at Pierce College in Puyallup, Washington. She will be working with adults to complete their high school diploma and transition into college level science courses. Outside of science, Shannon plays viola with the Puget Sound Symphony Orchestra, is an avid gardener and hiker, and enjoys organizing social dinner parties for others.