Hha and YdgT ACT THROUGH H-NS TO REPRESS HORIZONTALLY ACQUIRED GENES

by

James McKay Stevenson

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Molecular Genetics
University of Toronto

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University of Toronto

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Abstract

The bacterial protein H-NS acts to silence horizontally acquired genes. H-NS physically interacts via its N-terminus with two paralogous proteins, Hha and YdgT. Deletion of hha and ydgT results in derepression of a subset of H-NS repressed genes. I compared expression of hha/ydgT-dependent genes in Salmonella strains lacking hns and hha/ydgT/hns. Deletion of all three genes does not result in greater gene expression than deletion of hns alone, indicating that Hha and YdgT cannot act to repress genes in the absence of H-NS. Further, I used site-directed mutagenesis to generate H-NS proteins incapable of binding Hha. Complementing an hns deletion with an Hha-blind H-NS molecule, H-NS I11A, recapitulated the pattern of gene expression in the hha/ydgT strain. Indicating that elimination of the Hha-H-NS interaction is sufficient to result in derepression of hha/ydgT repressed genes. Hha and YdgT repress gene expression by acting through H-NS and cannot act independently of H-NS.
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<tr>
<td>amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
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<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>Cm</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-dithothreitol</td>
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<tr>
<td>EDTA</td>
<td>disodium ethylenediaminetetra-acetate</td>
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<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<tr>
<td>FPLC</td>
<td>fast performance liquid chromatography</td>
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<td>Hha</td>
<td>haemolysin expression modulating protein</td>
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<tr>
<td>H-NS</td>
<td>Heat-stable nucleoid structuring protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Km</td>
<td>kanamycin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Strep</td>
<td>streptomycin</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>YdgT</td>
<td>Hha paralogue (aka Cnu: OriC binding protein)</td>
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WT: wild-type
1 Introduction
1.1 Overview

Bacterial evolution is able to proceed more rapidly than that of eukaryotes, in part due to horizontal gene transfer events. Several mechanisms of horizontal gene transfer have been implicated in the evolution of antibiotic resistance (Barlow 2009) and many bacteria, including *Salmonella*, are believed to have evolved pathogenic potential due to horizontal gene transfer events (Groisman, Ochman 1997). However, most horizontal gene transfer events are detrimental to the recipient cell. As such, bacteria have developed mechanisms that allow them to take advantage of the opportunities afforded by the acquisition of foreign genes, while minimizing risks. One of the hallmarks of horizontally acquired genes is a characteristically high AT content, compared with the remainder of the genome (Daubin, Lerat & Perriere 2003). The protein H-NS silences foreign-acquired genes by binding DNA with characteristic high AT content (Navarre et al. 2006, Lucchini et al. 2006). In this way, H-NS allows the cell to regulate acquired genes without the need to recognize specific sequences and for this reason H-NS serves as the central regulatory molecule for most virulence and drug-resistance genes in enteric bacteria such as *E. coli* and *Salmonella enterica*.

Many H-NS regulated genes are also regulated by *hha* and *ydgT* (Vivero et al. 2008). The proteins encoded by these genes, Hha and YdgT respectively, are also known to bind to H-NS (Garcia et al. 2005). It is believed that Hha and YdgT may act through H-NS to silence expression of some, but not all, H-NS bound genes (Nieto et al. 2000). However, there is some debate as to the mechanism by which Hha and YdgT may influence H-NS mediated gene expression. This debate is complicated by evidence suggesting that Hha and YdgT may bind DNA directly (Kim et al. 2005, Olekhnovich, Kadner 2007). It should be noted that, like H-NS,
*hha* and *ydgT* regulate many important *Salmonella* virulence factors (Fass, Groisman 2009), in particular those of *Salmonella* Pathogenicity Island 1 (Olekhnovich, Kadner 2007, Fahlen et al. 2001). As such, an understanding the mechanism by which Hha and YdgT influence H-NS activity will advance our understanding of how *Salmonella* regulates many of its virulence genes.

This study aims to determine whether Hha and YdgT influence gene expression solely through their interaction with H-NS. Transcription of *hha/ydgT* dependent genes was investigated in *hha/ydgT/hns* and *hns* strains in order to determine whether *hha/ydgT* could repress genes in the absence of H-NS. A site-directed mutagenesis approach was also used to determine the residues critical for H-NS/Hha interaction, explore whether an H-NS protein that cannot interact with Hha could be generated, and find out how such a protein would influence the regulation of known Hha and H-NS regulated genes in *Salmonella*.

### 1.2 Genomic islands, pathogenicity islands, and horizontal gene transfer

Horizontal gene transfer between bacteria was first observed when it was demonstrated that virulence determinants could be transferred between pneumococci, permitting infection of mice (Griffith 1928). This process was subsequently termed transformation. Since then, the genetic basis for transformation and other means of horizontal gene transfer (conjugation, phage-mediated transduction) have been studied in great detail. Over evolutionary time, bacterial genomes are bombarded by potential horizontal gene transfer events. For example, bacteriophages have been shown to encode cholera toxin in *Vibrio cholerae* (Waldor, Mekalanos 1996) and plasmids harbour virulence determinants in *Shigella Flexneri* (Watanabe, Nakamura 1985), *Salmonella enterica* (Wu et al. 2010) and *Yersinia enterocilitica* (Tabrizi, Robins-Browne 1992). Evidence from sequenced bacterial genomes suggests that stably horizontally acquired
genes are biased towards cell surface, DNA binding and pathogenicity genes (Nakamura et al. 2004).

Pathogenicity islands are an important class of horizontally acquired genes. These large (>10-200kb) genomic insertions are often flanked by small directly repeated sequences and associated with tRNA genes. Transfer RNA loci often act as integration sites for horizontally acquired genes. Since pathogenicity islands occur at these integration hotspots, they are often generated by multiple insertion events, resulting in a mosaic-like structure. Pathogenicity islands frequently encode regulators of genes within the island, allowing rapid incorporation of virulence genes into the genetic pathways of the recipient bacteria. (Hacker, Kaper 2000).

1.3 Salmonella enterica

The facultative intracellular pathogen Salmonella enterica is capable of causing a range of life threatening diseases in human and animal hosts. Salmonella is estimated to cause approximately 21.6 million cases of typhoid fever, resulting in over 200 thousand deaths each year and a further 5.4 million cases of paratyphoid fever (Crump, Luby & Mintz 2004). Typhoid fever, caused by the human adapted Salmonella subspecies Typhi, is endemic to regions in sub-Saharan Africa and Asia. However, the spectrum of Salmonella serotypes associated with disease outbreaks is ever changing. Currently there are an increasing number of cases of S. Paratyphi A caused disease in Asia (Crump, Mintz 2010). Salmonella continues to evolve and its evolution is in part shaped by its interaction with human hosts, particularly with respect to development of antibiotic resistance (Aypak et al. 2010).
1.3.1 Evolution of *Salmonella enterica*

*Salmonella* and *Escherichia coli* (*E. coli*) are believed to have diverged approximately 100 million years ago (Lawrence, Ochman 1998). This relatively short period of evolutionary divergence makes it unsurprising that these bacterial species have maintained similar gene order (i.e. are “syntenic”) for large portions of their genomes with relatively few rearrangements. Any given housekeeping gene in *E. coli* is about 85% identical in sequence to any given *Salmonella* housekeeping gene. That said, any individual *E. coli* strain may diverge by more than a quarter of its gene content from an individual *Salmonella* strain. The observed divergence in terms of gene content is explained by the fact that both *E. coli* and *Salmonella* have acquired numerous large sections of DNA via horizontal gene transfer (Groisman, Ochman 1997). *Salmonella enterica* serovar Typhimurium has five large pathogenicity islands termed *Salmonella Pathogenicity Islands* 1-5 (SPI1-SPI5). These horizontally acquired genes have been incorporated into complex regulatory circuits that permit *Salmonella* to precisely regulate their expression during infection.

1.3.2 *Salmonella* are adapted for infection

As facultative pathogens, *Salmonella* are adapted not only to the many environments they experience during the course of infection, but also several non-host environments such as water, soil, or manure. When living outside a host, *Salmonella* experience nutrient limitation, as well as wide variations in osmolarity, pH and temperature (Rozen, Belkin 2001). Enteric bacteria such as *Salmonella* are capable of utilizing a wide range of carbon sources and activating the necessary catabolic pathways in the presence of the catabolite (Savageau 1974). After expulsion from the digestive tract of an infected animal, *Salmonella* are capable of surviving in manure for over two months (Shepherd et al. 2010). *Salmonella* are also capable of surviving predation,
utilizing virulence factors to lyse protozoans that have ingested them (Feng et al. 2009). This highlights the fact that some genes used by *Salmonella* to colonize animal hosts may have evolved as defences against protozoans.

After ingestion by a mammalian host *Salmonella* must survive both the acidic environment of the stomach and the basic environment of the small intestine in order to cause infection. *Salmonella* is capable of penetrating the intestinal epithelium and replicating intracellularly within macrophages, dendritic cells and epithelial cells. Survival in any of the niches within a mammalian host, requires both activation and silencing of genetic programs appropriate to each environment the bacteria encounter in the course of infection (Marcus et al. 2000). *Salmonella* that fail to properly activate and repress genes in response to the appropriate cues suffer a severe competitive disadvantage (Silphaduang et al. 2007). *Salmonella* have 169 identified genes that are required for resistance to bile salts (Langridge et al. 2009), these genes may serve other functions in other environments, but the sheer number of genes required for bile-resistance speaks to the difficulty of surviving in the environment of the small intestine as well as how well *Salmonella* has adapted to it.

*Salmonella* Pathogenicity Islands, SPI-1 and SPI-2 each encode a type III secretion system (T3SS) (Fig. 1). Activation of SPI-1 genes is essential for the uptake of *Salmonella* by mammalian cells, while SPI-2 is required for replication within the intracellular niche and establishment of systemic infection (Hansen-Wester, Hensel 2001). *Salmonella* Pathogenicity Islands act in a coordinated manner during infection. For instance, *Salmonella* requires the use of factors encoded in both SPI-1 and SPI-4 in order to invade polarized epithelial cells (Gerlach et al. 2008). SPI-2 activation is controlled by the two component systems PhoP/PhoQ and OmpR/EnvZ, as well as HilD (Fass, Groisman 2009), an AraC/XylS-type transcription factor.
encoded on SPI-1 and SlyA, a MarR-family transcription factor encoded in the core *Salmonella* genome. Activation of any of these genes requires specific combinations of environmental cues. For instance, it is known that magnesium limitation and low pH are cues used by the bacterium to recognize the intracellular environment and activate SPI-2 gene expression (Lober et al. 2006). Magnesium limitation is recognized by PhoQ and acidic pH is recognized by EnvZ, with both of these pathways activating SPI-2 transcription through SsrA/SsrB and PhoP/PhoQ acting also through the H-NS anti-repressor SlyA (Fass, Groisman 2009). However, these same magnesium and pH cues may be encountered in non-host environments. In order to avoid inappropriate gene expression, signals such as magnesium limitation must be integrated with other host-specific cues such as mammalian body temperature (37°C) in order to overcome repression of SPI-2 by Hha and H-NS (Duong et al. 2007). This complex and tightly controlled network of genes allows *Salmonella* to survive in the lumen of the digestive tract, colonize epithelial cells, replicate within the gut associated lymphatic tissue and ultimately occupy the liver, spleen (Tang et al. 2002) and brain (Bollen et al. 2008, Wickham et al. 2007).
Figure 1. *Salmonella enterica* ssp. *enterica* serovar *typhimurium* chromosome and pathogenicity islands. Note that the GC content of the horizontally acquired Salmonella Pathogenicity Islands is significantly lower than the genomic average. This results in H-NS repressing many of the genes found in the SPIs.
1.4 H-NS: a silencer of horizontally acquired genes

H-NS was identified in two independent biochemical screens for “histone-like” proteins in *E. coli* (Varshavsky et al. 1977, Bakaev 1981). The protein was named H-NS for heat-stable nucleoid structuring protein during structural and functional studies of “histone-like” proteins isolated from *E. coli* (Falconi et al. 1988, Friedrich et al. 1988). This is clearly a misnomer since, despite having been initially identified in a screen for “histone-like” proteins, neither the sequence nor function of H-NS resembles that of a histone or histone subunit. H-NS is a small (15.5kDa, 137 amino acids) neutral protein consisting of a C-terminal DNA binding domain connected by a flexible linker to an N-terminal domain responsible for dimerization, higher order oligomerization of H-NS molecules and interaction with H-NS associated proteins (Garcia et al. 2006). The structure of the N-terminal (Bloch et al. 2003, Esposito et al. 2002, Renzoni et al. 2001, Cerdan et al. 2003) domain of H-NS has been solved by X-ray crystallography and NMR analysis. The C-terminal domain of H-NS has been determined by NMR analysis (Shindo et al. 1995, Shindo et al. 1999). The full-length structure of H-NS or any identified homologue remains unsolved (Fig 2).
Figure 2) Structure of H-NS dimerization domain with the residues of the Hha signature highlighted. Ribbon structures of two H-NS dimerization domains bound to one another, highlighting residues in the Hha-signature in red, with side chains of those residues also shown. I mutated each of these residues in this study. The structure shown at the top displays the proposed anti-parallel dimer conformation, solved with *E. coli* H-NS 1-64 (Bloch et al. 2003). The bottom structure displays the proposed parallel dimer conformation, solved with *Salmonella* H-NS 1-89 (Esposito et al. 2002). The conformation taken by full length H-NS dimers is unclear. In both of the above conformations, the residues in the Hha-signature are surface exposed.
H-NS is not widely distributed in the bacterial kingdom. A search for sequence homologues to H-NS reveals that H-NS like molecules are restricted to subsets of the alpha-, beta- and gamma-proteobacteria (Tendeng, Bertin 2003). H-NS-like molecules show considerable divergence in terms of primary sequence, however, and sequence similarity alone is insufficient to identify proteins that may function in a manner similar to that of H-NS. For example, functional analogs capable of complementing hns mutations in *E. coli* and *Salmonella* have been identified outside the proteobacteria including the Lsr2 protein of the *Actinobacteria* (Gordon et al. 2008, Gordon et al. 2010, Tendeng et al. 2003). Lsr2 bears no sequence similarity to H-NS and is also structurally distinct.

The genome-wide binding sites of H-NS have been mapped using chromatin-immunoprecipitation on microarray (ChIP on chip) analysis in both *Salmonella* (Navarre et al. 2006, Lucchini et al. 2006) and *E. coli* (Grainger et al. 2006, Oshima et al. 2006). These experiments determined that the regions bound directly by H-NS possess significantly higher AT content than the genomic average. Loss of *hns* in *Salmonella* results in derepression of a host of horizontally acquired virulence determinants not found in *E. coli*. This may help to explain why *hns* mutants are lethal in *Salmonella* unless accompanied by compensatory mutations, while they are relatively well tolerated in laboratory strains of *E. coli*. Bacterial virulence determinants are largely horizontally acquired, not only in *Salmonella*, but in many other species. As such, H-NS has been identified as the master virulence regulator of many bacterial species (Muller et al. 2006, Cathelyn et al. 2007, Castang et al. 2008, Liu, Naka & Crosa 2009).

H-NS affects horizontal gene transfer through not only its repression of horizontally acquired genes, but also by modulating plasmid transfer and transposition rates. H-NS has been
known to both increase (Starcic-Erjavec et al. 2003) and decrease (Will, Lu & Frost 2004, Will, Frost 2006) the rate of plasmid transfer, depending on the plasmid in question. Many plasmids have been identified that encode their own H-NS homologues (Forns et al. 2005). It has been theorized that these plasmid encoded H-NS homologues provide additional H-NS to host cells, allowing AT-rich plasmids to occupy host cells without titrating host H-NS away from its normal chromosomal binding sites (Doyle et al. 2007, Dillon et al. 2010). H-NS also has both activating and repressory roles with relation to transposition. H-NS is known to directly positively affect Tn10 transposition (Swingle et al. 2004, Shiga et al. 2001), through stabilization of the transpososome (Wardle et al. 2005), and indirectly negatively regulate transposition by bacteriophage Mu, through gene repression (van Ul sen et al. 1996b).

1.4.1 Pleiotropic gene regulation by H-NS

Mutations in *hns* in either *E. coli* or *Salmonella* result in highly pleiotropic phenotypes. Historically, this resulted in *hns* carrying the names *bglY* (Defez, De Felice 1981), *osmZ* (May et al. 1990), *drdX* (Goransson et al. 1990), *virR* (Hromockyj, Tucker & Maurelli 1992), *cur* (Diderichsen 1980), and *pilG* (Spears, Schauer & Orndorff 1986). Recently, genome wide transcript analysis (Hommais et al. 2001) and chromatin immunoprecipitation (Grainger et al. 2006, Oshima et al. 2006) studies of *hns* mutants and H-NS respectively, have shed light on the pleiotropic nature of *hns* regulation. H-NS is present in approximately 20,000 copies in exponentially growing *E. coli* (Ali Azam et al. 1999). This allows H-NS to bind approximately 250 loci and regulate expression of approximately 5% of all *E. coli* genes. In *Salmonella* H-NS regulates approximately 13% of genes (including chromosomal and virulence plasmid encoded genes) and binds 745 loci (Navarre et al. 2006). Smaller scale studies have also finely mapped the footprint of H-NS at model H-NS regulated genes. In particular, studies of *proV* (Lucht et al.
and *bglG* (Stratmann, Madhusudan & Schnetz 2008, Nagarajavel et al. 2007, Schnetz 1995) in *E. coli* have added to our understanding of the mechanisms of H-NS mediated gene repression.

### 1.4.2 Gene silencing mechanisms of H-NS

H-NS can bridge adjacent DNA duplexes (Dame et al. 2005). This property has resulted in the theory that H-NS may not only block transcription through the occlusion of RNAP from promoter regions, but also trap RNAP at promoters in repressive loops (Schroder, Wagner 2000, Olekhnovich, Kadner 2006, Dame et al. 2002) (Fig. 3). This allows H-NS to repress gene expression through binding to regions well upstream or downstream of the -35 and -10 core promoter elements (Dattananda, Rajkumari & Gowrishankar 1991, Overdier, Csonka 1992, Wolf et al. 2006). H-NS is also known to play a role in structuring the bacterial nucleoid by maintaining looped domains (Noom et al. 2007) and constraining local supercoiling found in those domains (Mojica, Higgins 1997, Hardy, Cozzarelli 2005). This may also be a means of H-NS mediated gene silencing of supercoiling sensitive promoters (Owen-Hughes et al. 1992).

Biochemical investigations into the DNA bridging properties of H-NS have been largely focused on the nucleoid structuring properties of H-NS, but yield insights into the gene silencing mechanisms of H-NS. Optical tweezers have been used to manipulate DNA complexes bridged by H-NS and measure the force necessary to separate such complexes (Dame, Noom & Wuite 2006). It was found that each H-NS dimer creates a barrier to transcription of 7 pN, meaning an individual H-NS molecule may be easily displaced by translocating RNAP (25 pN) (Wang et al. 1998). A binding sequence overrepresented in genes bound by H-NS, to which H-NS has been found to bind with high affinity (Kd=15 nM), has recently been described (Bouffartigues et al. 2007). Combining this potential nucleation site on DNA with the known property of H-NS to...
form extended footprints on DNA, has led to a “bind and spread” model of H-NS binding to DNA (Bouffartigues et al. 2007, Lang et al. 2007). This helps to explain the fact that while an individual H-NS molecule bound to DNA provides little barrier to transcription, H-NS is capable of silencing many genes in vivo.
Figure 3) Diagram of H-NS trapping RNAP in a repressive loop. Note that sequences upstream of the promoter can serve as functional H-NS binding sites as has been observed for several H-NS regulated promoters including $bg{\_}l$, $proU$, $spv$ and $rpl$ (see text).
1.4.3 Countersilencing of H-NS

Activation of many H-NS repressed genes has been shown to be the result of H-NS antirepression (Navarre et al. 2005, Mellies et al. 2008), rather than activation per se via the direct recruitment of RNAP. Antirepression mechanisms range from the ability of RNAP associated with $\sigma^s$ to overcome H-NS silencing at specific promoters (Robbe-Saule et al. 1997) activating alterations in DNA structure by nucleoid associated proteins Fis (Falconi et al. 1996), HU (Dame, Goosen 2002) and IHF (van Ulsen et al. 1996a); competition for binding sites on DNA by sequence specific DNA binding factors (Navarre et al. 2005); to protein-protein interactions with H-NS itself (Liu, Richardson 1993). In many cases it has been demonstrated that productive transcription can occur without complete displacement of H-NS from the promoter. Direct testing in vitro has demonstrated that SlyA does not displace H-NS from pagC or ugtL promoters, but that its interaction with these promoters does result in transcription (Perez, Latifi & Groisman 2008). Further testing will be necessary to determine a mechanism, but it is assumed that in cases where transcription occurs without H-NS displacement, an alteration in the structure of the nucleoprotein complex must occur to enable RNAP binding, open complex formation and productive transcription.

1.4.4 The H-NS paralogue StpA

*Salmonella* or *E. coli* possess an H-NS parologue, StpA that shares 52% sequence identity (comparing *E. coli* K12 to *Salmonella enterica* serovar Typhimurium LT2, Fig. 4a) and is able to functionally substitute for H-NS for a variety of phenotypes (Shi, Bennett 1994). The *stpA* gene was initially identified based on its ability to suppress the mutant phenotype of a
plasmid encoded \textit{td-} gene from bacteriophage T4 (Zhang, Belfort 1992). It should be noted that while StpA is a parologue of H-NS, few phenotypes have been associated with mutations in \textit{stpA} without an additional mutation in \textit{hns}. Thus, while \textit{hns/stpA} double mutants have more severe growth defects than \textit{hns} mutants, StpA is not believed to modulate the function of H-NS in the same way as Hha or YdgT. Chromatin immunoprecipitation studies have shown that in WT cells the StpA binding profile overlaps that of H-NS, while it is reduced to one-third the binding sites in an \textit{hns} mutant (Uyar et al. 2009). In the absence of H-NS, StpA is known to be rapidly degraded by the Lon protease (Johansson, Uhlin 1999). This may help explain why \textit{hns} mutants have much more severe phenotypes than \textit{stpA} mutants and why the StpA binding profile is reduced in the absence of H-NS, since StpA does not accumulate beyond 10\% of wild-type levels in an \textit{hns} knockout (Sonnenfield et al. 2001).

\section*{1.5 Hha and YdgT modulate H-NS function}

Two paralogous proteins, Hha and YdgT (Fig. 4b), have been shown to bind to both H-NS and StpA (Paytubi et al. 2004). Hha mutants were originally identified by their enhanced expression of \textit{E. coli} alpha-haemolysin (Godessart et al. 1988, Nieto et al. 1991). Since then \textit{hha} and \textit{ydgT} have been associated with the regulation of many horizontally acquired virulence genes in both \textit{E. coli} (Sharma, Zuerner 2004) and \textit{Salmonella} (Vivero et al. 2008, Olekhnovich, Kadner 2007, Fahlen et al. 2001). Notably, mutations in \textit{hha} result in more severe phenotypes than mutations in \textit{ydgT} and the double \textit{hha/ydgT} mutant results in phenotypes of greater severity than the additive effects of the single mutants (Silphaduang et al. 2007, Paytubi et al. 2004). Taken together these data suggest that while Hha and YdgT show significant functional redundancy, Hha is the dominant molecule, with YdgT playing a “backup” role. Mutations in \textit{hha} or \textit{ydgT}
result in multiple phenotypes resembling those associated with *hns* including altered plasmid supercoiling (Carmona et al. 1993) and stimulation of transposition (Balsalobre et al. 1996).
a) Score = 121 bits (303), Expect = 4e-33, Method: Compositional matrix adjust.
Identities = 71/135 (53%), Positives = 91/135 (68%), Gaps = 2/135 (1%)

H-NS MSEALKILNINRTLRAQARECTLTEEMLEKLEVVNERREESSAAAEVEERTRKLQ
M+ L+ LNNIRTLRA ARE +++ LEEMLEK VV ERREEE ++ E+ K+
StpA MNMLQNINNIRTLRAMAREFSIDVLEEMLEKFRVVTKERREEEELQQRQLAEKQEKIN

H-NS QYREMLIADGIDPENNLLNSMAAKSGTKAKRAARPARKSYVVDENGETKTWTGQGRTPAV
+ E++ ADGI+P EL +M +A + KR RPAKY + D NGE KTWGQGRTP
StpA AFLELMKADGINPEELF-AMDSAMPRSAKKRPQPRAKYRFDFNGEKEKTWTGQGRTPKP

H-NS IKKAMEEQQKQLEDFLI
I +A+ GK L+DFLI
StpA IAQAL-AAGKSLDDFLI

b) Score = 53.5 bits (127), Expect = 3e-13, Method: Compositional matrix adjust.
Identities = 25/71 (36%), Positives = 49/71 (70%), Gaps = 4/71 (5%)

Hha LTKTDYLMRLRRCQTIDTELVIEKONYELSDN-ELAVFYSADHRLAEL-TMNKLYD-
+T DYL++ R+ +++LE++ + Y L+D+ ++ Y AADHR AEL + +L+D
YdgT MTVQDYLLKFRKISSLESLEKLFDFHLNYTLTDDMDIVNMRAADHRRAELVSGGLFDV

Hha -KIPSSVWKFIR
++P SVW++++
YdgT GQVPQSVRYVQ

Figure 4a) Alignment of H-NS and StpS protein sequences from *Salmonella enterica* ssp. Typhimurium LT2 and BLAST alignment scores.  b) Alignment of Hha and YdgT protein sequences from *S. enterica* ssp. Typhimurium LT2 and BLAST alignment scores.
1.5.1 Biochemical properties of Hha/YdgT

Hha and YdgT structurally resemble the N-terminal dimerization domain of H-NS (Bae et al. 2008) and a fusion protein with Hha in place of the N-terminal dimerization domain of H-NS can complement some hns phenotypes (Rodriguez et al. 2005). Hha and YdgT have been found to bind the N-terminal domain of H-NS (Garcia et al. 2006), potentially recognizing an “Hha signature” (NNIRTL) found therein (Madrid et al. 2007)(Fig. 2). This has led to speculation that Hha/YdgT may act as functional analogues of the N-terminal domain of H-NS, inserting themselves in H-NS-DNA complexes. It is unclear how Hha/YdgT might enhance H-NS mediated gene repression by acting as dimerization domain substitutes or binding to the dimerization domains of H-NS dimers or oligomers. This proposed mechanism is complicated by other evidence that Hha and YdgT may be capable of binding directly to DNA (Kim et al. 2005, Olekhnovich, Kadner 2007). So, while the protein-protein interaction between H-NS and Hha has been well characterized, the manner in which this relationship influences gene expression is still unknown.

1.5.2 H-NS variants that are “blind” to Hha/YdgT

In order to investigate the relationship between Hha/YdgT and H-NS, one tool we can turn to is H-NS mutant proteins which are incapable of binding Hha/YdgT. Two such mutants have already been described. An NMR study found that an R12H substitution in H-NS appeared to abrogate H-NS-Hha binding. NMR spectra were used as the readout in this study and no in vivo characterization of this H-NS mutant was carried out (Garcia et al. 2006). Another study by the same group investigated the fact that Vibrio cholerae possesses an H-NS homologue, but
lacks an Hha homologue. An amino acid alignment demonstrated that the Hha binding signature was present in *Vibrio* H-NS, but the asparagine at the ninth amino acid was substituted for a leucine. They then found that introducing the N9L substitution in *E. coli* H-NS abrogated Hha binding, while introducing an L9N substitution in *Vibrio* H-NS enabled it to bind Hha. Some preliminary phenotypic and biochemical analysis was performed in this study. *Vibrio* H-NS and *E. coli* H-NS-N9L were found to repress *hlyA* to a greater degree than WT *E. coli* H-NS. They were also found to bind an *hlyA* fragment with greater affinity than WT *E. coli* H-NS (Garcia et al. 2009).

1.6 Rationale and approach

It is presently unclear whether Hha and YdgT influence gene expression solely through their interaction with H-NS, or whether they are capable of acting independently. To address the necessity of H-NS in Hha/YdgT-mediated gene regulation I:

1. Examined gene expression in strains deficient in Hha, YdgT, H-NS and StpA alone and in combinations to determine if an effect of Hha and YdgT could be detected in the absence of H-NS. This approach was also used to determine which H-NS-regulated genes also required Hha/YdgT for repression.

2. Examined H-NS binding to several loci in the absence of Hha and YdgT

3. Generated point mutants in the “Hha-signature” motif of H-NS to determine which residues were critical for the interaction between Hha and H-NS.

4. Examined expression of Hha/YdgT-dependent genes in strains harboring H-NS molecules defective for their interaction with Hha.
5. Examined the DNA-binding and oligomerization properties of the “Hha-blind” H-NS mutant proteins.

For these studies I employed the model organism *Salmonella enterica* serovar Typhimurium to examine the relationship between H-NS and Hha/YdgT with relation to regulation of gene expression. This model has specifically been selected because *hha* and *ydgT* are known to be responsible for silencing virulence genes in SPI-2, prior to their activation in the intracellular environment (Silphaduang et al. 2007). Further, a recent study identified the *hha/ydgT* regulon in the *S. enterica* ssp. Typhimurium LT2 sequenced strain (Vivero et al. 2008). This study indicated that the *hha/ydgT* regulon is largely a subset of the well-characterized *hns* regulon, for which we have not only transcript but also H-NS binding site data (Navarre et al. 2006, Lucchini et al. 2006). This allows identification of genes controlled directly by H-NS in conjunction with Hha/YdgT and those controlled directly by H-NS alone. Such genes may be used to examine the hypothesis that Hha/YdgT act only through H-NS. In addition to the existing supporting data, *Salmonella* makes an excellent model for this study due to the wealth of available tools in this system. *Salmonella* is highly genetically tractable, with the ability to generate knockouts (Datsenko, Wanner 2000) and tagged alleles on the chromosome. The close relation of *Salmonella* to *E. coli* also allows ready comparison between the two species and gives us clues into the evolution of pathogenicity gene regulation in *Salmonella*. 
2 Experimental Procedures
2.1 Construction of *Salmonella* mutant strains used in this study

*Salmonella enterica* Serovar Typhimurium strain LT2 *hns::km, hha::cm* and *ydgT::cm* mutant strains were generated using P22-mediated transduction of disrupted alleles generated by Dr. William Navarre in previous studies (Navarre et al. 2006). Alleles were moved into the LT2 strain background by transduction using phage P22 HT105/1 *int-201* (Schmieger, 1971). The origins and properties of strains used in this study are outlined in the accompanying Table of Strains.

2.2 Generation of amino-acid substitution mutants

Parental plasmids pWN425, a low-copy plasmid containing the *hns* open-reading frame under control of its native promoter (Navarre et al. 2006), and pSSA4, with the *hns* open reading frame cloned into the *NdeI* and *XhoI* restriction sites of pET21b (generated by Sabrina Ali), were mutagenized using site-directed mutagenesis. Complementary PCR primers were designed using the formula $T_m = 81.5 + 0.41(\%GC) - 675/N$, where $T_m$ is melting temperature in degrees Celsius, $\%GC$ and $\%$ mismatch are whole numbers. Primers were designed such that the calculated melting temperature was less than or equal to 78 °C. The desired base substitution was located in the middle of each primer.

PCR was carried out using 5 μL 10X *Pfu* buffer with MgSO$_4$, 2.1 μL primer mix (complementary primers mixed and diluted in ddH$_2$O to 100 ng/μL), 3.2 μL parental plasmid (diluted to 1 ng/μL), 10 μL 1mM dNTPs and 1 μL *Pfu* DNA polymerase. PCR was carried out using the following program: 30s at 95 °C, 30s at 50 °C and 5:10min at 68 °C for 24 cycles.
PCR products were cleaned using the QIAGEN QIAquick PCR Cleanup Kit and were eluted using 45 μL of ddH₂O at 65 °C. Parental plasmid was then digested using DpnI, by adding 5 μL NEBuffer 4 and 1 μL DpnI to the eluted PCR products and incubating overnight at 37 °C. Following digestion, salts were dialyzed away from the PCR products using drop dialysis with Millipore 0.025 μm VSWP filters placed on 1.5 mL of ddH₂O for one hour.

Following cleanup, the mutagenized plasmids generated by PCR were electroporated into DH5α electrocompetent cells, those cells were incubated for one hour in LB at 37°C before being plated on selective media (10 μg/ml Cm for pWN425 derived mutant plasmids and 100 μg/ml amp for SSA2 derived mutant plasmids). Colonies were allowed to grow on selective LB agar overnight and five colonies from each plate were picked and grown in 4 mL LB with the appropriate antibiotic overnight. Each of these overnight cultures was used to purify plasmid using a Fermentas GeneJet Plasmid Miniprep Kit. The plasmids were then sent for sequencing at the SickKids Centre for Applied Genomics (TCAG) sequencing facility. Those with reads indicating successful mutation at the targeted sequence and a lack of mutations in the remainder of the sequence were then retransduced into DH5α and an LT2 hns::km strain (for pWN425 derivatives) or co-transduced with pCDFhha into BL21 hns::cm.

2.3 RT-QPCR analysis of transcription

Cultures were grown overnight in LB media, subcultured 1:200 in fresh LB media and grown to mid-exponential phase as determined by A₆₀₀ (A₆₀₀ = 0.45-0.6). At this point, 500 μL of culture was mixed with 1 mL of QIAGEN RNA Protect Bacterial Reagent in a 1.5 mL Eppendorf tube, vortexed for 10 seconds and incubated at room temperature for 30 minutes. Cells were then spun down at 14,000 rpm and the supernatant was removed by vacuum pump. If the pellet was not immediately processed for RNA extraction, then it was frozen at -20°C.
RNA was extracted from the cell pellets using the BioRad Aurum Total RNA Mini Kit protocol. The extracted RNA was run on a 1% agarose gel stained with ethidium bromide to verify that it had not become degraded during purification. Quantification and analysis of RNA purity was carried out by taking the absorbance of 260nm/280nm light in a spectrophotometer. RNA with a 260/280 ratio of 1.96-2.04 was considered pure and used for further analysis.

Reverse transcription was performed using the protocol for random hexamer primers for the BioRad iScript Select cDNA Synthesis Kit. Twenty microlitre reverse transcription reactions were allowed to proceed for 2 hours before the reactions were diluted with ddH₂O to a final volume of 500μL.

2.4 ChIP analysis of protein binding to genes in vivo

Cultures were grown overnight in LB media, subcultured 1:200 in 50 mL fresh LB media and grown to mid-exponential phase as determined by $A_{600} = 0.45-0.6$. At this point, 1.4 mL 30% formaldehyde was added to the culture. After incubating at room temperature for 15 minutes, 5.5 mL of 130 mM glycine was added to quench the formaldehyde. After incubating for at least 5 minutes at room temperature, the cells were centrifuged for 10 minutes at 5000 X g. The pellets were washed twice with ice cold PBS and spun down for 10 minutes at 5000 X g between each wash. These pellets were then frozen at -20 °C.

Pellets were resuspended in 500 μL of cell lysis buffer (10 mM Tris pH 8.0, 50 mM NaCl, 10 mM EDTA, 20% sucrose and 10 mg/mL lysozyme). Forty microlitres of Sigma “Protease Inhibitor Cocktail for use with bacterial cell extracts” was added to each resuspension. Five-hundred and forty microlitres of 2X RIPA buffer (100 mM Tris pH 8.0, 300 mM NaCL, 2% NP40 Alternative, 1% Sodium Deoxycholate and 2% SDS) was added to each resuspension and
samples were mixed by repeated pipetting. Samples were transferred to 15 mL polystyrene conical tubes with Diagenode sonicator probes. Samples were sonicated for 15 minutes using a 30 seconds on, 30 seconds off duty cycle in a Diagenode Bioruptor sonicating waterbath filled with ice water. Sonicated samples were transferred to 1.5mL tubes and spun down for 30 minutes at 14000 rpm at 4°C. Supernatant was removed, centrifuged again under the same conditions and the supernatant from this second centrifugation was separated for use in all later steps. Following this, 200 μL was removed and saved as a “total DNA” control. 80 μL of protein G-agarose beads and 9 μL of anti-FLAG antibody (Sigma-Aldrich anti-FLAG M2 monoclonal antibody, for FLAG-tagged H-NS) was added to the remaining supernatant and agitated overnight at 4 °C.

Beads were spun down at 5000 rpm at 4 °C and the supernatant was removed. The beads were then washed twice with 1 mL 1X RIPA buffer (50mM Tris pH 8.0, 150 mM NaCl, 1% NP40 Alternative, 0.5% Sodium Deoxycholate and 1% SDS), twice with 1 mL LiCl buffer (10 mM Tris pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP40 Alternative, 0.5% Sodium Deoxycholate) and once with 1 mL TE. The beads were centrifuged between each wash at 5000 rpm at 4 °C and the supernatant was removed. After all the above washes, the beads were resuspended in 110 μL elution buffer (50 mM Tris pH 8.0, 10 mM EDTA and 1% SDS) and incubated for 5 minutes in a 65 °C water bath. The beads were then centrifuged, the supernatant was collected, transferred to a new tube and incubated at 65°C for 5 hours. Following incubation, the samples were centrifuged for 15 minutes at 15,000 rpm. The supernatant was collected and transferred to a new tube.
At this point, 500 μL of Qiagen buffer PB was added to each sample and they were cleaned using the Qiagen Quiaquick PCR Cleanup kit and protocol. DNA eluted at the end of the protocol in 100 μL ddH₂O. Samples were then diluted to 500 μL total volume with ddH₂O.

Samples were used to examine enrichment of gene sequences bound by H-NS or RNAP by comparing the abundance of the gene in the total DNA control to the IP sample by Q-PCR. Enrichment data from each gene in each sample was normalized to the input of a given gene by multiplying enrichment by $\frac{\text{Gene } X_{\text{WT total DNA}}}{\text{Gene } X_{\text{mutant total DNA}}}$ (where “Gene X” is the gene of interest).

2.5 Western Blot

Blots were performed using polyclonal anti-FLAG rabbit antibodies (Sigma-Aldrich) or polyclonal anti-HA rabbit antibodies (Sigma-Aldrich), which were detected with goat anti-rabbit antibodies conjugated to horseradish peroxidase following standard Western blotting procedure (Burnette 1981).

2.6 Overexpression and purification of H-NS and Hha-H-NS

*Escherichia coli* BL21 (DE3) was transformed with a plasmid containing the *Salmonella* ssp. Typhimurium LT2 *hns* gene driven by a T7 promoter (pSSA4 or mutagenized derivatives thereof), other strains were also co-transformed with a plasmid containing the *Salmonella* ssp. Typhimurium LT2 *hha* gene also driven by a T7 promoter (pCDF-hha). These strains were grown to mid-log phase ($A_{600} = 0.5$) in LB media at 37 °C before T7 polymerase dependent gene expression was induced by the addition of 100 μM IPTG to the culture media. Induced cultures were grown for 3 hours before being centrifuged and cell pellets were frozen at -20 °C. Cells were resuspended in cell lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH
8.0) and lysed by sonication in 15 mL polystyrene conical tubes Diagenode sonicator probes and the Diagenode Bioruptor sonicating water bath filled with ice water. Whole cell lysates were then centrifuged for 45 minutes at 9000 rpm at 4 °C to separate soluble and insoluble fractions. Insoluble fractions were solubilised using a denaturing buffer (8 M Urea pH 8.0, 100 mM NaH₂PO₄, 10 mM Tris-Cl).

His₆-tagged proteins were bound by incubation with Qiagen Ni-NTA agarose resin at 4 °C in cell lysis buffer. The resin was then loaded onto a column and washed thoroughly with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). Finally, the proteins were eluted off the column using elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). Each fraction (incubation flow through, wash and elution) was collected and run on SDS-PAGE to assess protein recovery and protein purity. Protein concentrations were measured using a Bradford assay, as standardized using a serial dilution of BSA.

2.7 Electrophoretic mobility shift assays

DNA substrates were generated by PCR and gel extracted to ensure purity and remove residual unincorporated primers. DNA substrates were used at 15 ng per reaction for target DNA and 20 ng per reaction for control DNA. Protein and DNA were combined in 20 μL reaction volumes containing 40 mM KCl, 15 mM HEPES (pH 8), 1 mM EDTA, 0.5 mM DTT and 5% glycerol. The reaction mixtures were incubated at room temperature for 30 minutes before DNA loading dye was added and the samples were loaded in an acrylamide gel.

Gels contained 6% acrylamide (30% Acryl:1% Bis), 1 X TAE, 10% ammonium persulfate and 0.06% TEMED. Gels were pre-run for one hour at 100V at 4 °C. Gels were run for four hours at 65 V at 4 °C. Gels were stained with SYBR-green at room temperature for
thirty minutes and scanned on a Typhoon scanner at 100 μm resolution using a 488 nm filter and an exciting wavelength of 520 nm.

2.8 Gel filtration chromatography

Samples of H-NS at a concentration of 3 mg/mL were run on a Sephadex S-200 column with a flow rate of 1 mL per minute for 90 minutes. H-NS was stored and run at 4°C, in a buffer consisting of 10 mM Tris, 150 mM NaCl, 2.5 mM EDTA, 2 mM DTT and 5% glycerol. UV absorbance at 280 nm was recorded throughout the run and 1 mL fractions were collected.

2.9 β-galactosidase reporter assay

Reporter constructs were generated by ligation of PCR generated promoter fragments (Fig. 4) into the EcoRI and BamHI sites of a pRS1274 vector containing a promoterless LacZ gene.

Cells were grown overnight in LB at 37°C, subcultured into fresh LB and grown to $A_{600} = 0.5$ before they were centrifuged for 10 minutes at 5000 g. Pelleted cells were resuspended in 1mL of breaking buffer (0.1 M Tris, pH 8.0, 20% glycerol, 1 mM DTT), transferred to 1.5 mL tubes and disrupted by sonication for 5 minutes in ice water using a Diagenode Bioruptor sonicating water bath. The resulting protein extracts were then centrifuged for 30 minutes at 15000 rpm at 4°C. Protein concentrations in the protein extracts were determined by Bradford assay and β-galactosidase activities were determined as described by Miller (1972) and are given in units per microgram of protein in the whole cell extract.
Figure 5. Promoter regions inserted in β-galactosidase reporter constructs. The top diagram represents the fragment of the sifB promoter used and the bottom diagram the fragment of the ssrA promoter used. In both cases the fragment used ends with the nucleotides immediately preceding the translation start site of the gene.
2.10 Strain construction

I used and created a variety of *E. coli* and *Salmonella enterica* strains during the course of this study. A complete description of such strains can be found in the strain table on page 37. To create the strains necessary for the study of Hha and YdgT-mediated gene regulation I obtained knockouts in *hha* (*hha::Km-FRT*), *ydgT* (*ydgT::Cm*), *hns* (*hns::Km-FRT*) and *stpA* (*stpA::Cm*) from Dr. William Navarre. It has been determined through microarray analysis that single mutations in *hha* or *ydgT* do not result in a significant change in the transcriptional profile of *Salmonella* (Vivero et al. 2008), therefore I used P22 phage transduction to introduce the *ydgT::cm* mutation into the *hha::km-FRT* background. In order to investigate whether *hha/ydgT* may be capable of gene regulation independent of *hns* I constructed an *hha/ydgT/hns* triple mutant. To do this I used Flip recombinase to excise the km cassette which had replaced the *hha* gene and subsequently introduced an *hns::km* mutation via P22 phage transduction. I also introduced an *hns::km* mutation into a *stpA::cm* background, generating an *hns::km/stpA:cm* strain which could be expected to display greater derepression of *hns* regulated genes than the *hns* single mutant.
<table>
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3 Results
3.1 Growth of hha/ydgT mutant Salmonella

The double hha::km-FRT/ydgT::cm mutant displayed a marked growth defect both on solid and in liquid media. This strain also did not produce mucoid colonies on agar plates, a phenotype classically associated with hns mutants (Navarre et al. 2006). The hha/ydgT strain grew slightly faster than an hns mutant, supporting the hypothesis that hha/ydgT may regulate fewer genes than hns. The hha/ydgT/hns mutant strain grew at approximately the same rate as an hns mutant, while the hns/stpA strain was much slower in its growth. Both strains grew as mucoid colonies on agar plates (data not shown). These observations add to a large body of circumstantial evidence suggesting that Hha and YdgT affect gene expression only through their interaction with H-NS. However, there is contradictory evidence that Hha and YdgT may bind DNA in the absence of H-NS (Kim et al. 2005, Olekhnovich, Kadner 2007, Fahlen et al. 2001, Olekhnovich, Kadner 2006).

3.2 Identification of hha/ydgT repressed and independent genes

In order to investigate how hha and ydgT might affect gene expression through their relationship with H-NS, I first needed to identify a gene or set of genes that display the regulatory features that would make them ideal for mechanistic dissection. Genes known to be bound by H-NS (Navarre et al. 2006, Lucchini et al. 2006) and derepressed in the absence of hha and ydgT would be classified as “Hha/YdgT-dependent”. “Hha/YdgT-independent” genes are also repressed by H-NS, but their transcription is unaffected by the deletion of hha and ydgT. A recent identification of the hha/ydgT regulon through microarray analysis of the Salmonella hha/ydgT double mutant (Vivero et al. 2008) revealed that the overlap between the genes
repressed by hha/ydgT and those bound by H-NS was incomplete, yielding genes in both my “dependent” and ”independent” categories. Using the data from the hha/ydgT microarray study and the available H-NS ChIP-chip data, I identified the sifB and pipB genes as candidate Hha-dependent genes and the sifA, leuO and prov genes as independent genes to serve as negative controls.

In order to verify that these genes could be categorized in this way and used in subsequent studies, I used RT-Q-PCR to examine the transcription of each of these genes in WT, hha::km, ydgT::cm, hha::km/ydgT::cm and hns::km strains of Salmonella enterica LT2 (Fig. 5). I found that the microarray data was accurate in identifying genes upregulated in the hha/ydgT mutant strain. The sifB gene was approximately 9 and pipB approximately 16 fold more highly expressed in the hha/ydgT mutant strain than the wild-type strain, nearly matching the approximately 10 and 16 fold upregulation of these respective genes in the hns strain. However, the microarray data was inaccurate in identifying genes whose regulation was unaffected by hha/ydgT. I observed approximately 7 fold derepression of sifA and 4 fold derepression of leuO in the hha/ydgT mutant, resembling the 9 and 5 fold increase in transcription seen in the hns mutant strain as compared to wild-type (Fig. 5a). Of the genes I investigated, only the prov gene remained repressed when hha and ydgT were deleted (Fig. 5b). As such, prov was used as the sole negative control gene in all later experiments, while all of sifA, sifB, pipB and leuO were taken to be Hha-depdendent.
Figure 6) Identification of \textit{hha/ydgT} responsive and independent genes in \textit{Salmonella} gene knockout mutants, via RT-Q-PCR. a) Expression data for \textit{sifA}, \textit{sifB}, \textit{pipB} and \textit{leuO} genes, generated by RT-Q-PCR (N=3). Each gene retains repression in the \textit{hha} and \textit{ydgT} mutants and is derepressed to a similar degree in the \textit{hns} and \textit{hha/ydgT} strains. b) Expression
data for *proV*, generated by RT-Q-PCR (N=3). This gene is derepressed in an *hns* mutant, but repression is retained in the *hha, ydgT* and *hha/ydgT* backgrounds.
3.3 Analysis of *hha/ydgT* effects independent of *hns* using multiple mutants

Having identified one negative control and several genes regulated by both H-NS and *hha/ydgT*, I proceeded to examine the expression of these genes in *hha*, *ydgT*, *stpA*, and *hns* mutant strains as well as the *hha/ydgT*, *hha/ydgT/hns* and *stpA/hns* mutant backgrounds. I theorized that if *hha* and *ydgT* were capable of acting independently of H-NS, then an effect of Hha and YdgT on gene expression should be observed in the absence of H-NS. To test this I examined the expression of *hha/ydgT* repressed genes (*sifA*, *sifB*, *pipB* and *leuO*) in the *hns* strain and the *hha/ydgT/hns* strain using Q-PCR. I used a *hns/stpA* strain as a positive control, since *hns/stpA* mutants are known to show greater derepression of H-NS repressed genes than *hns* single mutants.

My results indicate that the *hha/ydgT/hns* strain did not display expression of any *hha/ydgT* repressed gene greater than that found in the *hns* strain, while the *hns/stpA* strain had greater expression levels for all *hha/ydgT* repressed genes tested (Fig 6). For example, *sifB* was derepressed approximately nine fold in the *hha/ydgT*, ten fold in the *hns*, seven fold in the *hha/ydgT/hns* and fifteen fold in the *hns/stpA* strain. This supports the hypothesis that Hha and YdgT can only act through H-NS to repress genes such as *sifB*.
Figure 7) Expression of hha/ydgT responsive and independent genes in *Salmonella* multiple gene knockout mutants, via RT-Q-PCR. Expression data for *sifA*, *sifB*, *pipB* and *leuO*, generated by RT-Q-PCR (N=3). All genes were repressed in the WT, *hha*, *ydgT* and *stpA* strains. Derepression is observed in the *hns*, *hha/ydgT*, *hha/ydgT/hns* and *hns/stpA* strains. The *hha/ydgT/hns* strain does not yield expression of any of these genes beyond the level seen in the *hns* strain, while the *hns/stpA* strain does display such further derepression.
3.4 Analysis of H-NS occupancy of hha/ydgT repressed genes in hha/ydgT mutants

Hha and YdgT bind H-NS (Garcia et al. 2005, Garcia et al. 2006), affect repression of a subset of the H-NS regulated genes (Vivero et al. 2008) and, from my work, appear to be unable to act independently of H-NS. This knowledge led me to question how Hha and YdgT might affect the ability of H-NS to bind the genes that it regulates, in vivo. Previous studies have employed in vitro approaches to examine the interactions of Hha and H-NS with genes or promoters of interest (Olekhnovich, Kadner 2007, Olekhnovich, Kadner 2006, Ellison, Miller 2006), however there has been no in vivo investigation into the effects of Hha and YdgT activity on H-NS DNA binding.

To address this, I used chromatin-immunoprecipitation of H-NS in Salmonella strains with a C-terminally FLAG-tagged copy of hns incorporated at the native hns locus on the chromosome; this strain acted as my wild-type for these experiments. From the tagged hns strain, I derived hha, ydgT and hha/ydgT mutants using P22-mediated transduction. Anti-FLAG antibodies were used to immunoprecipitate tagged H-NS cross-linked to DNA from each strain. I used Q-PCR to measure the amount of DNA of interest (sifB or proV) in the input to the ChIP and standardized the ChIP enrichment in each strain to its input. I hypothesized that if Hha/YdgT increase the affinity of H-NS for some sequences, I might observe depletion of H-NS binding at specific regions of the chromosome in the hha::km/ydgT::cm mutant strain. I further theorized that such regions of H-NS depletion would correspond to hha/ydgT repressed genes as assessed by transcript analysis. The sifB and proV genes were selected since the sifB gene was strongly derepressed in the hha/ydgT strain and I found regulation of the proV gene to be hha/ydgT independent. The proV gene also provided a good control, since its H-NS binding sites
have been well mapped on a fine scale using classical DNase footprinting approaches on template DNA in *vitro* (Lucht et al. 1994, Nagarajavel et al. 2007). In order to account for the fact that I could not predict *a priori* where H-NS occupancy of my candidate genes might change, I used Q-PCR with primer pairs covering 800 bp of the *sifB* and 400 bp of the *proV* promoters. Primers were designed to amplify several adjacent but non-overlapping ~100 bp regions comprising 400 bp upstream and 400 bp downstream of the *sifB* translation start site.

Since the *proV* promoter is much better characterized than that of *sifB*, particularly with respect to H-NS binding sites (Lucht et al. 1994, Overdier, Csonka 1992), I chose to examine only the 400 bp upstream of the *proV* translation start site. I also used the GC-neutral gene STM1033 as a negative control. STM1033 has been shown to not interact with H-NS *in vivo* (Navarre et al. 2006).

The entire 800 bp region comprising 400 bp upstream and downstream of the *sifB* translation start site was found to be depleted of H-NS binding in the *hha* and *hha/ydgT* strains as compared to the wild-type. More specifically, a region of the *sifB* promoter comprising the 300 bp immediately upstream of the translation start site displayed approximately three-fold depletion of H-NS binding in the *hha/ydgT* mutant as compared to the wild-type strain (Fig. 7a). The *ydgT* mutant strain displayed slightly (1.3-fold) greater H-NS enrichment to the entire 800 bp *sifB* region than the wild-type strain, consistent with the lack of difference in the expression of *sifB* in the wild-type and *ydgT* strains. The region of the *proV* gene comprising 400 bp upstream of the translation start site displayed a slight loss of H-NS occupancy in the *hha* strain and a slight increase in H-NS occupancy in both the *ydgT* and *hha/ydgT* strains, as compared to the wild-type (Fig. 7b). This is consistent with the lack of change in the transcription of *proV* observed in these strains in my earlier experiments.
Figure 8) H-NS occupancy of the *sifB* and *proV* promoters in *hha*, *ydgT* and *hha/ydgT* mutants relative to wild-type.  

**a)** ChIP of H-NS using RT-PCR as a readout, with primers against adjacent regions of the *sifB* promoter and gene (N=2). ChIP enrichment relative to that found in wild-type cells is shown for each region in each of the *hha*, *ydgT* and *hha/ydgT* mutants. The *hha/ydgT* mutant displays a marked depletion of H-NS relative to wild-type in the region comprising 300bp upstream from the translation start site of *sifB*.  

**b)** ChIP of H-NS using RT-PCR as a readout, with primers against adjacent regions of the *proV* promoter (N=1). ChIP
enrichment relative to that found in wild-type cells is shown for each region in each of the hha, ydgT and hha/ydgT mutants.

The data are consistent with a model where loss of Hha/YdgT leads to disruption of H-NS binding at sifB and that this is the primary event that causes the increase in sifB transcript levels observed in the mutant strains. Alternative explanations are also possible, including that H-NS may be displaced by the binding of a transcription factor that is directly or indirectly controlled by Hha/YdgT. Another possibility is that RNA polymerase itself is triggering localized H-NS depletion. However, these findings regarding H-NS gene occupancy indicate that Hha/YdgT have an effect on the binding profile of H-NS in vivo and that H-NS binding to hha/ydgT independent genes such as proV is likely to be unaffected by deletion of hha and ydgT.

3.5 Use of β-galactosidase reporters in E. coli to identify Hha/YdgT controlled genes

I could be certain that the genes I investigated were directly H-NS regulated, as a result of ChIP data on H-NS binding sites (Navarre et al. 2006, Lucchini et al. 2006). However, since effector encoding genes such as sifB are at the bottom of regulatory cascades in Salmonella (Fass, Groisman 2009) whose parts may themselves be hha/ydgT regulated, I could not be certain whether these genes were directly repressed by Hha/YdgT. In order to avoid the confounding effects of such upstream regulators I constructed β-galactosidase reporter constructs using the promoter regions of sifB and one of its upstream regulators, ssrA, and examined their expression in E. coli cells. I used E. coli because it lacks many of the virulence gene regulators present in Salmonella which may confound my results. Using E. coli also allowed me to take advantage of the Keio collection of single gene deletions in E. coli and rapidly obtain hha::km and ydgT::km strains.
While both the *sifB* and *ssrA* promoter fusion constructs proved active under wild-type conditions, only *ssrA* showed greater activity in the *hha::km* and *ydgT::km* contexts. The activity of the *sifB* promoter fusion construct was actually reduced to approximately one-third of the wild-type level in the *hha* and *ydgT* strains (Fig. 8a). This indicates that in the absence of *Salmonella enterica* specific activators of transcription, mutations in *hha* and *ydgT* do not have an activating effect on *sifB* transcription. Therefore, it is unlikely that *sifB* is directly controlled by Hha/YdgT. The activity of the *ssrA* construct, on the other hand, was enhanced in both the *hha* and *ydgT* strains, approximately 1.6 and 1.2 fold respectively (Fig. 8b). This indicates that *ssrA* is derepressed in the absence of *hha* or *ydgT*, even in a context where its upstream activators are absent. Though circumstantial, this provides evidence that *ssrA* may be directly controlled by Hha/YdgT. Due to this, I chose to use the same *ssrA* promoter fragment incorporated in the β-galactosidase assays for analysis of H-NS binding in subsequent EMSA experiments (section 3.9).
Figure 9) Expression of sifB and ssrA β-galactosidase promoter fusions in E. coli wild-type, hha and ydgT knockouts. a) β-galactosidase activity of sifB promoter fusion construct in E. coli Keio collection strains (N=2). Reporter activity is reduced by approximately one-third in the mutant strains, indicating sifB is not derepressed in this context. β-galactosidase activity of ssrA promoter fusion construct in E. coli Keio collection strains (N=2). Reporter activity is enhanced two-thirds fold in the hha background and one-fifth fold in the ydgT background, indicating that ssrA is derepressed in these contexts.
3.6 Identification of mutations in H-NS that phenocopy *hha/ydgT* mutants

HSQC-NMR studies on the binding of Hha to the N-terminal domain of H-NS (Garcia et al. 2005, Garcia et al. 2006) have yielded data on the residues in H-NS which may be involved in the binding of Hha. Mapping these residues on the solution structures of the *E.coli* (Bloch et al. 2003) and *Salmonella* (Esposito et al. 2002) H-NS N-terminal domains shows us that those in the Hha signature are surface exposed (Fig. 2). Both the *E. coli* and *Salmonella* domains are shown because their solution structures demonstrate different dimer conformations, antiparallel and parallel respectively, and it is unclear which of these conformations is formed in vivo. I constructed amino acid substitution mutants in each of the residues of H-NS in the Hha-signature (NNIRTL), substituting each with alanine. I also constructed the N9L mutation found in the Vibrio H-NS that was reported to make H-NS “Hha-independent” (Garcia et al. 2009) and the previously identified R12H mutation (Garcia et al. 2006). I constructed these mutations via site directed mutagenesis of a low copy vector (pWN425) with a C-terminally FLAG-tagged *hns* under the control of its native promoter. The C-terminal tag does not interfere with H-NS function as this construct is able to fully complement an *hns* mutation in *Salmonella* (Navarre et al. 2006). I used my plasmid encoded, tagged and mutated *hns* derivatives to complement an *hns* knockout and used RT-Q-PCR to examine the expression of the *sifB* and *proV* genes at mid-exponential growth (*A_{600} = 0.5*). I predicted that mutations in the H-NS protein that abolish its interaction with Hha will cause derepression of *sifB*, but not *proV*, phenocopying an *hha/ydgT* mutant. If the mutation in H-NS leads to a significant de-repression of *proV*, this shows that H-NS function is compromised, though this does not rule out loss of Hha binding.
I found that N9L and N10A mutations behaved similarly to wild-type H-NS, but the I11A, R12A and R12H mutations resulted in derepression of either sifB or proV and that only the I11A H-NS phenocopied an hha/ydgT mutant. I repeated this experiment, expanding the set of genes I examined to include sifA, sifB, pipB, leuO and proV. This confirmed that the I11A mutant derepressed all my identified hha/ydgT repressed genes (Fig 9a), but retained repression of proV (Fig. 9b). The R12A and R12H mutations resulted in derepression of all genes examined, indicating that these molecules may have deficiencies that extend beyond their inability to interact with Hha and YdgT. I also found that the N9L mutation previously reported to yield an Hha-independent H-NS protein (Garcia et al. 2009) did not derepress hha/ydgT repressed genes, but did result in derepression of proV. This does not necessitate that H-NS N9L can interact with Hha/YdgT, it may not require their activity to repress the genes I examined, consistent with the model proposed by Garcia et al. (2009). However, it does suggest that this H-NS molecule may be compromised with respect to repression of proV.

The derepression observed with these mutant H-NS constructs did not recapitulate the magnitude of gene expression seen in the hha/ydgT strain. For instance, pipB is expressed approximately four fold higher in the I11A, R12A and R12H H-NS strains as compared to wild-type, but is 16 fold higher in the hha/ydgT strain as compared to wild-type. It is possible that Hha and YdgT interact with StpA and that Hha/YdgT dependent StpA activity would be compromised in an hha/ydgT strain but would remain with the introduction of an Hha-blind H-NS.
Figure 10) Identification of Hha-blind H-NS variants using RT-Q-PCR of candidate genes.

a) RT-Q-PCR data for *sifA, sifB, pipB* and *leuO* gene expression in strains where *hns::km* is complemented by a low copy plasmid encoded copy of *hns* (wild-type or mutant, as noted, N=3). The I11A, R12A and R12H mutant H-NS constructs do not repress these genes.  

b) RT-Q-PCR data for expression of *proV*, in strains where *hns::km* is complemented by a low copy plasmid
encoded copy of hns (wild-type or mutant, as noted, N=3). The N9L, R12A and R12H constructs do not repress proV.

3.7 Western blotting of FLAG-tagged mutant H-NS proteins

In order to determine that the effects on transcription that I had observed with each of my H-NS mutants was not the result of alterations in protein expression, I used Western blots to measure the amount of each H-NS construct present in the cultures. I found that each construct resulted in expression of approximately the same amount of H-NS, indicating that differences in gene expression observed were likely not due to changes in levels of H-NS. The I11A, R12A and R12H constructs displayed a second FLAG-reactive band immediately below the H-NS band (Fig. 10). This second band was also present when blotting sample from an hha/ydgT mutant expressing H-NS tagged on the chromosome. This indicates that this secondary band is not an artefact from mutated plasmids. The lower band was unaffected by Cip treatment, indicating it is not the result of a phosphorylation event (data not shown).
Figure 11) Protein expression of H-NS variants as determined by Western blot. Anti-FLAG Western blot against FLAG-tagged H-NS performed on extracts from cell expressing WT, N9A, N9L, N10A, I11A, R12A and R12H FLAG tagged H-NS from a plasmid, as well as WT-FLAG-tagged H-NS expressed from the chromosome in an hha/ydgT mutant background and an hns::km mutant. Expression of H-NS is appears approximately equal in each strain. Strains in which H-NS cannot interact with Hha or YdgT display two FLAG-reactive bands, one corresponding to the migration of H-NS and one slightly lower.
To investigate whether this second, lower band was a degradation product of H-NS I purified H-NS from a *Salmonella hha/ydgT* knockout strain with a C-terminally FLAG-tagged *hns* allele incorporated on the chromosome using anti-FLAG resin. Coomassie stain of the gel verified the presence of two bands: one at approximately the same size as H-NS and a second band with lower molecular weight than H-NS. Both bands purified in this manner were submitted for N-terminal sequencing by Edman Degradation. The upper band was determined to start with the sequence MEALKI, corresponding to the N-terminus of full-length H-NS as predicted. We also obtained good sequence for the first five amino acids (KVFGGR) for the second band. This sequence could not be matched to anything found in *Salmonella*.

Similar experiments using immunoblot to examine the size of H-NS containing a C-terminal HA-tag (as opposed to a FLAG-tag) revealed only a single species in the *hha/ydgT* double mutant that appears to be H-NS. The Edman degradation and HA-immunoblot results indicate that an unlikely event had occurred. Namely that the second band observed in the anti-FLAG immunoblots is an artifact where an ≈12 kDa protein that cross-reacts with the FLAG antibody is present only when the Hha/H-NS interaction is compromised. However the fact that we could not definitively identify a protein containing a KVFGGR sequence in the *Salmonella* genome is perplexing.

### 3.8 Co-expression of Hha and mutant H-NS constructs

To examine the ability of the mutant H-NS proteins to interact with Hha, I generated C-terminally 6-His-tagged H-NS expression constructs (pSSA4 derivatives) by site-directed mutagenesis. I also cloned untagged Hha into an expression vector (pCDF-1b) compatible with
the H-NS expression plasmid. I exploited the fact that Hha expressed alone is known to form insoluble inclusion bodies, while co-expression of Hha with H-NS results in both proteins remaining soluble (Pons et al. 2004).

I found that Hha co-expressed with H-NS I11A, R12A or R12H was found almost entirely in the insoluble fraction (Fig. 11). Each of the H-NS proteins remained soluble, indicating that this effect was the result of the mutations in H-NS interfering with the Hha-H-NS interaction, rather than rendering H-NS insoluble and thus unable to interact with Hha. This experiment also indicates that Hha is unable to interact with the I11A, R12A and R12H H-NS mutants within the cell.
Figure 12) Protein solubility when Hha is co-expressed with H-NS variants. Soluble and insoluble fractions from Hha co-expressed with wild-type and mutant H-NS. Hha is found primarily in the insoluble fraction when expressed alone or with H-NS I11A, R12A or R12H. Co-expression of wild-type H-NS with Hha solubilises Hha.
3.9 Nucleoprotein complex forming properties of the I11A, R12A and R12H H-NS mutants

I wanted to ensure that the mutations I had introduced into the N-terminal domain of H-NS had not interfered with the ability to bind and form complexes on DNA. To this end, I performed EMSAs using purified WT, I11A, R12A and R12H H-NS proteins and a fragment of the ssrA promoter. It has been reported that a mutation (N9L) in the Hha-signature of H-NS can affect the ability of H-NS to bind DNA in EMSAs (Garcia et al. 2009). While I did not replicate the findings of this study with respect to the Hha binding properties of H-NS N9L, this report still indicates that one must account for the possibility that mutations in the dimerization domain of H-NS may affect the formation of H-NS-DNA complexes.

I found that the H-NS I11A protein shifted less of the ssrA fragment than wild-type H-NS at the same protein concentration (Fig 12a), indicating that it has reduced ability to form complexes on the DNA. The R12A and R12H proteins, shifted the ssrA DNA fragment at lower concentrations than wild-type H-NS and formed complexes which migrated higher in the gel than those generated with wild-type protein (Figs. 12b and 12c).
a) Bound ssrA

b) Bound ssrA

Unbound ssrA

Unbound STM1033
Figure 13) EMSAs using wild-type H-NS and H-NS I11A, R12A or R12H, binding a region of the ssrA promoter. EMSAs using ~250bp ssrA promoter fragment, bound by WT or mutant H-NS. a) H-NS I11A shifts the $p_{ssrA}$ fragment at a higher concentration than wild-type H-NS. The complexes formed by wild-type and H-NS I11A migrate at the same rate, indicating that they may be similar in size and structure. b) H-NS R12A shifts the $p_{ssrA}$ fragment at a lower concentration than wild-type H-NS and forms a complex which migrates higher in the gel. A control fragment from the STM1033 region of the Salmonella chromosome is visible below the ssrA fragment in each lane, this region is not bound by H-NS in vivo and remains unbound by either wild-type or H-NS R12A in this EMSA. c) H-NS R12H shifts the $p_{ssrA}$ fragment at a lower concentration than wild-type H-NS and forms a complex which migrates higher in the gel. A control fragment remains unbound by either wild-type or H-NS R12H in this EMSA.
3.10 Analysis of the oligomerization properties of the I11A and R12A H-NS mutants

Since the mutations I made in H-NS were located in the dimerization/oligomerization domain of H-NS and the binding of H-NS to DNA is dependent in part on apparent cooperativity between H-NS molecules (Ellison, Miller 2006, Falconi et al. 1998, Badaut et al. 2002), I sought to investigate the oligomerization properties of the I11A and R12A mutant H-NS proteins. H-NS is known to form higher order complexes in solution, which can be detected through their elution profile through a gel-filtration matrix (Ueguchi et al. 1996). I exploited this property of H-NS, using FPLC-gel-filtration with an S200 column to examine oligomerization of the WT, I11A and R12A H-NS proteins at a concentration of 3.5mg/mL. I theorized that the apparent enhanced binding of H-NS R12A to DNA may be the result of enhanced oligomerization of this protein. Conversely, the apparent loss of affinity for DNA displayed by H-NS I11A may be the result of decreased oligomerization of this protein.

The wild-type H-NS protein passed through the S200 column with a characteristic broad elution profile (Ueguchi et al. 1996) (Fig. 13a). Both the I11A and R12A H-NS proteins displayed similarly broad elution profiles, however the amount of protein present in each fraction was different from the wild-type (Figs. 13b and 13c). Wild-type H-NS was present in approximately equal amounts in each fraction collected across its broad elution profile, indicating that each size of oligomer formed by the protein was present in approximately equal amounts. The I11A and R12A H-NS proteins displayed elution profiles skewed towards larger oligomeric complexes. This indicates that these proteins formed a greater proportion of high order complexes than did wild-type H-NS. This may help to explain why the H-NS R12A protein formed apparently larger nucleoprotein complexes and did so at lower concentrations
than wild-type H-NS in the EMSA. However, these oligomerization data are not easily reconciled with the apparent loss of DNA binding observed with the I11A mutant. One explanation would be that while the I11A, R12A and R12H mutations all enhance oligomerization of H-NS in solution, the I11A mutation results in the formation of H-NS complexes whose structure hinder DNA binding.
Figure 14) Gel filtration chromatography analysis of in solution oligomerization of wild-type H-NS, H-NS I11A and R12A.  a) The characteristically broad elution profile of wild-type H-NS, indicating that the protein is able self associate to form higher order complexes in solution.  b) Elution profile of H-NS I11A from size exclusion FPLC.  H-NS I11A elutes in an earlier fraction and its elution profile is shifted forward as compared to wild-type H-NS.  This indicates that H-NS I11A forms larger complexes and a higher proportion of it is found in large complexes as compared to wild-type H-NS.  c) Elution profile of H-NS R12A from size exclusion FPLC.  H-NS R12A elutes in an earlier fraction and its elution profile is shifted forward as compared to wild-type H-NS.  This indicates that H-NS R12A forms larger complexes and a higher proportion of it is found in large complexes as compared to wild-type H-NS.
4 Discussion
4.1 Hha and YdgT act primarily through H-NS and cannot repress genes independently

One of the central questions I sought to address in beginning this project was why a subset of H-NS repressed genes required the activity of Hha and/or YdgT for their repression. Put another way, why can H-NS effectively repress some genes alone, while it requires the additional activity of Hha or YdgT to repress others? I have not been able to answer this question, however I have disproven one existing theory. It has been suggested that Hha and YdgT may act to regulate horizontally acquired genes, while H-NS can act alone to repress expression of core genome genes (Vivero et al. 2008). I have shown that previous studies have underestimated the number of H-NS repressed genes which are also Hha/YdgT repressed. One such gene which I examined is leuO, which is a core genome gene, shared by both Salmonella enterica and E. coli. This leads me to conclude that Hha and YdgT do not interact with H-NS only in the regulation of horizontally acquired genes. As such, the necessity for Hha/YdgT activity at some H-NS regulated genes remains unexplained.

It is known that Hha and YdgT bind H-NS (Garcia et al. 2005, Bae et al. 2008) and it has been hypothesized that they may influence gene expression through this interaction. However, there is also some suggestion that Hha and YdgT may be capable of binding DNA independently of H-NS (Olekhnovich, Kadner 2006). As such, it has been unclear whether Hha/YdgT may act independently of H-NS. I addressed the hypothesis that Hha and YdgT repress genes in Salmonella solely through their interaction with H-NS by examining transcription in hha/ydgT/hns strains as compared to hns strains and by examining transcription in hns strains complemented with Hha-blind H-NS proteins.
My data comparing transcription in *hns* mutants and *hha/ydgT/hns* mutants is entirely consistent with a model whereby Hha and YdgT act solely through H-NS to repress gene expression (Fig. 14). Further, I was able to recapitulate the pattern of transcription observed in an *hha/ydgT* mutant by complementing an *hns* knockout with an Hha-blind H-NS I11A protein (Fig. 9). This lends credence to the theory that Hha and YdgT act through H-NS to repress gene expression, since they were present in the cell, but unable to repress expression of genes such as *sifB* and *pipB*. Given that Hha and YdgT have been found to form complexes with the H-NS parologue, StpA (Paytubi et al. 2004), it is possible that they exert some gene repressive activity through StpA. This may account for the diminished magnitude of gene activation seen in the strains complemented with H-NS I11A. However, since StpA relies in part on H-NS for its own activity and is a relatively poor repressor of transcription (Uyar et al. 2009), the primary activities of Hha and YdgT are likely exerted through H-NS. This knowledge will enable further studies on Hha/YdgT to focus on the effects that they may exert on H-NS, since the mechanism by which Hha and YdgT repress gene expression will also involve H-NS mediated gene repression.
Figure 15) H-NS is displaced from some regions of the genome in the absence of Hha and YdgT. Note that we do not know whether Hha interacts with H-NS at all H-NS bound sequences (as shown at right) and is only required for the repression of some genes or whether Hha only interacts with H-NS at a subset of genes (as shown in the centre), potentially only at those locations where Hha/YdgT activity is required. We do know that in the absence of Hha and YdgT, H-NS is displaced from some sequences it would otherwise occupy (as shown at left).
4.2 Characterization of Hha-blind H-NS mutants

H-NS mutations previously identified to abolish Hha binding (H-NS R12H and N9L) provide a useful tool for dissecting the H-NS-Hha relationship (Garcia et al. 2006, Garcia et al. 2009). However, both of the existing studies used *E. coli* H-NS as their focus and they did little characterization of how these “Hha-blind” H-NS molecules affected gene expression and the mechanisms by which such molecules might affect gene expression. I identified two novel Hha-blind H-NS molecules (H-NS I11A and R12A), characterized their effects on gene expression, as well as their DNA binding and oligomerization properties. I found that each of the mutations in H-NS which abolish Hha binding also have effects on protein-DNA complex formation and oligomerization.

An examination of the two previously described Hha-blind H-NS mutants as well as the two others I identified in my study revealed that only the I11A mutation resulted in the same gene expression effects as deletion of *hha* and *ydgT* (Fig. 9).

I found that the I11A and R12A proteins both formed higher order oligomers in solution more readily than WT H-NS (Fig 13). However, they behaved differently from one another with respect to nucleoprotein complex formation. H-NS R12A and R12H form complexes on DNA at lower concentrations than WT H-NS and form complexes which migrate more slowly through a gel (Figs. 13b and 13c). H-NS I11A, on the other hand, formed complexes at higher concentrations than WT H-NS and formed complexes qualitatively similar to WT H-NS (Fig. 13a). This indicates that the conformation, and thus the protein structure, required for H-NS to form oligomers in solution is different from that required to form oligomers on DNA. Since H-
NS R12A and R12H form complexes on DNA more readily than WT H-NS, but are defective for gene repression, one can also conclude that formation of an H-NS-DNA complex is insufficient in and of itself to repress gene expression.

Previous studies have used DNA footprinting to demonstrate that H-NS need not be fully displaced from a promoter for expression of the downstream gene to occur (Perez, Latifi & Groisman 2008). It is possible then that the nucleoprotein complexes formed by H-NS R12A or R12H are more easily displaced by transcription factors or RNAP than WT complexes. In order to further examine this, ChIP could be used to examine the binding profile of these mutant H-NS proteins. I predict that H-NS I11A, R12A and R12H will display binding profiles that resemble either the WT H-NS profile or that of an hha/ydgT mutant. If the binding profile resembles that of WT Salmonella, then these proteins need not be displaced from DNA in order to derepress genes such as sifB. If their H-NS binding profile resembles that of an hha/ydgT mutant, then it is likely that these H-NS mutants are more easily displaced from DNA and that this plays a role in allowing transcription. DNA footprinting could also be used to examine at higher resolution whether H-NS I11A, R12A or R12H form complexes with DNA that differ from those formed by WT H-NS. The susceptibility of these complexes to displacement by transcription factors could also be examined by DNA footprinting. For instance, WT H-NS may either occupy a binding site for a transcription factor or be resistant to displacement by that, while the mutant proteins may allow the transcription factor access to its binding site and/or be displaced by the transcription factor.

Techniques such as ChIP and DNA footprinting may also allow further characterization of the properties of these mutant H-NS proteins. At present, we cannot rule out the possibility that complementing an hns mutant with H-NS I11A results in a transcriptional profile which
resembles that of an hha/ydgT mutant as a result of a mechanism independent from the abolition of Hha binding. It may be possible that the H-NS I11A protein simply cannot form protein-DNA complexes of the same size, density or robustness as WT H-NS. This may be tested via ChIP, comparing the occupancy of promoters by WT and I11A H-NS proteins. If ChIP enrichment of H-NS I11A is less than that of WT H-NS across the entire chromosome, then derepression of specific genes may simply be the result of their having a higher threshold of H-NS occupancy necessary to achieve repression. However, though this may may indicate that the H-NS I11A phenotype is Hha independent, it may still shed light on Hha function. If the set of genes derepressed when hns is complemented by H-NS I11A overlaps significantly with the hha/ydgT regulon and it can be proved that H-NS I11A mediated derepression is the result of reduced protein-DNA complex formation, then this will indicate that the reason H-NS requires Hha/YdgT activity at some genes and not others is that those genes require this higher threshold of H-NS occupancy for silencing. Hha activity, acting on WT H-NS, may help to establish the necessary robust H-NS complex. If the DNA binding profile of H-NS I11A mirrors that of WT H-NS it will suggest that gene derepression in the H-NS I11A strain is the result of loss of Hha binding or there is some alteration to H-NS I11A which mirrors this part of the hha/ydgT phenotype as well.

4.3 hha/ydgT repress a subset of H-NS repressed genes

My work has shown that the full set of hha/ydgT regulated genes has not yet been identified. I have found that several genes identified by the Vivero et al. (2008) microarray study as unaffected by deletion of hha/ydgT are, in fact, significantly upregulated in the absence of hha/ydgT. The only H-NS repressed gene which I was able to identify as hha/ydgT independent was proV. Vivero et al. (2008) noted that others have identified horizontally
acquired sequences for which H-NS is not an effective silencer (Navarre et al. 2006) and it has been speculated that Hha/YdgT may assist H-NS in silencing horizontally acquired genes, while ancestral genes would be silenced by H-NS alone (Banos et al. 2009). While my findings expand the number of hha/ydgT-repressed genes, they do not further our understanding of what separates a gene regulated by H-NS and hha/ydgT from a gene regulated by H-NS alone.

It is interesting to note that the genes I found to be hha/ydgT regulated were more highly expressed than proV in the hns strain. This may indicate H-NS repressed genes with strong promoters require hha/ydgT activity, while those with weaker promoters do not. However, in the case of the genes studied here, we cannot isolate promoter strength from the activity of upstream regulators. For example, it is possible that pipB is much more highly upregulated than proV in an hns mutant because it falls at the bottom of a regulatory cascade where one or more of its activators are themselves H-NS repressed. A synergistic effect may be seen where both pipB and its upstream activators are derepressed in an hha/ydgT or hns strain. It is also possible that while pipB repression remains effective in an hha/ydgT strain, its upstream activators are derepressed and are able to counteract H-NS mediated silencing. In order to address this, genes would need to be isolated from their activators. One method of accomplishing this for Salmonella virulence genes such as pipB is to generate reporter fusions with the promoters of such genes and examine their expression in E. coli hns and hha/ydgT mutants. Another method would be to generate an in vitro transcription system, where transcription could be examined in the presence and absence of H-NS and Hha.

4.4 hha/ydgT influence the H-NS binding profile on the chromosome

Given that Hha and YdgT bind H-NS and act primarily through H-NS to repress gene expression, it is unsurprising that Hha and YdgT may influence the binding of H-NS to some
gene sequences. I have found that H-NS occupancy of a region of the sifB promoter is hha/ydgT dependent. I have also demonstrated that for the proV gene, whose transcription is unaffected by the deletion of hha and ydgT, this deletion also does not affect the binding of H-NS to the proV promoter. These data suggest that the degree of de-repression observed at a given gene in hha/ydgT mutants may inversely correlate with the degree of H-NS occupancy at many promoters.

This knowledge could be used to further our understanding of how Hha and YdgT exert their influence on gene expression through H-NS, and help to determine why some H-NS repressed genes are hha/ydgT repressed and others are not. A genome wide tiling ChIP-chip experiment examining H-NS occupancy of the genome of hha/ydgT mutant cells may shed light on which sequences become H-NS depleted in the absence of Hha and YdgT. Such an experiment would be unable to distinguish direct from indirect effects. H-NS may be displaced from many sequences by other DNA binding proteins rather than by the loss of Hha or YdgT per se. In order to address this issue, ChIP may be performed on the promoter fusion constructs in E. coli; in a context where upstream activators are absent. I have already established that genes active in a Salmonella hha/ydgT mutant are not necessarily directly dependent on Hha/YdgT and that the use of promoter fusion reporter constructs in E. coli can determine whether a gene is likely directly controlled by Hha/YdgT. Alternatively, finer scale changes in the H-NS-DNA complex may be mapped using DNA footprinting on the same sequences used for in vitro transcription.

These approaches are not mutually exclusive and would likely be most effective if used as a workflow. Genome wide tiling ChIP-chip could be combined with existing transcript data (Vivero et al. 2008) to identify candidate genes. Such genes could then be cloned into reporter
constructs, whose expression could be examined in *E. coli* to determine whether they are likely to be controlled directly or indirectly by Hha/YdgT (candidate genes would be those whose known activators are *Salmonella* specific and, thus, absent in *E. coli*). ChIP could be performed on the promoter sequences of those reporters which are overexpressed in *E. coli hha, ydgT* and/or *hha/ydgT* cells, to verify that H-NS is unable to repress these sequences without Hha/YdgT. Finally, the promoters of those genes which are depleted of H-NS binding in *E. coli hha/ydgT* cells could be used in *in vitro* transcription and footprinting experiments to further examine the mechanism by which H-NS and Hha-H-NS complexes bind to and silence these genes.

### 4.5 Identifying chromosomal locations of H-NS/Hha/YdgT complexes

It remains unknown whether Hha and H-NS interact only at genes where Hha/YdgT are required for repression or whether Hha/YdgT interact with H-NS at all genes, but are only required at some. This question could be most easily resolved by performing ChIP using antibodies against Hha. However, successful ChIP is difficult with proteins that do not bind directly to DNA. Furthermore, purification of large quantities of soluble Hha in the absence of H-NS is difficult (Pons et al. 2004). My attempt to develop antibodies against Hha complexed to H-NS using a phage-display panning approach resulted only in antibodies that recognized H-NS. It is possible that some of these antibodies are capable of recognizing both H-NS and Hha, due to the structural similarity between Hha and the N-terminus of H-NS (Rodriguez et al. 2005). Generation of a tagged *hha* allele would avoid these problems. My data suggest that an N-terminally 6-His-tagged Hha is capable of interacting with and pulling down H-NS, indicating that an N-terminal tag may not interfere with Hha function. While the tag does not interfere with the binding of Hha to H-NS, we cannot rule out the possibility that it may prevent the Hha-H-NS complex from adopting the proper conformation to effect gene repression. Should ChIP of Hha
or YdgT prove impossible, more indirect strategies may be employed. I have already proposed a genome wide tiling ChIP-chip of H-NS in an hha/ydgT background as an experiment to detect the effects of Hha on H-NS, as well as aiding in identifying genes directly regulated by Hha. I hypothesize that if H-NS binding were found to be depleted globally in the hha/ydgT strain, then Hha is bound to H-NS at all genes. It is possible that Hha/YdgT act to enhance H-NS binding at all or nearly all genes, but that loss of Hha/YdgT results in derepression of only those genes where this depletion of H-NS crosses a threshold where transcription is no longer repressed. It may be that it is this threshold of H-NS occupancy that determines which genes are Hha/YdgT repressed. If, however, H-NS depletion is limited to genes that are activated in hha/ydgT mutants, then it will remain an open question whether Hha and YdgT are only bound to H-NS at such sites or whether they are bound to H-NS everywhere, but that H-NS only requires the activity of Hha/YdgT to bind some sequences.

4.6 Possible mechanisms of Hha activity

Given that Hha and YdgT act primarily through H-NS and that they bind the N-terminal dimerization domain of H-NS (Garcia et al. 2006, Paytubi et al. 2004), it is likely that Hha and YdgT influence H-NS oligomerization or the structure of the H-NS nucleoprotein complex. Properties such as cooperativity of binding (Dame, Noom & Wuite 2006) and structure of the H-NS-DNA complex (Perez, Latifi & Groisman 2008) are believed to be crucial for proper gene repression by H-NS. One can envision several mechanisms whereby Hha/YdgT might modulate H-NS function through influencing these factors. Determination of where Hha/YdgT act will shed light on the plausibility of some of these hypotheses.

Determining which genes are directly regulated by Hha and YdgT will allow us to examine the correlation between Hha/YdgT-mediated regulation and the structure of those genes.
For instance, I theorized that Hha and YdgT may be necessary for RNAP trapping by H-NS. In order for H-NS to form a repressive loop and trap RNAP, there must be two H-NS binding regions with the loop formed between them (Fig. 3). If we map the Hha/YdgT binding regions of the chromosome and find that they correspond to such regions, then this will provide evidence that there is some feature of repressive loop structures that is recognized by and requires Hha/YdgT. An alternative outcome is that even if Hha/YdgT are present at nearly all H-NS bound genes but are only required for silencing at a subset of loops. Should this be the result of mapping Hha/YdgT binding locations, it will shed no light on where Hha/YdgT activity is required for H-NS mediated gene repression. This is one reason why identification of Hha/YdgT regulated genes may require means other than ChIP, some of which have been outlined above.

Should any method identify one or more genes where Hha/YdgT activity is directly necessary for their repression, this will allow fine scale study of the binding of H-NS. DNA footprinting studies of genes directly regulated by Hha/YdgT may reveal that the regions protected by H-NS alone differ from those protected by H-NS in complex with Hha. If H-NS-Hha protects regions which are unprotected by H-NS alone, analysis of these regions may reveal structural or sequence determinants common to Hha/YdgT regulated genes. If there is no difference in the nucleotides protected by H-NS alone or H-NS in complex with Hha, then Hha may act to help establish or maintain the H-NS nucleoprotein complex, rather than altering its form. Titration of H-NS and Hha-H-NS into footprinting experiments may reveal that Hha alters the affinity of H-NS from some or all regions it binds. If H-NS affinity for DNA is unchanged by Hha activity, then Hha may aid to maintain H-NS complexes. One means of testing this would be to use the Q-TRAP device previously used to study the biophysical properties of H-NS-DNA complexes (Dame, Noom & Wuite 2006). This device allows manipulation of individual strands of DNA while they are bridged by H-NS complexes. Dame et al. determined
the force necessary to pull apart one H-NS link in a bridged DNA complex to be 7 pN. It is possible that the binding of Hha to the dimerization domain of H-NS may stabilize the interaction of H-NS monomers in dimers or result in greater cooperativity between dimers in oligomers bound to DNA. Measurement of the force necessary to displace H-NS-Hha complexes from DNA may determine whether Hha activity has an effect on the stability of the nucleoprotein complex. Biophysical analysis of this nature may also give insight into the properties of the Hha-blind H-NS mutants and how their oligomerization properties have been affected by the mutations.

4.7 Conclusion

In conclusion, this study has led to the development of tools which may shed light on the mechanism by which Hha/YdgT act through H-NS to mediate gene silencing. For example, I have found that H-NS is depleted at the promoter of sifB, an hha/ydgT responsive gene, and that *E. coli* reporter constructs can be used to determine that this gene is not directly controlled by Hha/YdgT. While neither of these findings sheds light on the mechanism by which Hha/YdgT influence H-NS function, they can be used as part of later studies to identify genes directly controlled by Hha/YdgT and the study of those genes may, in turn, allow determination of the mechanism of action of Hha/YdgT.

Because establishment of a mechanism of action will require model genes to study, the first goal of any study following up this work should be to find at least one gene directly regulated by Hha/YdgT. Approaches to do so are outlined in greater detail above; such approaches include ChIP of Hha or YdgT, ChIP of H-NS in *hha/ydgT* mutants and use of *Salmonella* promoter fusions in *E. coli*. Once a bone fide Hha/YdgT target gene has been established, biochemical studies can determine whether Hha has an effect on the affinity of H-
NS for sequences therein or whether Hha may alter the structure of the H-NS-DNA complex.

These possibilities may be examined using EMSAs and DNA footprinting. These are the techniques most likely to yield information regarding the mechanism by which Hha and YdgT influence H-NS mediated gene repression.
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