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CHARACTERIZATION AND REGULATION OF THE *ICSP* AND *OSPZ* LOCUS IN *SHIGELLA FLEXNERI*

by

Krystle Lashell Pew

Bachelor of Science
Hampton University
2008

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science Degree in Biological Sciences
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College of Sciences

Graduate College
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ABSTRACT

Characterization and Regulation of the *icsP* and *ospZ* locus in *Shigella flexneri*

by

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Dr. Helen Wing, Examination Committee Chair
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Shigella flexneri is a gram negative, rod shaped bacterium that is the causative agent of bacillary dysentery, which is characterized by fever, abdominal pain, and bloody diarrhea. Genes essential to the pathogenicity of *S. flexneri* are encoded by a virulence plasmid. *Shigella* has evolved a complex regulatory system to regulate transcription of virulence genes. This involves two regulators, VirF and VirB, which allow the bacterium to respond to environmental stimuli and maximally exploit host niches. An additional factor impacting virulence gene regulation is H-NS, a histone nucleoid structuring protein that globally represses transcription. This work addresses the transcriptional regulation of *icsP* and *ospZ*, divergent virulence plasmid encoded genes. Analysis of the intergenic region shared by *icsP* and *ospZ* has identified remote VirB binding sites essential to *icsP* promoter activity. Analyses of the requirements for VirB-dependent regulation of *icsP* promoter activity identified the VirB binding sites as a *cis*-acting element with small spacing requirements. In addition, analyses of H-NS-dependent regulation of the *icsP* promoter showed that intrinsic curvature does not have a role in H-NS mediated repression. Together, these data indicate that the VirB and H-NS interact to modulate *icsP* promoter activity, demonstrating that transcriptional regulation is an intricate process.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 The genus *Shigella*

Shigella spp. are gram-negative, non-motile, non-sporulating, rod shaped bacteria that are members of the *Enterobacteriaceae* family. *Shigella* was first isolated and characterized by Japanese scientist, Kiyoshi Shiga, in 1896. The *Shigella* genus contains four species: *S. sonnei*, *S. flexneri*, *S. boydii*, and *S. dysenteriae* (Ewing, 1949). These species are further divided into serotypes according to differences in biochemistry and O-antigen variability; *S. flexneri* has 13 serotypes (Jennison & Verma, 2004).

This genus shows high sequence similarity with *Escherichia coli,* in fact the two cannot be distinguished in DNA hybridization studies. *S. flexneri* and *E. coli* genome sequences only differ by 1.5% (Lan & Reeves, 2002). Multiple studies using various techniques have collected data indicating that *Shigella flexneri* should be included in the species *Escherichia coli* (Fukushima, Kakinuma, & Kawaguchi, 2002; Ochman, Whittam, Caugant, & Selander, 1983; Pupo, Karaolis, Lan, & Reeves, 1997; Rolland, Lambert-Zechovsky, Picard, & Denamur, 1998). Interestingly, *Shigella flexneri* and *E. coli* have distinct phenotypes.

1.2 Prevalence of *Shigella*

Shigella species are facultative, intracellular pathogens of human and primate hosts. *Shigella* is one of the most prevalent causes of bacillary dysentery. It is a communicable disease spread by the fecal-oral route through contaminated food, water,

and poor hygiene. Although, the previous mentioned routes are most common, *Shigella* has also been shown to be transmitted via an insect vector, *Musca domestica* (the common house fly), which transmits *Shigella* in areas where improper disposal of human feces is common practice. It is estimated that there are 163.2 million episodes of endemic shigellosis in developing countries and 1.5 million episodes in industrialized countries per year. Of these cases, children comprise an overwhelming majority with 69% of all cases and 61% of the approximately 1.1 million deaths caused by shigellosis each year in both developed and developing countries (Kotloff et al., 1999). More recently, the global burden of shigellosis is reported to be higher than the aforementioned estimates, especially in resource-poor areas (von Seidlein et al., 2006).

1.3 Shigellosis

Shigellosis, bacillary dysentery, is an acute inflammation of the human colon resulting from infection with *Shigella* spp. The incubation period ranges from 1-4 days up to 8 days. The clinical manifestations of shigellosis include painful abdominal cramps, nausea, fever, and the hallmark of this disease, mild to severe bloody diarrhea. These manifestations are reflective of the invasive nature of *Shigella* infection. Those presenting with shigellosis can have mild infections characterized by watery diarrhea or severe dysentery typified by acute inflammatory colitis with the passing of bloody, mucoid diarrhea. Shigellosis is diagnosed through the isolation and identification of shigellae from the feces using differential/selective media such as MacConkey or *Shigella-Salmonella* agar (Niyogi, 2005).

1.4 Pathogenesis of *Shigella*

The hallmark of *S. flexneri* pathogenesis is the invasion of colonic epithelium (Fig.1). This invasion triggers the inflammatory response and severe cramping characterized by *Shigella* infections. The gene products involved in the pathogenesis of *S. flexneri* are encoded within a 30 kb fragment of the virulence plasmid, known as the entry region. This region codes for a type III secretion system which is a needle-like projection from the bacterial cell that mediates bacteria-host cell contacts and facilitates the delivery of bacterial proteins to host cells.

Once ingested, *S. flexneri* gains access to the intestinal mucosa by triggering its uptake into microfold cells (M cells) present in the mucosal layer of the human gut. Shigellae trigger this uptake via a membrane ruffling process (Sansonetti & Phalipon, 1999). M cells are specialized epithelial cells that continuously sample the gut lumen, delivering particles to lymphoid mucosal tissue and subsequently, the host immune system (Man, Prieto-Garcia, & Nicoletti, 2004). M cells deliver sampled material to an intraepithelial pocket formed by the basolateral membrane. The intraepithelial pocket contains macrophages and lymphocytes that are capable of activating the immune system in response to presented antigens (Neutra, Pringault, & Kraehenbuhl, 1996).

Shigella is endocytosed by macrophages where it escapes the phagocytic vacuole gaining access to the macrophage cytoplasm (Fig. 1). A secreted protein, IpaB, then initiates an apoptotic pathway via caspase-I activation (Chen, Smith, Thirumalai, & Zychlinsky, 1996); following apoptosis of the host cell the bacterium gains access to the basolateral membrane. Macrophage cell death has additional consequences. The death of the host cell releases the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18

(Sansonetti et al., 2000). IL-1β and IL-18 mediate the acute inflammatory response consistent with infection by *S. flexneri*. IL-1β triggers inflammation of the intestines. IL-18 functions to generate an antimicrobial response by activating natural killer cells and facilitating the production and release of gamma interferon (IFN-γ), leading to the overall amplification of the immune response (Le-Barillec et al., 2005; Way, Borczuk, Dominitz, & Goldberg, 1998).

The uptake of *Shigella* into M cells and presentation to macrophages serves two purposes: first, it allows the bacterium access to the basolateral membrane of colonic epithelial cells by providing a route across the impermeable layer of epithelial lining (Wassef, Keren, & Mailloux, 1989), and secondly, it induces inflammation and recruits PMN cells which compromises the integrity of the mucosal layer, allowing *Shigella* within the lumen access to the sub-mucosa (Perdomo, Gounon, & Sansonetti, 1994).

Shigellae enter epithelial cells by inducing membrane ruffling. *S. flexneri* induces rearrangements in the host cell cytoskeleton which engulfs the bacterial cell forming a vacuole. It is important to note that this process is not mediated by adhesion factors or flagellum, as *Shigella* has no known adhesion factors nor are genes encoding a flagellum expressed. Following uptake into the host cell the secreted protein IpaB lyses the vacuole containing the bacterium, similarly to the mechanisms used to escape the phagocytic vacuole. IpaB is an effector molecule secreted by the type III secretion system.

Additional factors critical to *Shigella* pathogenesis include effector molecules delivered to the host cell cytoplasm via the type III secretion apparatus. Virulence plasmid genes encode this type III secretion system and the effector proteins crucial to the invasive phenotype. The *ipa* operon encodes the invasion plasmid antigens IpaA,

IpaB, IpaC, and IpaD. Complementing the *ipa* operon is the *mxi-spa* operon which encodes the type III secretion needle which delivers the effectors to the host cell cytoplasm, including the Ipa invasion antigens (Menard, Sansonetti, & Parsot, 1993; Sasakawa et al., 1988).

Once within the cytoplasm *Shigella* continues to exploit the host cell cytoskeleton by forming an actin tail that is crucial for intracellular mobility and intercellular spread. The actin tail localization at one bacterial pole generates a propulsive force allowing the invading *Shigella* to propel themselves throughout the host cell and into adjacent cells. Furthermore, actin tails allow the organism to take advantage of replicative niches (Gouin, Welch, & Cossart, 2005; Stevens, Galyov, & Stevens, 2006).

The outer membrane protein IcsA (VirG) mediates the actin mobility of *S. flexneri*. It is localized to the old bacterial pole by two internal regions (Charles, Perez, Kobil, & Goldberg, 2001). IcsA maintenance within the outer membrane is essential for motility as mutant strains lacking IcsA display non-localization of the protein and lack intercellular movement (Bernardini, Mounier, d'Hauteville, Coquis-Rondon, & Sansonetti, 1989; d'Hauteville & Sansonetti, 1992; Lett et al., 1989; Sansonetti, Arondel, Fontaine, d'Hauteville, & Bernardini, 1991). The outer membrane protease IcsP is critical to the polar localization of IcsA within the outer membrane. IcsP cleaves IcsA at specific arginine residues, releasing the α domain from the bacterial surface (Fukuda et al., 1995). In IcsP mutant strains, bacteria remained trapped within microcolonies in the host cell cytoplasm, actin polymerization occurred over the entire surface of the bacterial cell, and actin tails were abnormal in shape and length as compared to the wild-type (Egile, d'Hauteville, Parsot, & Sansonetti, 1997). In contrast, when IcsP is over-expressed, actin-

tail assembly is less efficient, formed actin-tails are stunted, and there is a marked decrease in intracellular spread of the bacterium (Wing, Goldman, Ally, & Goldberg, 2005). These findings indicate the important role of the IcsP protease in *Shigella* pathogenesis.

It should be noted that the intracellular lifestyle of *Shigella* within the host provides a form of seclusion from the host immune system and other mechanisms aimed at recognizing, responding, and clearing foreign invaders such as bacteria. Rather it is the cellular disruption caused by *Shigella* cell invasion and cell-to-cell spread that recruits the host immune response, resulting in the severity of symptoms. Despite this robust inflammatory response, *Shigella* infections trigger symptoms of disease, such as bloody diarrhea.

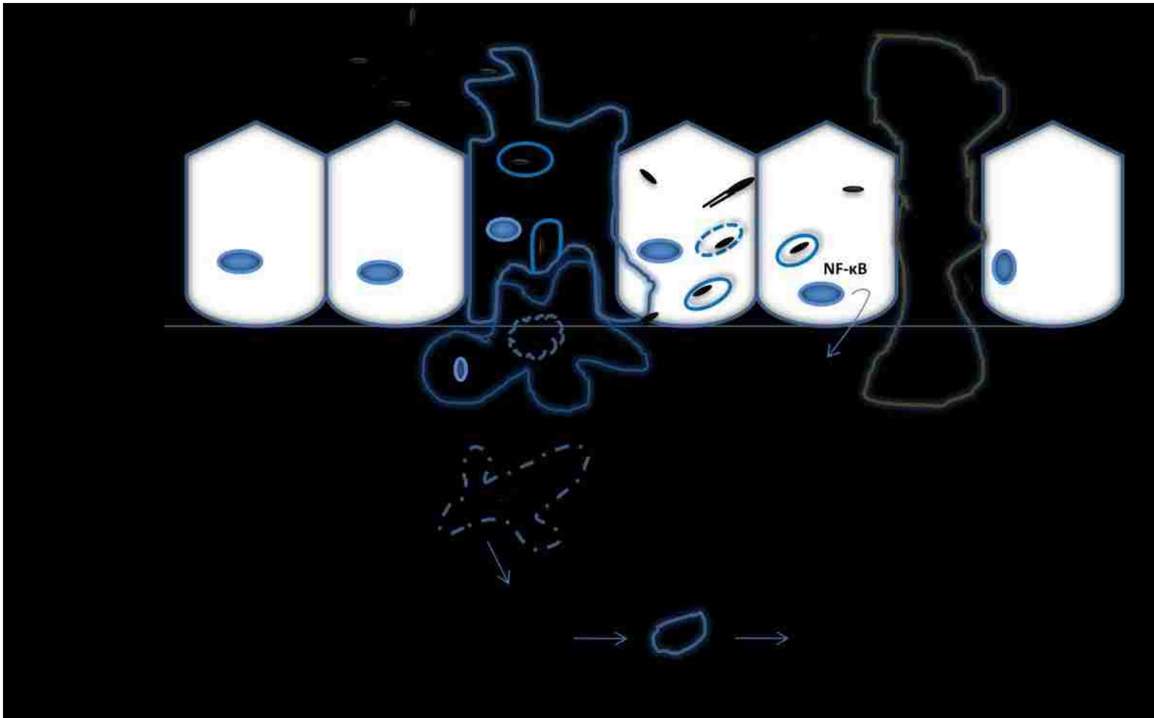

FIGURE 1: *Shigella* Pathogenesis. *Shigella* invades the colonic epithelium by utilizing M cells to access the basolateral membrane. M cells present sampled *Shigella* to macrophages, within which *Shigella* induces apoptosis and escapes. The induction of apoptosis leads to the secretion of chemokines and cytokines that activate natural killer cells, and thus the host immune sysyem. Once free to access the basolateral membrane

Shigella induces membrane ruffling of epithelial cells and is engulfed by endocytosis. Once within the cytoplasm, *Shigella* replicates and moves laterally intra and intercellularlly by use of an actin tail.

1.5 The *Shigella* Regulatory Cascade

The genes encoded by the *Shigella* virulence plasmid are regulated at the level of transcription by environmental cues such as temperature, osmolarity, and pH (Beloin & Dorman, 2003). Regulatory proteins that regulate expression of virulence genes are encoded both chromosomally and on the virulence plasmid. *S. flexneri* has an intricate regulatory mechanism that involves two regulatory proteins, VirF and VirB (Fig. 2).

The *Shigella* virulence plasmid encodes approximately 100 genes alongside numerous insertion sequences. The sequences encoding these genes have a G + C content ranging from 30% to 60% indicating that their sources vary (Jin et al., 2002). A 30 kb pathogenicity island exhibits a G+C content of 34%. This cluster of genes is known as the entry region and encodes a type III secretion system, its effector proteins, and transcriptional regulators. The secreted Ipa invasion effectors and the Mxi-Spa type III secretion apparatus are divergently transcribed within the entry region (Sasakawa et al., 1988).

A temperature shift from the non-permissive temperature of 30 °C to the permissive temperature of 37 °C is the primary environmental signal in activation of genes located within the pathogenicity island of the *Shigella* virulence plasmid (Hromockyj & Maurelli, 1989; Maurelli, Blackmon, & Curtiss, 1984). *virF* encodes a 30 kDa protein, VirF, that is critical to positive regulation of the virulence cascade. VirF is required to activate *virB*; VirB then activates structural gene promoters (Tobe et al.,

1991; Tobe, Yoshikawa, Mizuno, & Sasakawa, 1993). VirB has been found to function mainly as an antagonist of H-NS, a global repressor of transcription. H-NS binds to promoters at the non-permissive temperature of 30 °C. Such a complex regulatory mechanism could allow the bacterium to tightly regulate responses to environmental stimuli and also maintain virulence gene repression under conditions where virulence gene products are not needed. Invasion gene products are only necessary inside the host and, specifically within the lower digestive tract (Porter & Dorman, 1994).

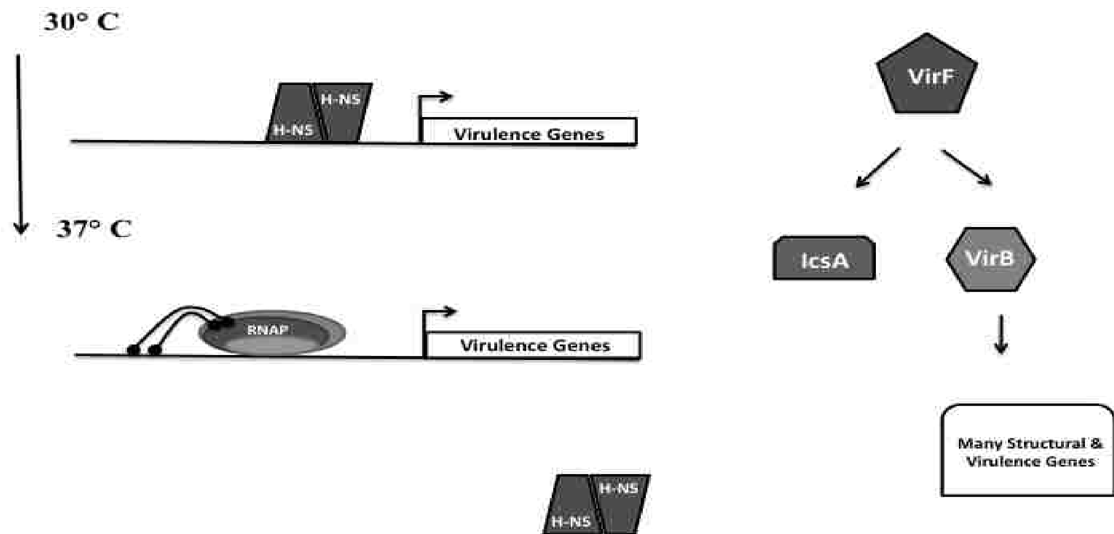

FIGURE 2: Schematic of *Shigella* virulence cascade. Genes encoded on the virulence plasmid are thermo-regulated. Upon a switch from the non-permissive temperature of 30 °C to 37 °C, H-NS mediated repression of *virF* is alleviated, allowing the transcription factor VirF to initiate transcription of *virB*, whose gene product in turn activates structural genes present on the *Shigella* virulence plasmid.

1.6 Aims of Work

The aim of this study is to improve understanding of mechanisms of virulence gene regulation in *Shigella flexneri*, with specific focus on the transcriptional regulation of the *icsP* gene. Furthermore, this work also seeks to characterize the promoter of a divergent gene, *ospZ.* This thesis is divided into two main chapters (chapters 2 and 3) encompassing four major objectives:

Aim 1: To determine the helical orientation and spacing requirements for VirB-dependent regulation of the *icsP* promoter from the most distal VirB binding sites.

Aim 2: To determine the role of intrinsic curvature in the regulation of the *icsP* promoter.

Aim 3: To determine H-NS binding sites in the *icsP* promoter region.

Aim 4: To determine the transcription start site of *ospZ.*

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CHAPTER 2

ROLE OF TRANSCRIPTION FACTOR BINDING SITES AND INTRINSIC CURVATURE OF DNA IN *ICSP* PROMOTER REGULATION

2.1 Introduction

2.1.1 Bacterial Transcription Overview

Transcription is the process of transcribing a DNA sequence into RNA. This process is carried out by RNA polymerase (RNAP). RNA polymerase has a multi-subunit structure that is the catalytic unit of transcription. It consists of $\beta\beta'\alpha_2$ and ω subunits. The β and β' subunits form a claw structure that clamps DNA in addition to forming the RNAP active site (Korzheva et al., 2000). The two identical α subunits are joined by a polypeptide linker and contain a domain that facilitates the assembly of the β and β' subunits. The ω subunit is required to stabilize the β' subunit in non-aggregated forms and recruit the α2β assembly to the promoter (Ghosh, Ishihama, & Chatterji, 2001). Together these units form the apoenzyme. An additional subunit, σ, interacts with RNAP to form the holoenzyme. σ has three main functions when in complex with the RNAP holoenzyme: recognizing specific promoter sequences; the correct positioning of RNAP at target promoters; and facilitating the unwinding of the DNA duplex near the transcription start site (Gross et al., 1998).

The canonical bacterial promoter consists of a transcription start site, and -10 and -35 sequences (Fig. 3). The -10 and -35 hexamers are conserved sequences located approximately -10 bp and -35 bp upstream of transcription start site, respectively. These

sites are recognized by domains 2 and 4 of the σ subunit of RNAP, serving as docking points (Gross et al., 1998).

Additional promoter elements include the extended -10 and UP elements (Fig. 3). An extended -10 region is a 3-4 bp motif adjacent upstream of the -10 hexamer and is recognized by the σ subunit (Murakami, Masuda, Campbell, Muzzin, & Darst, 2002; Sanderson, Mitchell, Minchin, & Busby, 2003). UP elements are approximately 20 bp sequences further upstream of the -35 hexamer and it is recognized by the C-terminal domains of the RNAP α subunits (Ross, Ernst, & Gourse, 2001). Both the extended -10 and UP elements contribute to the initial binding of RNAP to the target promoter. The intrinsic strength of a promoter is dependent on the extent to which the -10 and -35 sequences match the consensus sequences (Kobayashi, Nagata, & Ishihama, 1990; Szoke, Allen, & deHaseth, 1987). It is important to note that not all promoters possess a UP element or extended -10 site, these sequences function to enhance the binding affinity of RNAP in the absence of -10 and -35 sequences that display high sequence similarity to the consensus sequences.

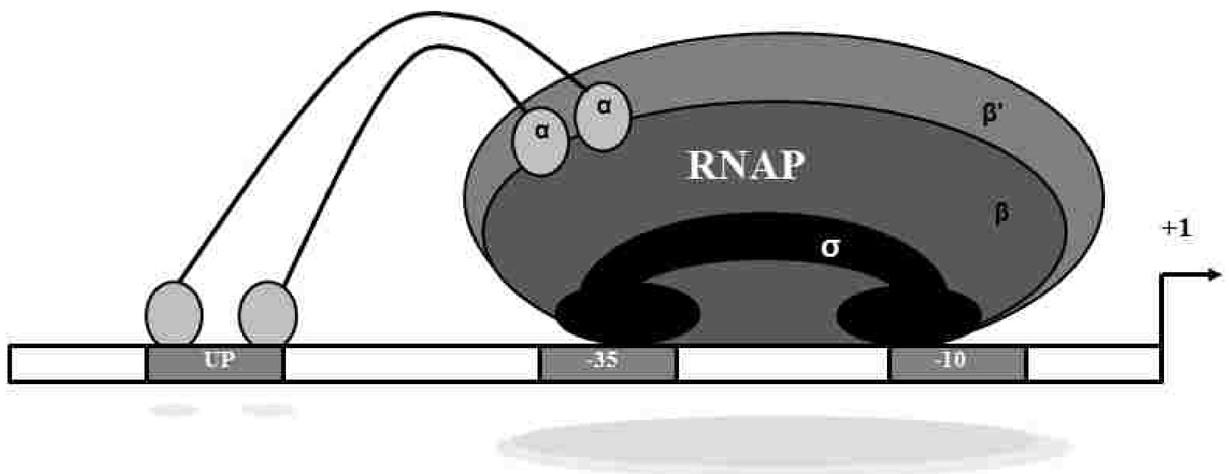


FIGURE 3: Schematic demonstrating the organization of a bacterial promoter.

The process of transcription can be summarized as the binding of RNA polymerase, open complex formation, transcription initiation, elongation of mRNA, promoter clearance, and termination. The binding of RNA polymerase is contingent upon the presence of -10, -35, extended -10, and/or UP element promoter sequences. At this point in transcription, the RNAP and bound DNA are in a closed complex. The DNA is unwound and becomes single-stranded in the region of the transcription start site, +1, forming the open complex. RNAP begins to transcribe the DNA, producing several abortive transcripts before elongation proceeds. The σ dissociates, leaving the exit channel clear for the transcribed mRNA to exit the RNA polymerase. Termination of transcription can be achieved via two mechanisms: Rho-independent transcription termination and Rho-dependent termination. Rho-independent termination involves palindromic terminator sequences within the mRNA that signal the RNA polymerase to stop. This sequence forms a stem-loop hairpin structure, promoting the dissociation of RNAP from the DNA template. Rho-dependent termination requires the ρ (rho) factor protein. It functions to inhibit mRNA synthesis by binding to specific sites on the nascent RNA strand and then moving along the mRNA towards RNAP. RNAP is paused by a stem-loop structure upstream. Once the ρ (rho) factor protein reaches the RNAP it causes RNAP to dissociate, effectively terminating transcription (reviewed in Madigan, Madigan, & Brock, 2009).

As the amount of RNA polymerase present in the cell is limited, in conjunction with a limited supply of σ factors, the sequence of the promoter becomes important in the recruitment of RNAP for transcription. To counter these limitations, there are several mechanisms that have evolved to ensure the recruitment and distribution of RNAP to

promoters of varying strength. Various promoter architectures, different σ factors, small ligands, DNA conformations, and transcription factors all have a role altering gene expression in response to environmental stimuli (Browning & Busby, 2004).

2.1.2 Regulation by Transcription Factors

The *E. coli* genome has more than 300 genes predicted to encode transcription factors that bind to promoter sequences (Madan Babu & Teichmann, 2003; Perez-Rueda & Collado-Vides, 2000). The majority of transcription factor proteins are sequence specific DNA-binding proteins that can influence transcription at one or multiple promoters. Transcription factors can be grouped into families based on sequence analysis and function (Perez-Rueda & Collado-Vides, 2000). Examples include the LacI, CRP, OmpR, and AraC families of transcription factors. Regulation of promoters by transcription factors occurs in response to environmental stimuli. Transcriptional regulation often requires transcription factor binding to DNA and interacting with RNAP or promoting sigma factor binding (van Hijum, Medema, & Kuipers, 2009a). Approximately 65% of annotated *E. coli* K-12 operons and genes are described as being regulated by at least one of these encoded transcription factors, with many genes being regulated by multiple transcription factor binding sites for that particular transcription factor (van Hijum, Medema, & Kuipers, 2009a).

Transcription factors themselves are regulated either by controlling their activity or their gene expression. Mechanisms to regulate transcription factor activity include modulation of DNA-binding affinity by small ligands, covalent modification,

transcription factor concentration regulation, and finally, transcription factor sequestration by a regulatory protein (Browning & Busby, 2004).

The typical transcription factor binding site is between 12 and 10 bp long appearing as a direct repeat or inverted repeat (Rodionov, 2007). The location of these sites within the promoter has the ability to influence whether the transcription factor functions as an activator or repressor of transcription. More specifically, repression or activation of transcription by a transcription factor is dependent on the sequence of the transcription factor binding site, its proximity to the sigma-factor binding site or its position with respect to the transcription start site (Perez-Rueda & Collado-Vides, 2000). Additionally, the repression or activation effects of transcription factor binding sites are influenced by site orientation on the DNA helix. Commonly, in order for a transcription factor (TF) to exert an effect both the transcription factor binding site and RNAP site have to be on the same face of the DNA helix (van Hijum, Medema, & Kuipers, 2009a).

Transcriptional activators typically bind upstream of transcription start site sequences of target promoters, mediating transcription via several mechanisms. Transcription factor mediated activation can occur via transcription factor binding upstream of the core promoter and interacting with the alpha subunit of RNAP; TF binding adjacent to core promoter and facilitating sigma factor of holoenzyme binding; or, TF binding initiating a DNA conformational change facilitating holoenzyme binding (Barnard, Wolfe, & Busby, 2004; Browning & Busby, 2004; Lloyd, Landini, & Busby, 2001; Rodionov, 2007; Smits, Hoa, Hamoen, Kuipers, & Dubnau, 2007) **.**

Alternatively, transcription factors can function to repress transcription instead of functioning as activators. Transcription factors repress transcription by binding to core

promoter elements such as -10 or -35 sequences, effectively preventing RNAP association with the promoter. TF binding at the beginning of coding region blocks the process of elongation. Other mechanisms include TF binding mediating DNA looping by binding regions both upstream and downstream of the core promoter, or by modulating the activity of a transcriptional activator (van Hijum, Medema, & Kuipers, 2009b). Repressor sites are typically located -60 to +60 with regards to transcription start site (Collado-Vides, Magasanik, & Gralla, 1991; Espinosa, Gonzalez, Vasconcelos, Huerta, & Collado-Vides, 2005; Madan Babu & Teichmann, 2003; Moreno-Campuzano, Janga, & Perez-Rueda, 2006). There are exceptions, as transcription factors that function as repressors have been found to bind to sites further upstream of the transcription start site (Lanzer & Bujard, 1988).

2.1.3 Role of H-NS in *Shigella* Virulence

Nucleoid structuring proteins aid in the packing of bacterial chromosomal DNA into the nucleoid region of the cell. These proteins are abundant in bacterial cells and are indiscriminate in which DNA sequences they bind, thus nucleoid structuring proteins have the potential to influence transcription of the bacterial genome. These proteins not only can bind DNA, but can alter DNA architecture by looping and bridging DNA or changing DNA supercoiling. One such protein, H-NS, regulates *Shigella* virulence by mediating repression of virulence genes.

H-NS is a chromosomally encoded histone nucleoid-structuring protein that typically functions as a global repressor of transcription. H-NS can recognize, bind, and influence the DNA topology of bacterial promoters. H-NS can be present in the cell as

monomers (approximately 10^5 per cell) (Williams & Rimsky, 1997) or as dimers at low concentrations within the cell (Smyth et al., 2000). It has been found to bind to DNA in two ways: specifically, by binding to A-T rich sequences or intrinsically curved DNA, or non-specifically (Tupper et al., 1994). Upon binding to DNA, H-NS can function to silence transcription by several mechanisms (Fig. 4). It has been proposed that H-NS nucleoprotein complexes prevent open complex formation (Nagarajavel, Madhusudan, Dole, Rahmouni, & Schnetz, 2007) or traps open complexes once formed (Shin et al., 2005).

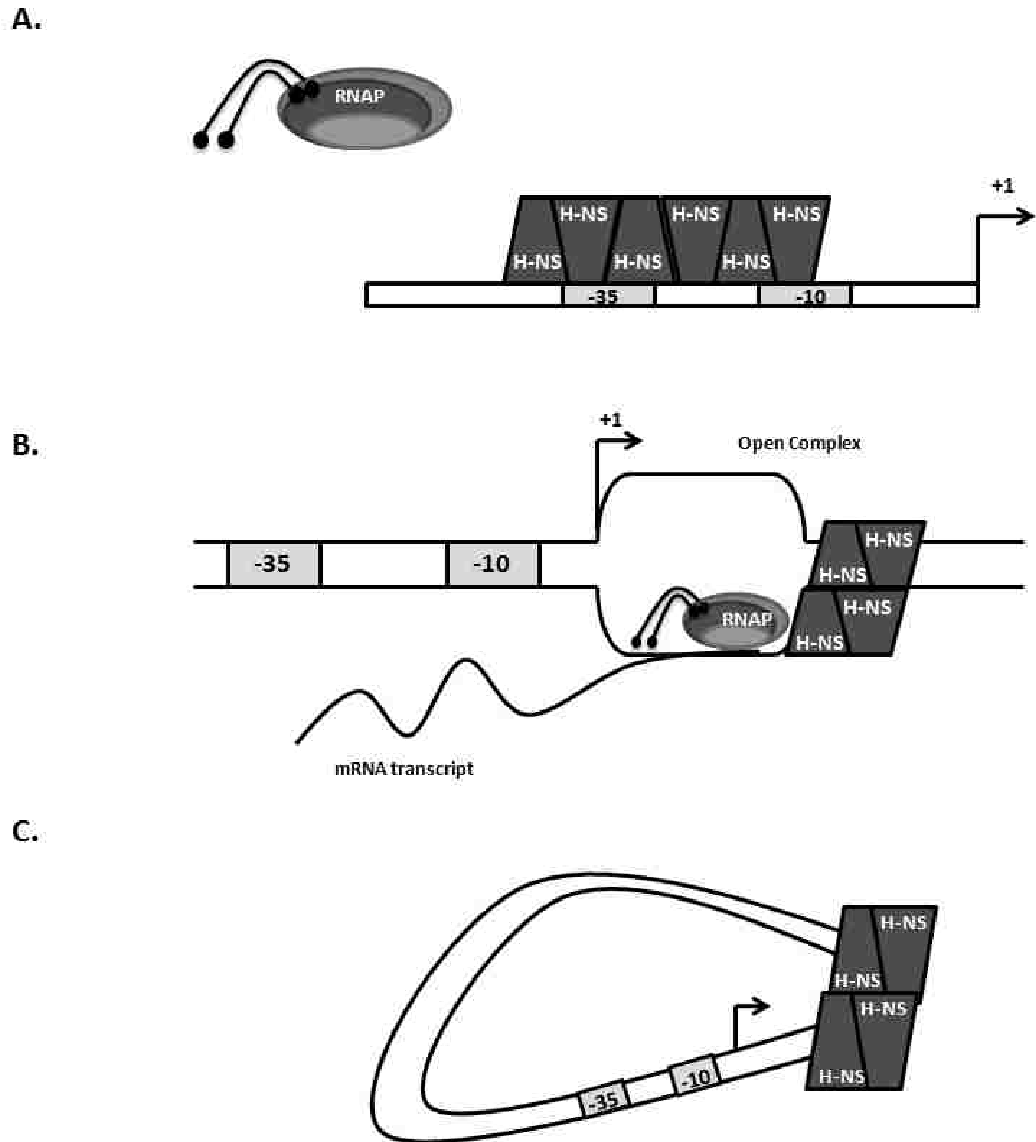


FIGURE 4: Schematics of H-NS mediated repression of transcription, in panel A the binding of H-NS to DNA prevents RNA polymerase (RNAP) association with promoter sequences, B, H-NS binding to promoter sequences traps the open complex, preventing RNAP from proceeding, or, C, H-NS mediates repression of transcription by binding an upstream and downstream region of DNA within the promoter, forming DNA loops.

H-NS is a ~15 kDa protein (Atlung & Ingmer, 1997) with a DNA-binding motif within the carboxyl-terminus and an oligomerization domain within the amino-terminus (Ueguchi, Suzuki, Yoshida, Tanaka, & Mizuno, 1996; Williams, Rimsky, & Buc, 1996); these functional domains are connected through a flexible linker (Smyth et al., 2000). H-

NS preferentially binds planar curves within DNA sequences, which are often A-T rich sequences. It is commonly reported that H-NS has no consensus binding site, but one group has identified an H-NS DNA binding motif with high sequence specificity. This motif was identified within the *E. coli proU* operon by footprinting analysis and H-NS-DNA ChIP-on-chip experiments (Lang et al., 2007). Therefore the role of H-NS as a repressor is feasible given that A-T rich DNA sequences and planar curved DNA are characteristic of bacterial promoters (Pedersen, Jensen, Brunak, Staerfeldt, & Ussery, 2000).

The presence of curved DNA in bacterial promoters and its role in gene expression has been investigated for several genes present in *E. coli*. Although the specific mechanisms that allow curved DNA to influence biological processes is unknown, its role in transcription initiation has been documented and several possible routes proposed. Firstly, intrinsically curved DNA could potentially form large loops around RNA polymerase, enhancing the affinity of the complex for promoter DNA sequences (Matthews, 1992; Perez-Martin, Rojo, & de Lorenzo, 1994). Secondly, it has been demonstrated that minimal intrinsic curvature may enhance the affinity of protein-DNA contacts (Suzuki & Yagi, 1995). Furthermore, there is evidence to suggest that DNA curvature and/or looping can place distant transcriptional components in close proximity (Matthews, 1992).

2.1.4 VirB: Transcription Factor or H-NS Antagonist?

lym use to propel itself within the host cell cytoplasm and laterally into adjacent host cells. IcsP cleaves IcsA, maintaining a tight polar cap of IcsA on the bacterial surface that allows for directed movement of the bacterium. Proteolytic modification of IcsA by IcsP therefore has the potential to control *Shigella* virulence.

IcsP protein production requires VirB, which regulates *icsP* at the level of transcription (Wing, Yan, Goldman, & Goldberg, 2004). Analysis of the region upstream of the *icsP* promoter has identified 9 putative VirB-binding sites (Fig. 4). Previous work has shown that mutagenesis of the two most distal VirB-binding sites, located -1144 and -1130 relative to the transcription start site, results in the complete loss of VirB-dependent regulation of the *icsP* promoter (Castellanos, 2009). This suggests that these two sites are indispensible for the VirB-dependent regulation of the *icsP* promoter.

FIGURE 5: Graphical representation of the *icsP* promoter. The gray arrows represent the location of a VirB binding site. The match to consensus sequence 5′-(A/G)(A/T)G(G)AAAT-3′ is given (Castellanos et al., 2009; Taniya et al., 2003; Turner & Dorman, 2007b) . The white arrows indicate a center of intrinsic curvature as predicted by MUTACURVE software.

Considering classical transcriptional activators typically function at sites within 200 bp of transcription start site, it is unusual that VirB binding sites located over 1 kb upstream of *icsP* are required for *icsP* expression. Thus, we chose to further analyze the role of remote VirB binding sites in VirB-dependent regulation of the *icsP* promoter. To accomplish this, we sought to determine the spacing and helical orientation requirements of the VirB binding sites, to establish if the binding sites are required *in cis* with respect to downstream promoter elements, and to elucidate binding site organization necessary to mediate possible VirB DNA binding at these sites. Our experiments address the mechanism of VirB influence from these sites by providing insight into whether VirB can function *in trans* to mediate VirB-dependent regulation; if the distal location or helical orientation of VirB binding sites is a critical component to VirB-dependent regulation;

and finally, determine the relative spacing requirements between the most distal VirB binding sites that are organized as an inverted repeat. Our experiments also address the mechanism of H-NS mediated repression of *icsP* promoter activity by examining the role of intrinsic curvature and the contribution of H-NS binding sites to the transcriptional regulation of *icsP*.

2.2 Materials and Methods

2.2.1 Bacterial Strains and Media

S. flexneri serotype 2a strain 2457T was isolated by Lt. Col. Oscar Felsenfeld in Tokyo, Japan, and is both serologically and biochemically a typical culture of *S. flexneri* (Formal et al., 1958). The isogenic *virB* mutant strain AWY3 was created by moving the kanamycin-resistant locus from YSH6000 *virB*::Tn5 into the *S. flexneri* wild-type strain 2457T by P1 transduction (Adler et al., 1989; Wing et al., 2004). Strain BS103 is a derivative of 2457T lacking the virulence-associated plasmid, pSf2a140 (Maurelli, Blackmon, & Curtiss, 1984).

S. flexneri 2a strains were routinely grown at 37 ˚C in Trypticase Soy Broth (TSB) with aeration or on Trypticase Soy Agar (TSA) (TSB containing 1.5% [wt/vol] agar). To verify the virulence phenotype, *Shigella* strains were streaked on TSA plates containing 0.01% (wt/vol) Congo red (Sigma Chemical Co., St. Louis, Mo.) *E. coli* strains were grown at 37 ˚C in Luria-Bertani broth with aeration or on LB agar (LB broth containing 1.5% [wt/vol] agar). Where appropriate, antibiotics were used in the following concentrations in *Shigella* and *E. coli* strains, chloramphenicol 25 µg ml^{-1}, ampicillin 100 µg ml^{-1}, tetracycline 20 µg ml^{-1}.

2.2.2 Plasmid Constructions

The starting point for this work was pHJW20, which carries the *icsP* promoter region transcriptionally fused to *lacZ* in pACYC184 (Castellanos et al., 2009). Briefly, pHJW20 carries 1,232 bp of wild-type DNA sequence upstream of the *icsP* transcription start site with a unique *XbaI* site upstream of *lacZ* gene.

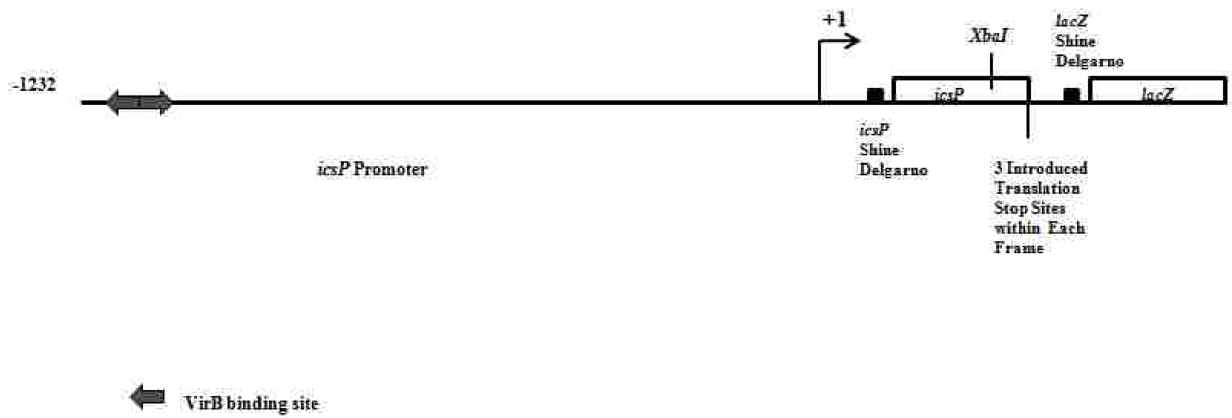


FIGURE 6: Schematic of the *icsP-lacZ* transcriptional fusion pHJW20.

pMIC21 is a pHJW20 derivative lacking all *icsP* promoter sequences, resulting in a promoter-less *lacZ* reporter plasmid (Castellanos et al., 2009).

Description of Constructions Relocating VirB Binding Sites

The following P*icsP-lacZ* transcription fusion constructs contain 5, 10, or a 50 bp deletion within the *icsP* promoter, relocating the VirB binding sites closer to *icsP* transcription start site.

The starting point for these constructs was pJS01, a pHJW20 derivative that carries 1040 bp of the *icsP* promoter region, cloned upstream of the *icsP* transcription start site using *Pst*I and *Pac*I.

pKLP03 is a pJS01 derivative carrying the *icsP* promoter with a 5 bp deletion 92 bp downstream of the most distal VirB binding sites. The *icsP* promoter fragment was PCR amplified using oligonucleotidesWing 98 and Wing 226, the resulting product was digested with *Nru*I and *Bgl*II, and ligated into *Nru*I and *Bgl*II digested pJS01.

pKLP04 is a pJS01 derivative carrying the *icsP* promoter with a 10 bp deletion 82 bp downstream of the most distal VirB binding sites. The *icsP* promoter fragment was PCR amplified using oligonucleotides Wing 98 and Wing 227, the resulting product was digested with *Nru*I and *Bgl*II, and ligated into *Nru*I and *Bgl*II digested pJS01.

pKLP05 is a pJS01 derivative carrying the *icsP* promoter with a 50 bp deletion 42 bp downstream of the most distal VirB binding. The *icsP* promoter fragment was PCR amplified using oligonucleotides Wing 98 and Wing 228, the resulting product was digested with *Nru*I and *Bgl*II, and ligated into *Nru*I and *Bgl*II digested pJS01.

Sequences of oligonucleotides used in plasmid construction are listed in Table. 2.

Description of Constructions Carrying VirB Binding Sites in trans

The following plasmids were created to clone the most distal VirB binding sites *in trans* to the downstream *icsP* promoter sequence.

pBR322 is a commonly used plasmid cloning vector in *E. coli*, conferring resistance to ampicillin and tetracycline.

pKLP09 is a pBR322 derivative carrying sequences -1232 to -664 of the *icsP* promoter. The *icsP* promoter sequence was PCR amplified using oligonucleotides Wing 93 and Wing 234, the resulting product was digested with *Sal*I and *BamH*I, and ligated

into *Sal*I and *BamH*I digested pBR322. This construct does not contain a reporter transcriptional fusion.

pDH01 is a pHJW20 derivative carrying 663 bp of the *icsP* promoter upstream of transcription start site fused to the *lacZ* gene (Castellanos et al., 2009).

Sequences of oligonucleotides used in plasmid construction are listed in Table. 2.

Description of Constructions Carrying Insertions between Most Distal VirB Binding Sites

The following constructs were created to introduce base pair insertions between the most distal VirB binding sites using site-directed mutagenesis (Stratagene).

The starting point for this work was the high-copy plasmid pNE01, a pBluescript derivative carrying the full length *icsP* promoter. To create pNE01 *icsP* promoter fragment was isolated from pHJW20 using *Pst*I and *Xba*I, and ligated into *Pst*I and *Xba*I digested pBluescript/KSII+.

pKLP11 carries the *icsP* promoter with a 2 bp insertion between the most distal VirB binding sites. Insertions were introduced into pNEO1 using oligonucleotides Wing 265 and 266, the resulting plasmid was digested with *Pst*I and *Pac*I and ligated into *Pst*I and *Pac*I digested pHJW20.

pKLP12 carries the *icsP* promoter with a 3 bp insertion between the most distal VirB binding sites. Insertions were introduced into pNEO1 using oligonucleotides Wing 263 and 264, the resulting plasmid was digested with *Pst*I and *Pac*I and ligated into *Pst*I and *Pac*I digested pHJW20.

pKLP13 carries the *icsP* promoter with a 4 bp insertion between the most distal VirB binding sites. Insertions were introduced into pNEO1 using oligonucleotides Wing

261 and 262, the resulting plasmid was digested with *Pst*I and *Pac*I and ligated into *Pst*I and *Pac*I digested pHJW20.

Sequences of oligonucleotides used in plasmid construction are listed in Table. 2.

In silico analysis of icsP promoter

The MUTACURVE program was used to i) evaluate the amplitude of the intrinsic DNA curvature of every nucleotide in the sequence immediately upstream of the *icsP* transcription start site, and ii) predict the effect of double point mutations on the intrinsic curvature of the DNA. The MUTACURE program predicts DNA curvature using the algorithm of Goodsell & Dickerson (Goodsell & Dickerson, 1994), with the addition of Satchwell's contribution matrixes for rotational and spatial displacements (Satchwell, Drew, & Travers, 1986). It also generates curvature profiles of the original and mutated sequences for comparative analysis (Olivares-Zavaleta, Jauregui, & Merino, 2006).

Centers of curvature were identified at regions -435, -663, -842, and -935 with respect to the transcription start site of *icsP*. To evaluate the effect of curvature on *icsP* promoter activity, a series of constructs were created carrying base pair substitutions in regions predicted to disrupt or restore curvature respectively.

Description of constructs carrying base-pair substitutions in regions of predicted DNA curvature

The following constructs are P*icsP-lacZ* fusions that contain base-pair substitutions in regions of predicated DNA curvature within the *icsP* promoter, with a copy number of 10-12 plasmids per cell. Substitutions were introduced using site-directed

mutagenesis. The starting point for this work was the high-copy plasmid pNEO1 a pBluescript derivative carrying the full length *icsP* promoter. The following table outlines the identified regions and base pair substitutions predicted to disrupt or restore curvature.

Base Pair Substitutions Introduced to Reduce Curvature

Position of curved region	**Predicted curvature value (deg/helix turn) prior to substitution**	**Predicted curvature value (deg/helix turn) following substitution**	**Base pair substitutions responsible**
-842	9.65	4.82	A>C at -876 C>A at -880
-935	9.36	5.19	G>T at -968 A>C at -874
-663	8.09	2.67	C>A at -961 T>G at -696
-435	7.88	2.99	T>G at -454 A>C at -463

TABLE 1: Table of the identified MUTACURVE predicted regions of intrinsic curvature within the *icsP* promoter and base pair substitutions predicted to disrupt curvature (Harrison, 2010).

pMUT1 is a pHJW20 derivative carrying a 2 bp substitution made by site directed mutagenesis in the -842 region of the *icsP* promoter. Mutation 1 is located at -876 (T → G) and mutation 2 is located at -880 (G → T) with respect to the *icsP* transcription start site. Mutations were introduced into pNEO1 using Wing 206 and Wing 207.

pJC04 is a pMUT1D derivative carrying 4 bp substitutions in the *icsP* promoter, present at -876 (T → G), -880 (G → T), -968 (C → A), and -974 (T → G) with respect to the *icsP* transcription start site. Mutations were introduced using Wing 208 and Wing 209.

pKLP14 is a pDH01 derivative carrying the *icsP* promoter sequence present in pJC04. The *icsP* promoter insert was created by digesting pJC04 with *Pst*I and *Pac*I, and ligated into *Pst*I and *Pac*I digested pDH01.

pMUT3D is a pHJW20 derivative, carrying a 2 bp substitution made by site directed mutagenesis in the -663 region of the *icsP* promoter. Mutation 1 is located at -691 (G→ T) and mutation 2 is located at -696 (A → C) with respect to the *icsP* transcription start site. Mutations were introduced into pNEO1 using Wing 210 and Wing 211.

pJC05 is a pMUT3D derivative carrying 4 bp substitutions in the *icsP* promoter. Mutations are present at -691 (G→ T), at -696 (A → C), at -455 (C → A), and at -464 (G → T) with respect to *icsP* transcription start site. Mutations were introduced into pMUT3D using oligonucleotides Wing 212 and Wing 213.

pKLP15 is a pDH01 derivative carrying the *icsP* promoter sequence present in pJC05. The *icsP* promoter insert was created by digesting pJC04 with *Pst*I and *Pac*I, and ligated into *Pst*I and *Pac*I digested pDH01.

pJC06 carries all base pair substitutions to reduce MUTACURVE predicted intrinsic curvature. The *icsP* promoter fragment insert was created by digesting pJC05 with *Nsi*I and *Pst*I, and ligated into *Nsi*I and *Pst*I digested pJC04.

pKLP16 is a pDH01 derivative carrying the *icsP* promoter sequence present in pJC05. The *icsP* promoter insert was created by digesting pJC04 with *Pst*I and *Pac*I, and ligated into *Pst*I and *Pac*I digested pDH01.

Sequences of oligonucleotides used in plasmid construction are listed in Table. 2.

Description of constructs carrying deletions in the icsP promoter to rotate H-NS binding sites

The following P*icsP-lacZ* transcription fusion constructs contain 5 or 10 bp deletion within the *icsP* promoter, rotating the H-NS binding sites with respect to each other. The starting point for this work was the high-copy plasmid pNEO1 a pBluescript derivative carrying the full length *icsP* promoter. Deletions were introduced using site-directed mutagenesis (Stratagene).

pKLP23 carries the *icsP* promoter with a 5 bp deletion centered at -310. Deletions were introduced into pNEO1 using oligonucleotides Wing 286 and 287, the resulting plasmid was digested with *Pst*I and *Pac*I and ligated into *Pst*I and *Pac*I digested pHJW20.

pKLP24 carries the *icsP* promoter with a 10 bp deletion centered at -310. Deletions were introduced into pNEO1 using oligonucleotides Wing 296 and 297, the resulting plasmid was digested with *Pst*I and *Pac*I and ligated into *Pst*I and *Pac*I digested pHJW20.

Sequences of oligonucleotides used in plasmid construction are listed in Table. 2.

All plasmids were sequenced at the Nevada Genomics Center using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1, and plasmids with correct sequence were used.

	Oligonucleotides Used In Plasmid Construction
Primer	Sequence 5'-3'
WING 93	TGGGTTGAAGGCTCTCAAGGGC
WING 98	CCCGTTCCATGTGCTCGCC
WING 206	CCTTGAAAACGCCGTATCGGGAGAGAGTTCACTCTATTG
WING 207	CAATAGAGTGAACTCTCTCCCGATACGGCGTTTTCAAGG
WING 208	CCACTACTGAACTATTCTGGGATTTAAATTCAATACAATGGTTGGTTGAAGGTC
WING 209	GACCTTCAACCAACCATTGTATTGAATTTAAATCCCAGAATAGTTCAGTAGTGG

WING 210	GTTATCCTCTACACATTTTATCTAATGTGTACTTGGACAATTCATTGGTGC
WING 211	GCACCAATGAATTGTCCAAGTACACATTAGATAAAATGTGTAGAGGATAAC
WING 212	GTATAGTTTTTCAGATTTTGTTTGTCCCGTGACATATTATGGAAATGGCAGAAGC
WING 213	GCTTCTGCCATTTCCATAATATGTCACGGGACAAACAAAATCTGAAAAACTATAC
WING 214	CCTTGAAAACGCCGTATTGGGGGAGAGTTCACTCTATTG
WING 215	CAATAGAGTGAACTCTCCCCCAATACGGCGTTTTCAAGG
WING 216	CCACTACTGAACTATTCTGGGATTTAAATCCAATATAATGGTTGGTTGAAGGTC
WING 217	GACCTTCAACCAACCATTATATTGGATTTAAATCCCAGAATAGTTCAGTAGTGG
WING 218	GTTATCCTCTACACATTTTATCTGATGTATACTTGGACAATTCATTGGTGC
WING 219	GCACCAATGAATTGTCCAAGTATACATCAGATAAAATGTGTAGAGGATAAC
WING 220	GTATAGTTTTTCAGATTTTGTTTATCCCGTGATATATTATGGAAATGGCAGAAGC
WING 221	GCTTCTGCCATTTCCATAATATATCACGGGATAAACAAAATCTGAAAAACTATAC
WING 227	TGAGAGATCTAAAGAGGCTTGGCAGTTTGG
WING 228	TGAGAGATCTCACTAATACAAACTTTTTGTAAAG
WING 234	CCGGGGATCCTCAGTTATCCTCTACACATTTTATCTC
WING 261	TGATTGAATACTTCCGGGGATTTCAGAGACTATGAAATGAAGTATATTTAATATACT
WING 262	AGTATATTAAATATACTTCATTTCATAGTCTCTGAAATCCCCGGAAGTATTCAATCA
WING 263	GATTGAATACTTCCGGGGATTTCAGAGATATGAAATTGAAGTATATTTAATATACT
WING 264	AGTATATTAAATATACTTCATTTCATATCTCTGAAATCCCCGGAAGTATTCAATCA
WING 265	TGATTGAATACTTCCGGGGATTTCAGAGTATGAAATGAAGTATATTTAATATACT
WING 266	AGTATATTAAATATACTTCATTTCATACTCTGAAATCCCCGGAAGTATTCAATCA
WING 287	GCTATTGCAGCCGTGATGATATTGTCTTATCGTTGTTC
WING 288	GAACAACGATAAGACAATATCATCACGGCTGCAATAGC
WING 296	GGCTATTGCAGCCGTGATGATCTTATCGTTGTTCT
WING 297	AGAACAACGATAAGATCATCACGGCTGCAATAGCC

TABLE 2: Oligonucleotides

2.2.3 Promoter Activity of P*icsP*-*lacZ* Transcriptional Fusions

Activity of the *icsP* promoters were determined by measuring β-galactosidase activity using the Miller protocol in strains carrying pHJW20 or derivatives. Routinely, transcription was analyzed in three independent transformants in early stationary phase cultures. Cells were routinely back-diluted 1:100 from 5ml overnight cultures grown with shaking at 30 °C. Freshly inoculated cultures were subsequently grown for 5 h in TSB at 37 °C.

2.3 Results and Discussion

2.3.1 VirB binding sites *in trans* do not influence transcription at the *icsP* promoter

Remote VirB binding sites are essential for VirB-dependent regulation of the *icsP* promoter. These sites named Box 1 and Box 2, are located between positions −1144 and −1130 with respect to *icsP* transcription start site, and are organized as an inverted repeat. Mutagenesis of Box 1 and Box 2 results in a complete loss of VirB-dependent *icsP* promoter activity (Castellanos et al., 2009). In the presence of VirB, promoter activity of a P*icsP-lacZ* fusion increases 17-fold for the full length promoter. Considering the contribution of the remote location of the VirB binding sites to this significant increase in *icsP* promoter activity, we sought to determine if VirB directly regulates *icsP* expression. Specifically, we wanted to establish if VirB can function *in trans* to mediate VirB-dependent regulation and if VirB regulates another factor, such as a small RNA produced from this intergenic region, that directly controls *icsP* expression.

To test this, we cloned the VirB binding sites into a separate DNA molecule, pKLP09. This allowed us to place the VirB binding sites *in trans* to the *icsP* promoter carried by pDH01 (Fig. 7). pDH01 is a P*icsP-lacZ* fusion extending to -664 lacking the VirB binding sites. As a positive control, pHJW20 was used which carries the full length *icsP* promoter fused to *lacZ*, and pMIC21 served as a negative promoter-less control.

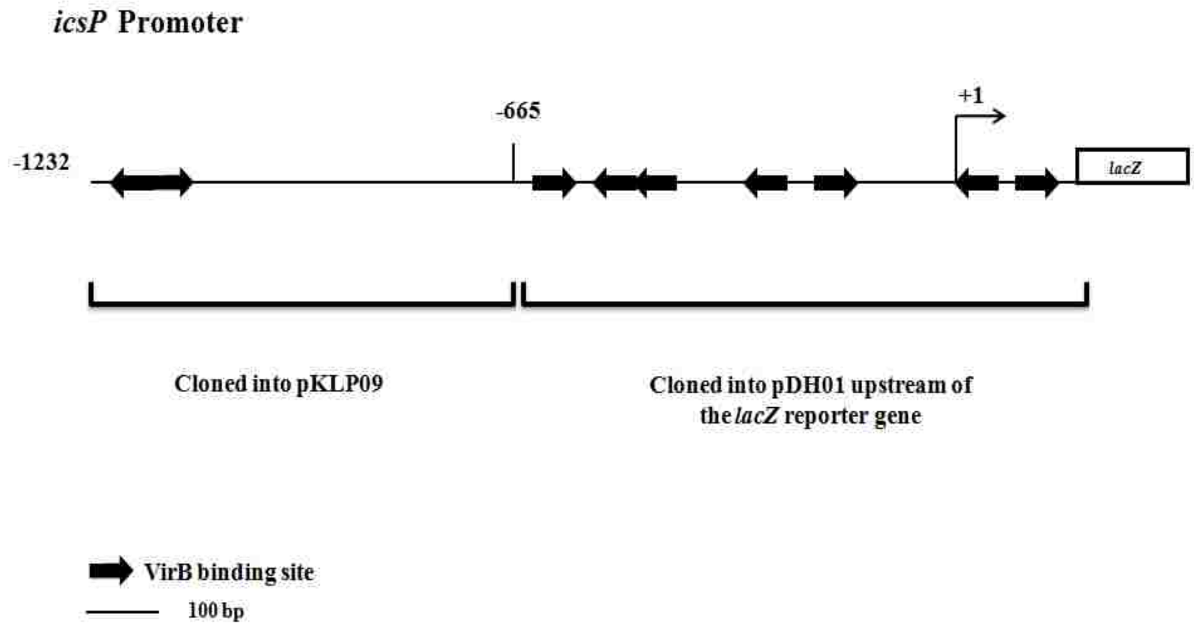


FIGURE 7: Graphical representation of the *icsP* promoter region with VirB binding sites annotated. DNA sequences cloned *in trans* are marked by brackets with the respective plasmid labeled below.

Constructs carrying *icsP* promoter fragments were introduced into wild-type *Shigella flexneri* strain 2457T and *virB* mutant *S. flexneri* strain AWY3 to determine promoter activity using the β-galactosidase assay.

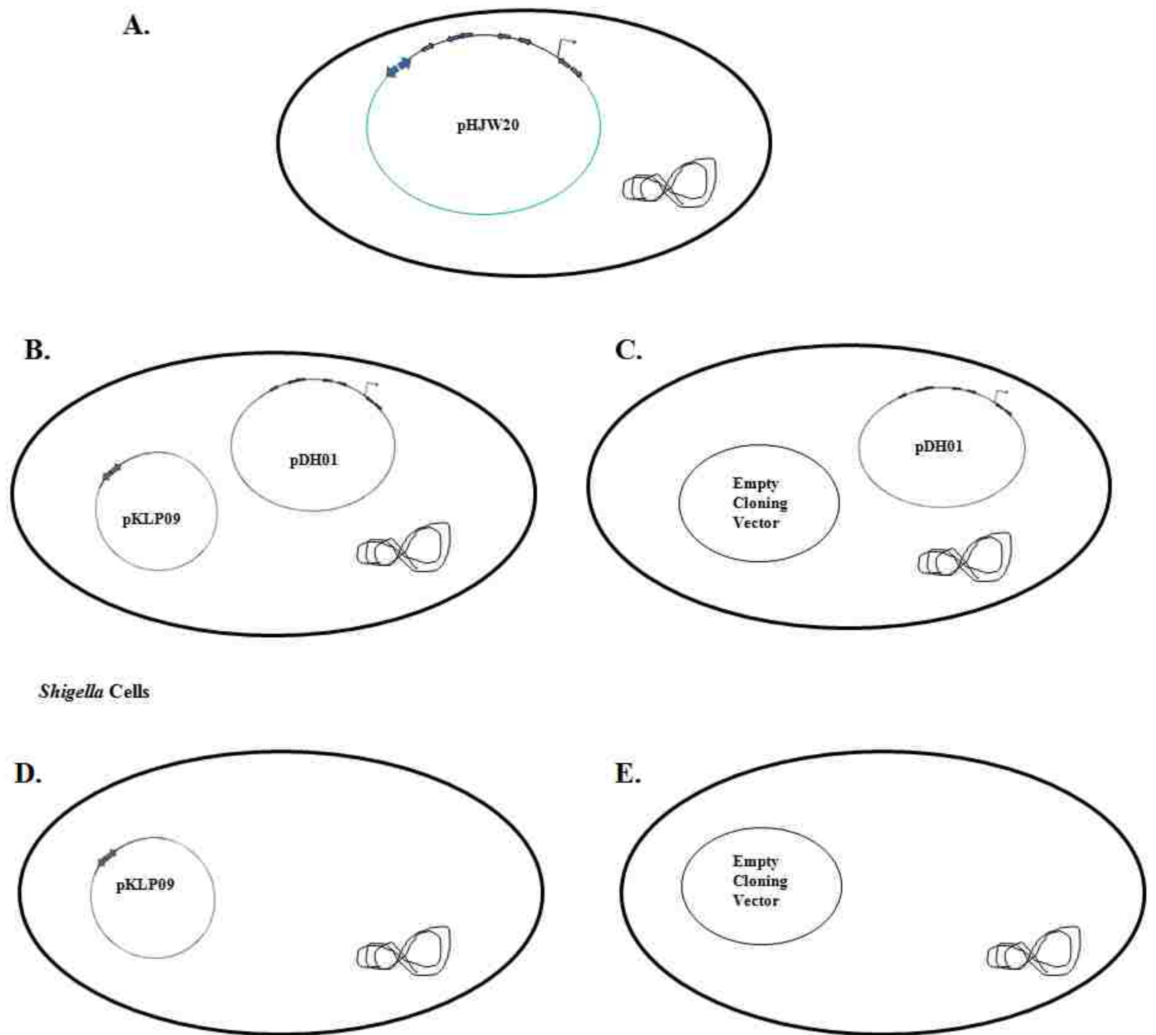


FIGURE 8: The wild-type *icsP* promoter containing the VirB binding sites *in cis* was introduced into *Shigella* cells (A),. The *icsP* promoter was cloned *in trans* to the VirB binding sites (B), or the promoter was cloned *in trans* to the empty cloning vector (C) as a control. Additionally, the VirB binding sites alone (D) or the empty cloning vector (E) was introduced into *Shigella* cells as further controls.

In the presence of VirB the full-length *icsP* promoter showed a significant increase in promoter activity (Fig. 9), confirming previously published results (Castellanos et al., 2009). For strains carrying the VirB binding sites *in trans* to the *icsP* promoter, there was a loss of VirB dependency because promoter activity was comparable in both wild-type and *virB* mutant *Shigella* strains. This same trend was

mirrored in strains carrying the empty cloning vector and the P*icsP-lacZ* reporter bearing sequences downstream of -665. Both wild-type and *virB* mutant strains carrying the empty cloning vector or the VirB binding sites alone displayed no promoter activity.

Based on these findings we conclude that VirB binding sites Box 1 and Box 2 are required *in cis* for VirB-dependent regulation of the *icsP* promoter, which strongly suggests that VirB directly regulates *icsP* promoter activity rather than modulating the activity of another factor which in turn regulates the *icsP* promoter.

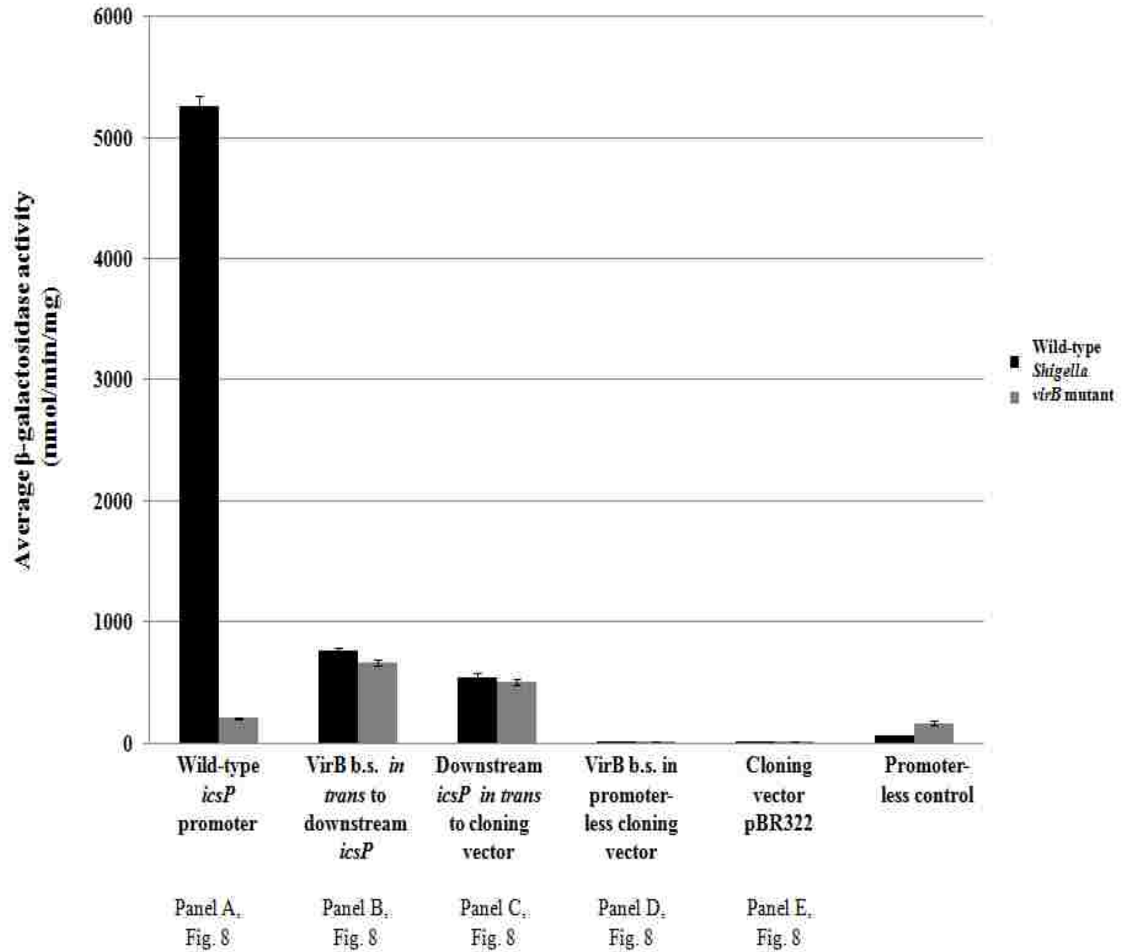


FIGURE 9: Activities of wild-type *icsP* promoter, most distal VirB binding sites *in trans* with respect to downstream promoter elements*, icsP* promoter sequences downstream of the most distal binding sites *in trans* with the cloning vector, the distal sites alone, the cloning vector, or the promoter-less control in wild-type *S. flexneri* 2a (2457T) and the *virB* mutant (AWY3). Assays were run in triplicate and the means and deviations are shown.

2.3.2 Minor Helical Orientation and Spacing Requirements for VirB-dependent Regulation of the *icsP* Promoter From Distal VirB Binding Sites

Considering the organization of the *icsP* promoter, we sought to determine if the location of VirB binding sites Box 1 and Box 2 within the *icsP* promoter is important for VirB-dependent regulation. The required VirB binding sites are located over 1 kb upstream of the transcription start site, which is unusual, as classically binding sites for

transcription factors are considered to be within 200 bp of +1. In addition to establishing spacing requirements, we also sought to determine the helical orientation requirements of these sites. Typically, DNA-binding proteins are able to recognize and bind specific DNA sequences located on a precise face of the DNA helix. To determine the spacing and helical-orientation requirements for VirB-dependent regulation from the distal VirB binding sites, a series of deletions were created downstream of these sites within the *icsP* promoter, relocating them progressively closer to the *icsP* transcription start site (Fig. 10). P*icsP-lacZ* fusions were created with the *icsP* promoter carrying a 5 bp, 10 bp, or 50 bp deletion. By deleting 5 bp downstream of the VirB binding sites, the sites were placed on the opposite face of the DNA helix. A 10 bp deletion places the sites on the same DNA face as in the wild-type promoter, and a 50 bp deletion brings the sites within closer proximity to *icsP* transcription start site.

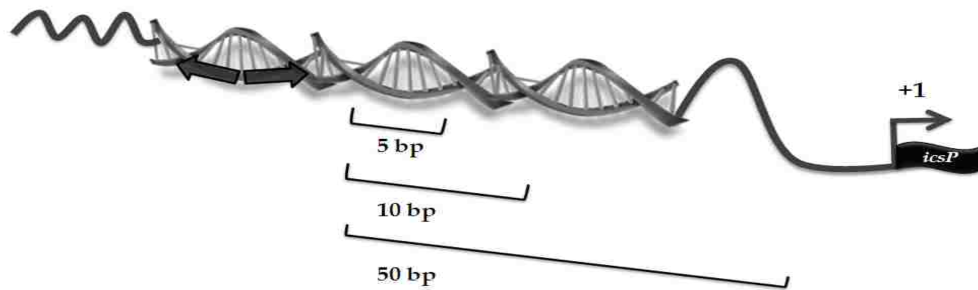


FIGURE 10: Schematic of deletions created downstream of most distal VirB binding sites in the *icsP* promoter.

Constructs carrying P*icsP-lacZ* transcription fusions with deletions in the *icsP* promoter downstream of the VirB binding sites were introduced into wild-type *Shigella flexneri*

strain 2457T and *virB* mutant *S. flexneri* strain AWY3 to determine promoter activity using the β-galactosidase assay.

Our data show that deletions of 5 bp or 10 bp downstream of the most distal VirB binding sites do not significantly alter transcriptional activity of the *icsP* promoter (Fig. 11). Promoter activity for these constructs in wild-type *Shigella* strains is comparable to promoter activity for the full-length *icsP* promoter. In the presence of VirB, *icsP* promoter sequences with relocated distal VirB binding sites show a significant increase in promoter activity, indicating that *icsP* expression is VirB-dependent. Similarly, *icsP* promoter sequences carrying a 50 bp deletion downstream of the most distal VirB binding sites demonstrate a VirB-dependency for *icsP* expression. However, there is a small but statistically significant decrease in promoter activity of *icsP* promoter sequences carrying a 50 bp deletion when compared to wild-type *icsP* promoter activity (Fig. 11). All constructs displayed similar levels of activity in *virB* mutant strains, demonstrating that the introduction of deletions within the promoter has no effect on VirB-independent promoter activity. These data indicate that there is a small spacing requirement for VirB-dependent regulation from these sites, yet there is not a face of helix dependency. Interestingly, relocating the VirB binding sites to the opposite face of the helix has no significant effect on *icsP* promoter levels.

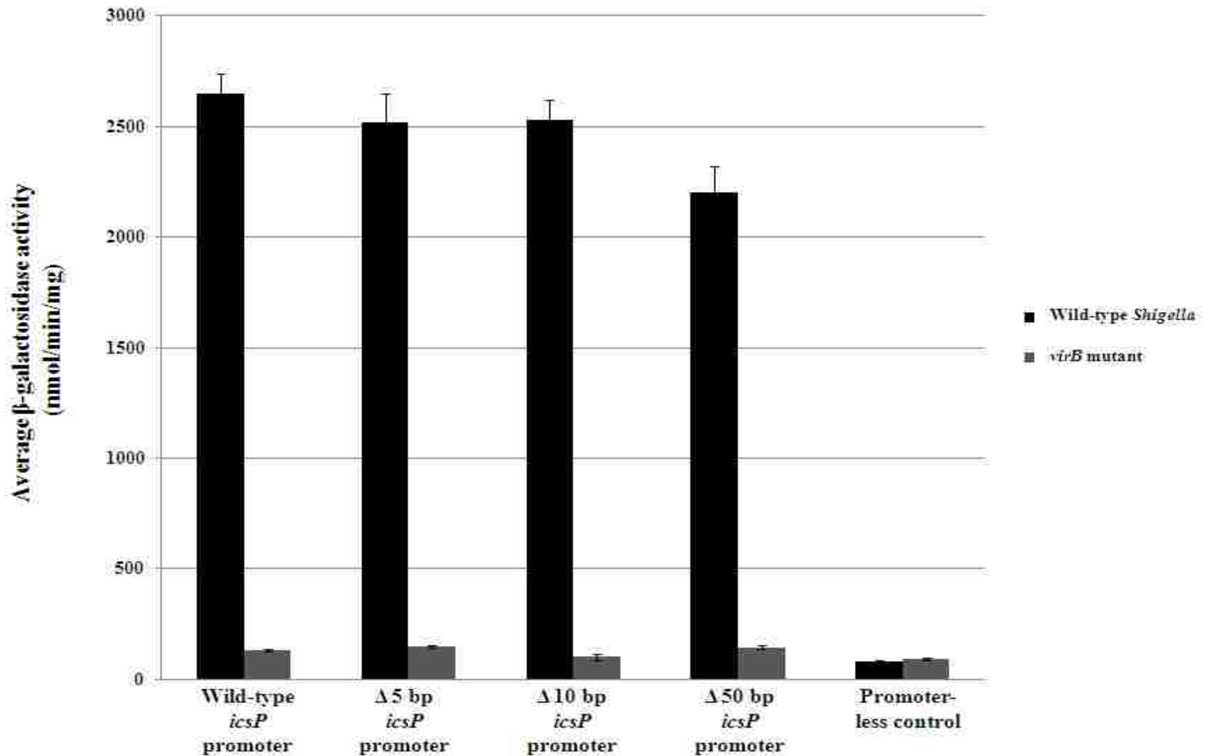


FIGURE 11: Activities of wild-type *icsP* promoter or *icsP* promoter carrying deletions downstream of the most distal VirB binding sites with respect to transcription start site in wild-type *S. flexneri* 2a (2457T) and the *virB* mutant (AWY3). Assays were run in triplicate and the means and deviations are shown.

2.3.3 Strict Spacing Requirements of Box 1 and Box 2 VirB Binding Sites with Respect to Each Other

In the wild-type *icsP* promoter, the most distal VirB binding sites, Box 1 and Box 2 are organized as an inverted repeat that is separated by 1 nucleotide, representing a space of 3.3 Å (Mandelkern, Elias, Eden, & Crothers, 1981). The VirB protein contains a helix-turn-helix motif, which in conjunction with the N-terminus, mediates DNA-binding. VirB has been shown to form dimers *in vivo* and *in vitro,* independent of DNA binding. It is been proposed that VirB dimerization and oligomerization has a role in DNA binding (Beloin, 2002).

We sought to determine the tolerance of base pair insertions between the essential VirB sites. Insertions of 2, 3, or 4 bp were placed between Box 1 and Box 2 using site-directed mutagenesis, for a total spacing between the sites of 3 bp, 4 bp, and 5 bp respectively (Table 3). P*icsP-lacZ* transcription fusions with base pair insertions between the VirB binding sites were introduced into wild-type *Shigella flexneri* strain 2457T and *virB* mutant *S. flexneri* strain AWY3 to determine promoter activity using the β-galactosidase assay.

***icsP* Promoter Fragment**	**Sequence**
Wild-type	5'- ATTTCAGTATGAAAT -3'
2 bp Insertion	5'- ATTTCAGT**CG**ATGAAAT -3'
3 bp Insertion	5'- ATTTCAGT**CGA**ATGAAAT -3'
4 bp Insertion	5'- ATTTCAGT**CGAT**ATGAAAT -3'

TABLE 3: Base pair insertions made between Box 1 and Box 2. Sequence of Box 1 and Box 2 is underlined; base pair insertions are in bold font.

Our data show that there is no tolerance for base pair insertions between the remote VirB binding sites. (Fig. 12). The wild-type *icsP* promoter displays VirB-dependent promoter activity. Constructs carrying base pair insertions between the distal VirB binding sites display extremely low levels of *icsP* promoter activity that are similar to those seen in the *virB* mutant. These data indicate that there is a 1 bp requirement between the VirB binding sites for VirB-dependent regulation of the *icsP* promoter, and these data suggest that VirB binds as a dimer to Box 1 and Box 2.

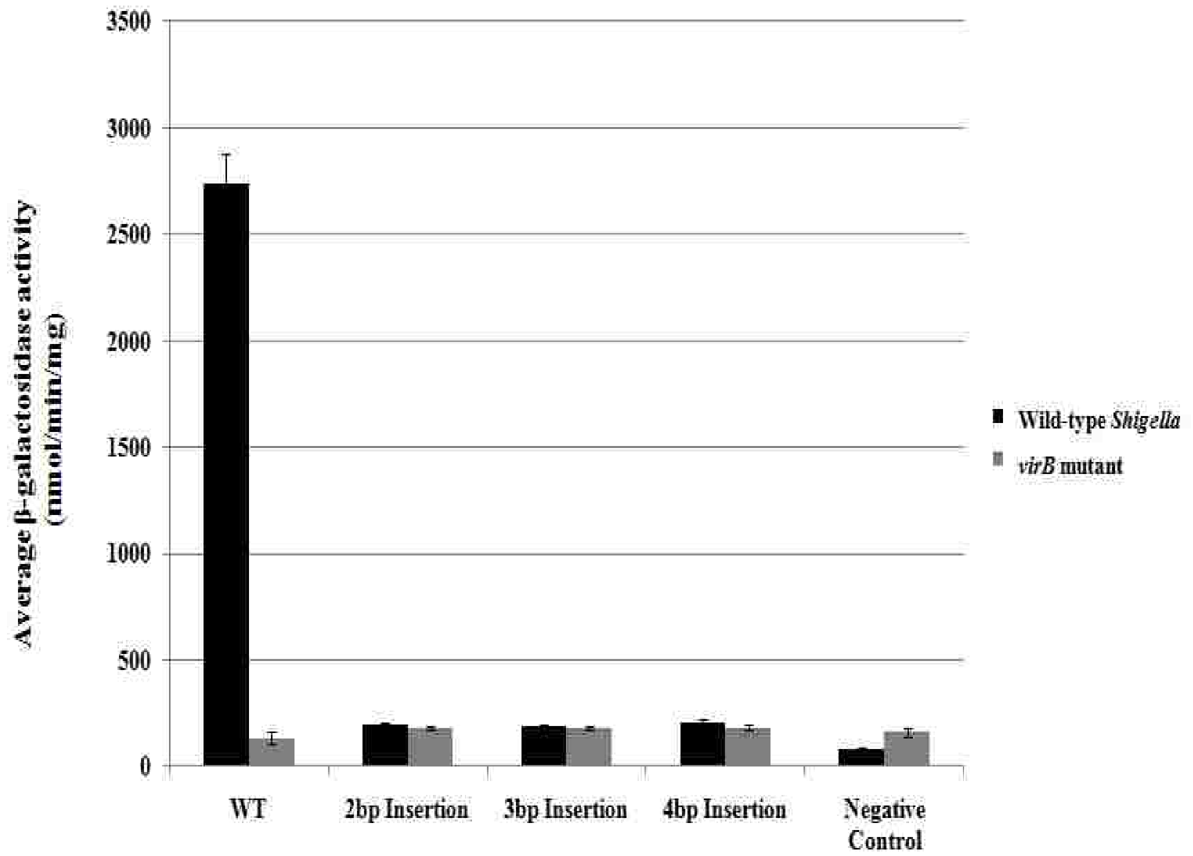


FIGURE 12: Activities of wild-type *icsP* promoter or *icsP* promoter carrying base-pair insertions between the most distal VirB binding sites in wild-type *S. flexneri* 2a (2457T) and the *virB* mutant (AWY3). Assays were run in triplicate and the means and deviations are shown.

2.3.4 Predicted Intrinsic Curvature of DNA at the *icsP* Promoter Does Not Contribute to VirB-Dependent Regulation

Histone nucleoid structuring protein H-NS is a well-established repressor of transcription that recognizes intrinsically curved DNA sequences. H-NS mediates repression of the *icsP* promoter (Castellanos et al., 2009). VirB functions to antagonize H-NS, alleviating repression and facilitating transcription at the *icsP* promoter (Castellanos et al., 2009). *In silico* analysis of the *icsP* intergenic region identified four regions of predicted curvature within the *icsP* promoter (Harrison, 2010). Base pair mutations were introduced into predicted regions of curvature in the *icsP* promoter to

disrupt or reduce the curvature at those positions in order to determine the role of intrinsic curvature in the regulation of *icsP* expression. Promoter activity was measured using β-galactosidase assays. Previous data collected show that promoter activity in the presence of mutations in single regions of predicted curvature is comparable to wild-type *icsP* in both a wild-type *Shigella* background and a *virB* mutant (Harrison, 2010). We therefore chose to further analyze the role of predicted intrinsic curvature in the transcriptional regulation of the *icsP* promoter by combining these mutations. Mutations in predicted regions of curvature were combined in the two most promoter proximal curves centered at -435 and -663, the most distal curves centered at -842 and -935, and lastly, all curves (Fig. 13). P*icsP-lacZ* transcription fusions with base pair substitutions in regions of predicated curvature were introduced into wild-type *Shigella flexneri* strain 2457T and *virB* mutant *S. flexneri* strain AWY3 or wild-type *E. coli* strain MC4100 and an isogenic *h-ns* mutant strain to determine promoter activity using the β-galactosidase assay.

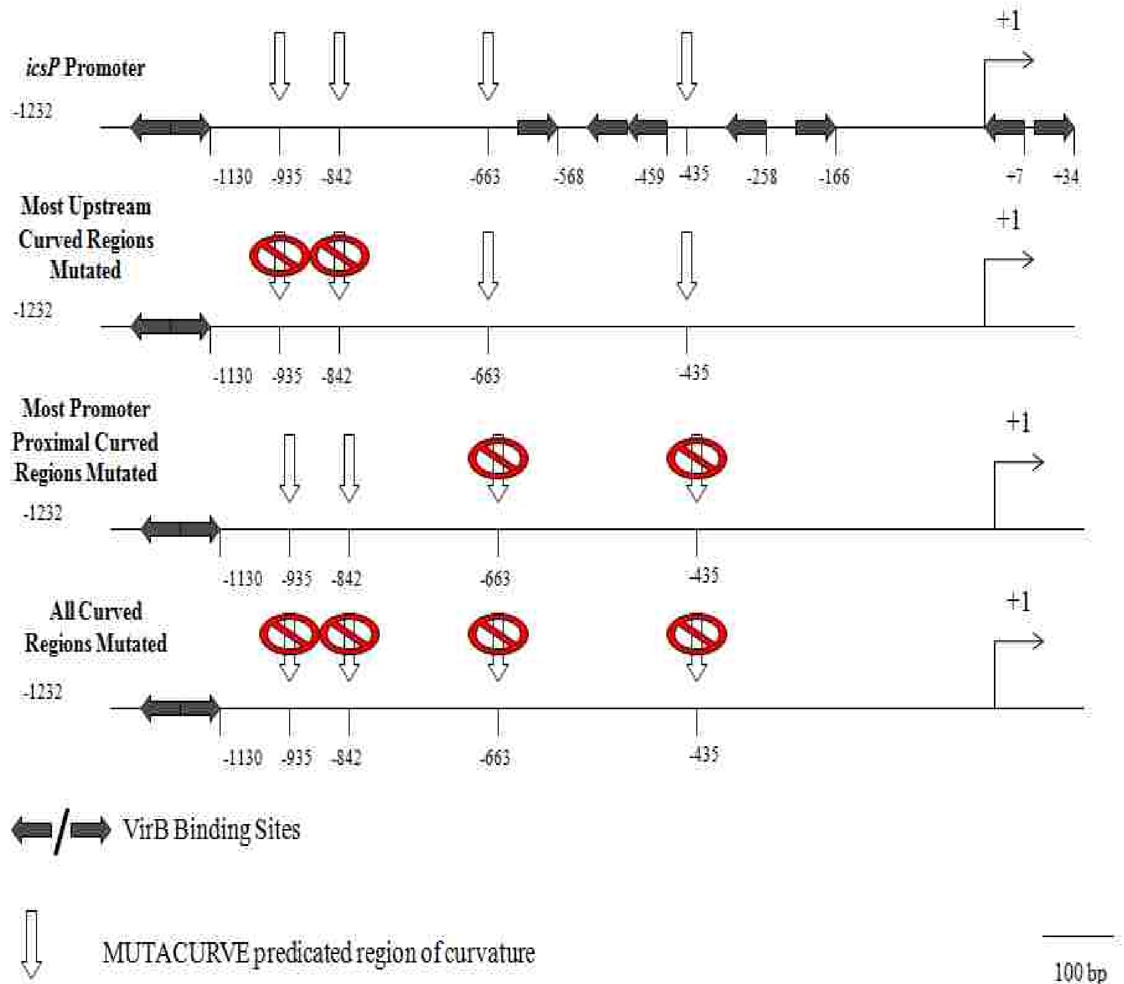


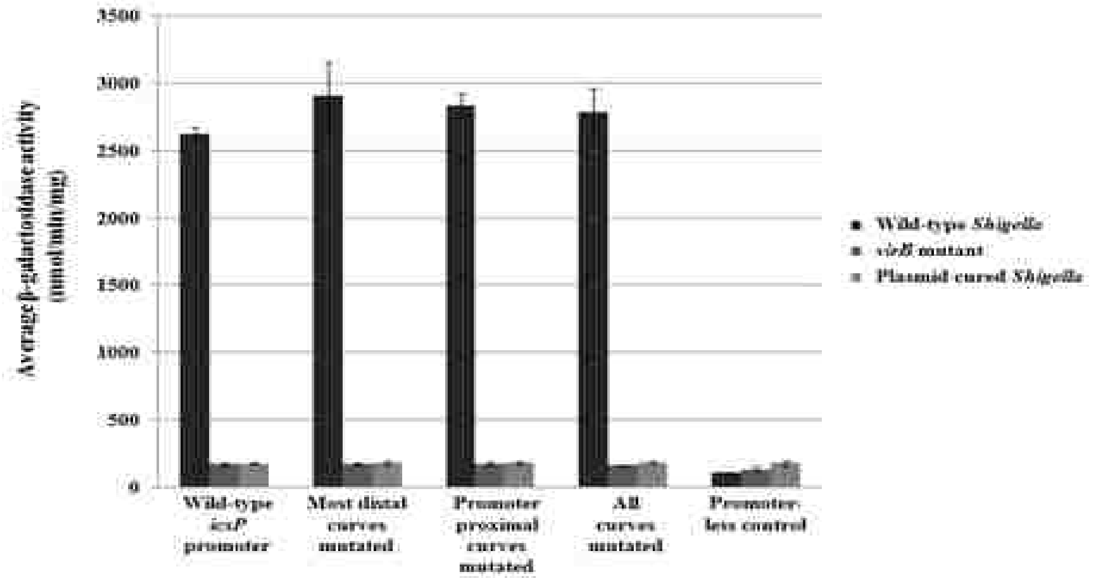
FIGURE 13: Graphical representation of the *icsP* promoter region with VirB binding sites annotated. White arrows indicate center position of regions of curvature within *icsP* promoter. Slash symbols indicate regions of curvature containing base pair mutations predicted to reduce curvature.

In a *Shigella* background, constructs carrying mutations that disrupt regions of predicted curvature that are promoter proximal, promoter distal, or within all regions of curvature, display VirB-dependent promoter activity that is comparable to wild-type *icsP* promoter activity (Fig. 14A). These data suggest that the predicted curvature does not play a role in the VirB-dependent regulation of *icsP*. This is interesting given that the role of VirB is to de-repress H-NS mediated repression of the *icsP* promoter. In the context of

more linear DNA, VirB binding is still required for DNA remodeling into a conformation conducive to transcription. These data suggest that in the presence of more linear promoter DNA, H-NS maintains the ability to mediate repression of *icsP* promoter activity. Therefore, we chose to assay promoter activity of constructs carrying mutations to disrupt curvature within the *icsP* promoter in the presence and absence of H-NS.

In wild-type *E. coli* strains carrying constructs with mutations that disrupt regions of predicted curvature that are promoter proximal, promoter distal, or within all regions of predicted curvature, display promoter activity comparable to wild-type *icsP* promoter levels (Fig. 14B). In *hns* mutant strains, these constructs show an increase an *icsP* promoter activity to levels similar to the wild-type *icsP* promoter. From these data we conclude that in the presence of reduced curvature, H-NS retains the ability to repress the *icsP* promoter.

A



B

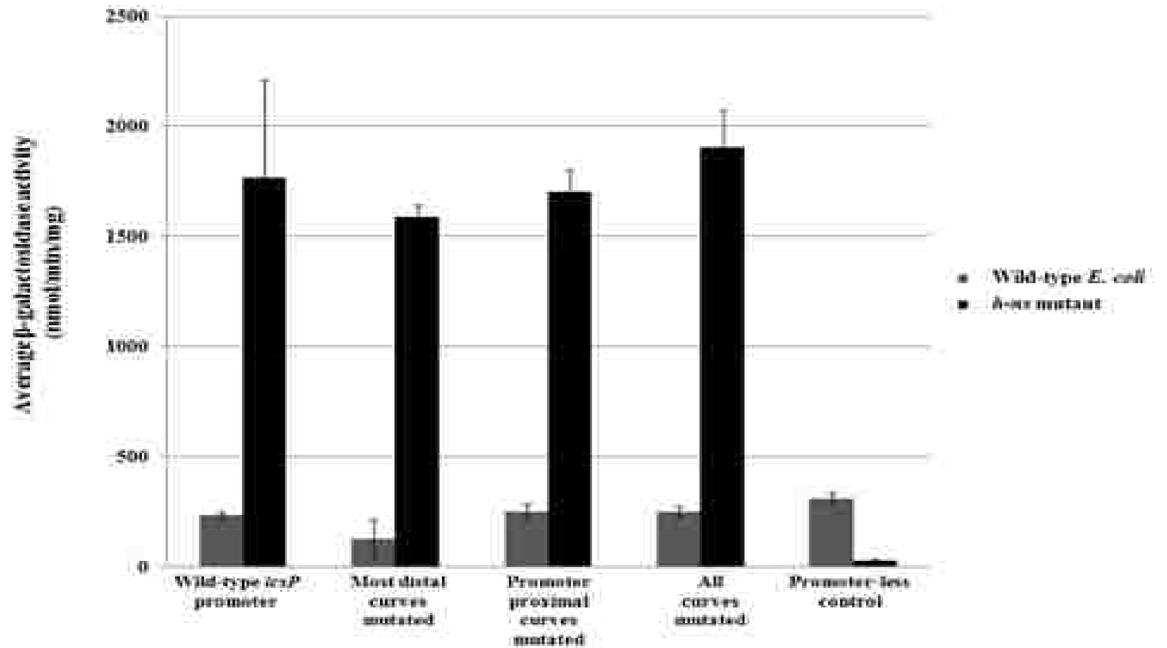


FIGURE 14: Activities of wild-type *icsP* promoter, *icsP* promoter with base pair mutations centered at -842 and -935, *icsP* promoter with base pair mutations centered at -435 and -663, *icsP* promoter with base pair mutations present in all regions of curvature, and promoter-less control in A) wild-type *S. flexneri* 2a (2457T), the *virB* mutant (AWY3), and plasmid cured strain (BS103) or B) wild-type *E. coli* (MC4100) and the *h-ns* mutant. Assays were run in triplicate and the means and deviations are shown.

The ability of H-NS to mediate repression of *icsP* in the presence of more linear DNA sequences is striking because H-NS has been shown to bind intrinsically curved DNA. These data indicate that curvature of the *icsP* promoter does not have a role in H-NS mediated repression of the *icsP* promoter. It has been shown that H-NS not only recognizes intrinsically curved DNA, but also binds to A-T rich DNA tracts and mediates DNA looping. Perhaps H-NS mediates repression of *icsP* by altering the topology of the DNA and looping the *icsP* promoter.

It is understood that our analysis of the predicted regions of curvature within the *icsP* promoter has limitations. Our experimental design introduced mutations to reduce areas of curvature predicted by computer software, however, so far, we have not used a biochemical approach to analyze the intrinsic curvature of the promoter or to further investigate the specific regions of predicted curvature.

2.3.5 Rotation of H-NS Binding Sites at the *icsP* Promoter Does Not Alleviate H-NS Mediated Repression

Previous data have identified two distinct regions of the *icsP* promoter that are bound by H-NS. Electrophoretic mobility shift assays with purified His-tagged H-NS identified DNA sequences upstream of the *icsP* gene bound by H-NS, sequences designated targets 1, 4, and 5 (Harrison, 2010). Target 1 extends from +24 to -213 relative to *icsP* transcription start site, and targets 4 and 5 overlap, extending from -523 to -1058 relative to *icsP* transcription start site. It should be noted that the upstream H-NS binding site contains 3 of the predicted regions of curvature, but does not include the most distal VirB binding sites (Fig. 15).

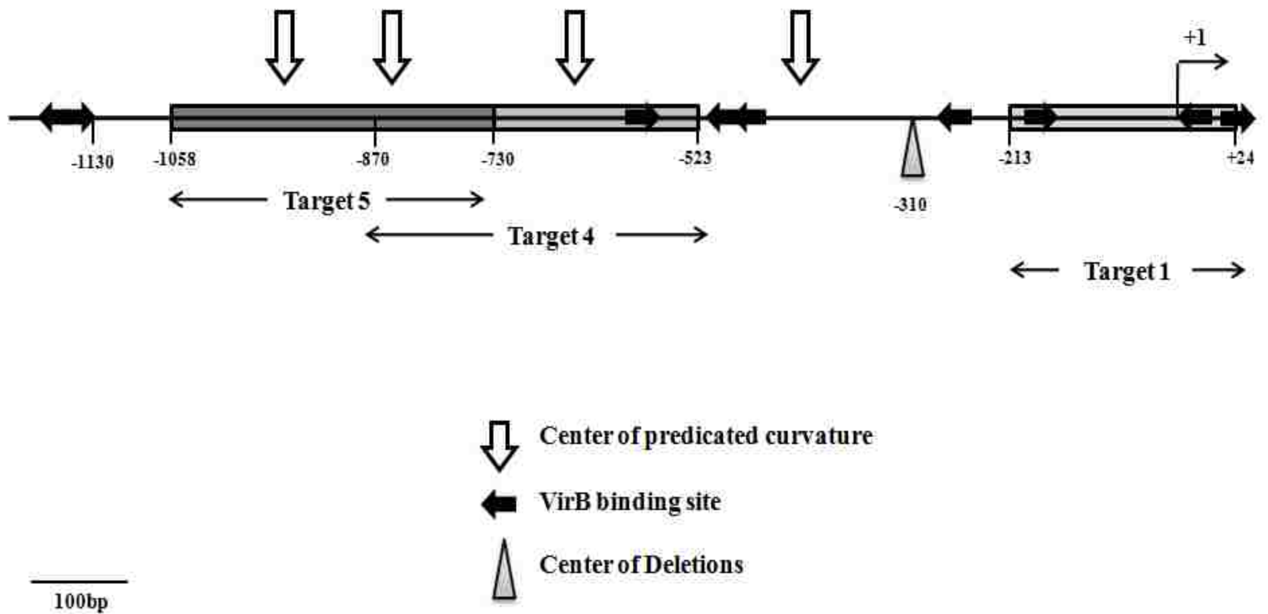


FIGURE 15: Schematic depicting the H-NS binding sites present in the *icsP* promoter. Target 1, 4, and 5 identify regions of H-NS binding in the *icsP* promoter region. Associated VirB binding sites and regions of predicted curvature are denoted by arrows.

Similar to *icsP,* published studies on the H-NS dependent repression of the *virF* promoter reported two distinct regions of H-NS binding within the *virF* promoter. These studies demonstrated that rotating the DNA helix between the two H-NS binding sites negatively impacted thermoregulation of *virF* and reduced H-NS mediated repression of *virF* (Prosseda et al., 2004). These findings provide evidence for the idea that two H-NS binding sites are not independent and that H-NS dimers can form extensive protein-protein interactions over long distances (Esposito et al., 2002; Falconi, Colonna, Prosseda, Micheli, & Gualerzi, 1998; Schneider et al., 2001; Spurio, Falconi, Brandi, Pon, & Gualerzi, 1997). We sought to determine if the H-NS sites have helical orientation requirements for H-NS mediated repression of the *icsP* promoter.

To do this, we created P*icsP-lacZ* fusions carrying a 5 bp deletion, pKLP23, or a 10 bp deletion, pKLP24, in between the identified H-NS binding regions within the *icsP*

promoter, rotating the sites on the DNA helix with respect to each other. These constructs were introduced into wild-type *E.coli* strain MC4100 and an isogenic *h-ns* mutant strain or into wild-type *Shigella flexneri* strain 2457T and *virB* mutant *S. flexneri* strain AWY3; promoter activity was measured using the β-galactosidase assay.

In an *E. coli* background, in the presence of either a 5 bp or a 10 bp deletion, H-NS is able to mediate repression of the *icsP* promoter (Fig. 16). However, in the absence of H-NS, *icsP* promoter activity of pKLP23 increases with respect to wild-type *icsP* promoter activity and promoter activity of pKLP24 is slightly higher than the wild-type. Our data indicate that the rotation of the DNA helix between the H-NS sites does not reduce H-NS mediated repression of the *icsP* promoter.

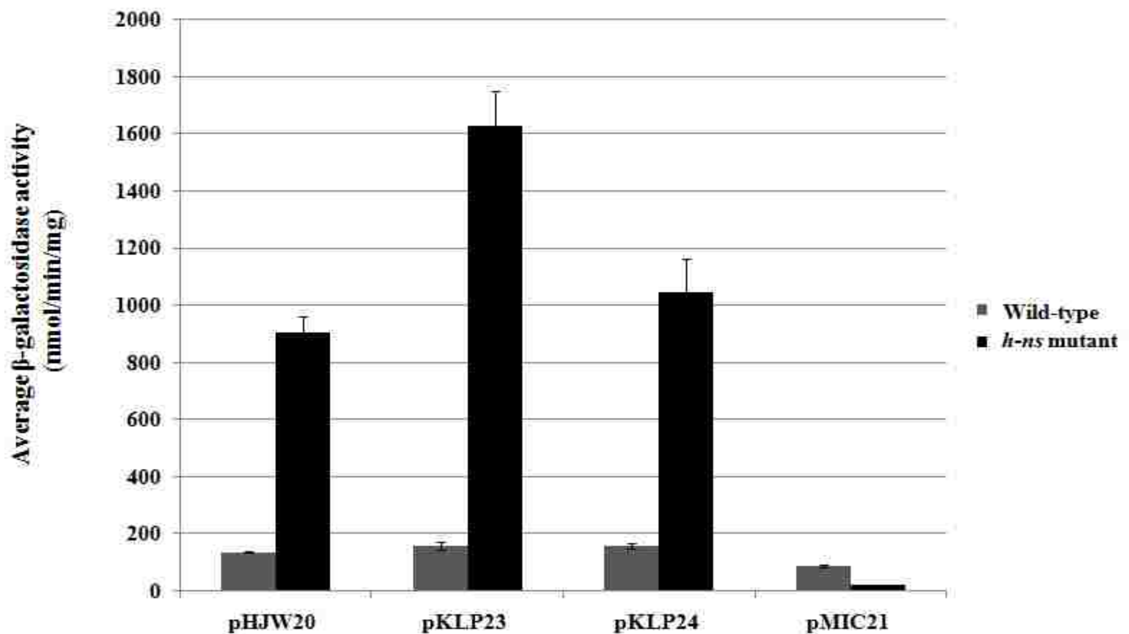


FIGURE 16: Activities of wild-type *icsP* promoter, *icsP* promoter with 5 bp deletion between H-NS binding sites (pKLP23), *icsP* promoter with 10 bp deletion between H-NS binding sites (pKLP24), and promoter-less control in wild-type *E. coli* (MC4100) and the *h-ns* mutant. Assays were run in triplicate and the means and deviations are shown.

In a *Shigella* background, the presence of either a 5 bp or a 10 bp deletion between the H-NS binding sites does not alter VirB-dependent regulation of *icsP* promoter activity (Fig.17). Furthermore, these deletions relocate the most distal VirB binding sites in closer proximity to the *icsP* transcription start site. In either construct carrying a deletion between the H-NS binding sites, there is an increase in *icsP* promoter activity as compared to wild-type *icsP* promoter activity. This effect is remarkable, given that in constructs carrying similar deletions, but in a different region downstream of the VirB binding sites within the *icsP* promoter, the introduction of deletions reduced *icsP* promoter activity. These findings emphasize that the location of the sites within the promoter is important to VirB dependent regulation; perhaps shifting the binding sites modulates the extent of VirB-dependent *icsP* promoter activity.

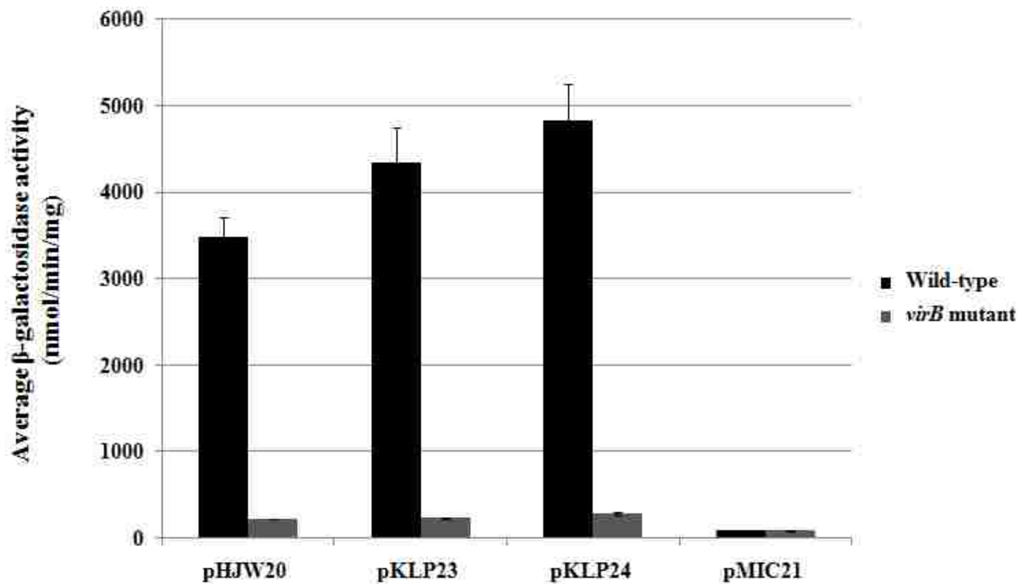


FIGURE 17: Activities of wild-type *icsP* promoter, *icsP* promoter with 5 bp deletion between H-NS binding sites (pKLP23), *icsP* promoter with 10 bp deletion between H-NS binding sites (pKLP24), and promoter-less control in wild-type *Shigella* (2457T) and the *virB* mutant (AWY3). Assays were run in triplicate and the means and deviations are shown.

We show that rotation of the DNA helix between the H-NS binding sites in the *icsP* promoter does not alter the ability of H-NS to repress the *icsP* promoter. In fact, our data indicates that placing these sites on the opposite face of the DNA helix actually increases basal promoter activity in the absence of H-NS. These findings are in contrast to studies of H-NS mediated repression of transcription that underscore the interdependence of H-NS binding sites as a mechanism to regulate promoter activity. From our data it is unclear what accounts for these observations, but perhaps this rotation allows for a more stable DNA conformation that is permissive to transcription of the *icsP* gene. The formation of a more stable structure as a result of rotating the DNA helix has the potential to stabilize DNA-H-NS interactions and to stabilize any DNA looping or long distance H-NS-H-NS interactions as well.

2.4 Conclusion

The remote regulation of the *icsP* promoter by VirB binding to sites located over 1 kb upstream of the *icsP* transcription start site is reminiscent of enhancer sequences present in eukaryotic cells. Eukaryotic enhancer sequences are distally located DNA sequences specifically bound by transcription factors that either activate or repress transcription. Enhancer sequences have been found to function upstream or downstream of promoters and in either orientation. Studies have shown that the presence of an enhancer cannot direct RNA Pol II, the transcribing enzyme of eukaryotes, to a specific promoter, but it functions by working in conjunction with other factors assembled at the promoter. The removal of an enhancer sequence does not prevent transcription initiation

from targeted eukaryotic promoters; binding of transcription factors to enhancer sequences up-regulate the rate of open-complex formation (reviewed in (Blackwood & Kadonaga, 1998).

Although enhancers are typically associated with eukaryotic promoters, there have been examples of enhancers reported for bacterial promoters. A common example is the interaction between alternative RNAP σ^{54}-holoenzymes and enhancer-binding proteins such as NtrC. This system of transcriptional regulation has been found in a variety of species including NtrC from enteric bacteria and NifA of *Klebsiella pneumonia*. The most well studied bacterial enhancer binding protein, NtrC, does not function by facilitating binding of RNAP to the promoter, but rather induces a DNA conformational change once RNAP is bound, initiating transcription by contacting RNAP via DNA looping, and consequently allowing the formation of an open complex (Porter, North, Wedel, & Kustu, 1993; Su, Porter, Kustu, & Echols, 1990; Weiss, Batut, Klose, Keener, & Kustu, 1991).

This is in contrast to the role of VirB as a transcription factor, and more specifically, the role of the remote VirB binding sites. VirB does not contact RNAP nor has it been shown to recruit RNAP to promoters of *Shigella* virulence genes (Turner & Dorman, 2007). Mutagenesis of the most distal VirB binding sites abolishes *icsP* promoter activity, demonstrating that VirB is essential to transcription at the *icsP* promoter and is not merely a facilitator up-regulating transcription.

Our studies show that VirB-dependent regulation from the most distal VirB binding sites is a unique system. We have shown that VirB functions to facilitate transcription of *icsP* by antagonizing H-NS mediated repression (Castellanos et al.,

2009). How VirB mediates VirB-dependent regulation from these sites was intriguing not only because of their location within the promoter, but the profound negative effect of mutagenesis of these sites on *icsP* promoter activity. In this work we have focused on the determining the spatial and helical orientation requirements for VirB-dependent regulation from these sites.

We have demonstrated that VirB directly regulates promoter activity of a P*icsP-lacZ* fusion, and have provided evidence suggesting that VirB binds as a dimer to the distal VirB binding sites. We have also shown through deletion analysis that while there is not a helical orientation requirement, there is a small spacing requirement for VirB-dependent regulation of *icsP*. These data implicate the importance of the location of the VirB binding sites in the promoter. The necessity of the VirB binding sites to lie *in cis* with the *icsP* promoter elements and the seemingly fluid spacing and helical requirements, emphasize the role of VirB as an antagonizer of H-NS, and not as a enhancer nor as a classic transcription factor.

H-NS, a known global repressor of bacterial transcription, mediates repression of the *icsP* promoter. We have identified four regions of predicted curvature within the *icsP* promoter, and disruption of these curves does not alter H-NS mediated repression or thermoregulation of *icsP* expression. Furthermore, rotation of the DNA helix between these H-NS sites does not interfere with H-NS mediated repression of *icsP*. This indicates that H-NS does not solely recognize intrinsic curvature present in the *icsP* promoter to mediate repression, but perhaps via another mechanism such as DNA looping or modulation of DNA supercoiling.

Our studies of the silencing-anti-silencing of *icsP* transcription by VirB and H-NS are important to further our understanding of mechanisms regulating bacterial gene expression. We have gathered evidence that supports our model of silencing-anti-silencing at the *icsP* promoter by demonstrating that there is no absolute on-off switch for transcription. Rather instead, our data suggest that VirB and H-NS interactions with the DNA reconfigure its conformation allowing for transcription, and modulations in the DNA sequence alter the nascent stability of the DNA.

In conclusion, our studies have provided insight into how VirB influences *icsP* expression from remote distances and also increase our understanding of how H-NS mediates repression of *icsP* expression. Our work also demonstrates the role of DNA topology in gene regulation and the complexity of systems evolved to regulate transcription of virulence genes. Our work contributes to our understanding of silencing-anti-silencing regulatory systems in *Shigella* and other enteric pathogens.

2.5 References

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CHAPTER 3

TRANSCRIPTION START SITE MAPPING OF *OSPZ* PROMOTER

3.1 Introduction

3.1.1 Type III Secretion Systems

The type III secretion systems (T3SSs) have been identified in many pathogenic and commensal gram-negative bacteria that live in close association with mammals, plants, and insects (Troisfontaines & Cornelis, 2005). These complex bacterial nanostructures are primarily used to deliver bacterial effector proteins directly into the cytoplasm of host cells, to manipulate the host cell's physiology. T3SSs are on average comprised of 20 different proteins that form a two ring structure and a protruding needle that typically makes contact with the target cell (Fig. 18). Some bacteria possessing a T3SS, such as *E. coli*, have been shown to have filaments extending from the needle, purportedly to mediate attachment to host cells through the glycocalyx layer (Coburn, Sekirov, & Finlay, 2007).

The T3SS system is composed of three classes of proteins: structural proteins, translocators, and effectors. The structural proteins actually comprise the apparatus, the two rings and the needle. Translocators are proteins that facilitate the transfer of effector proteins into the host cell. Translocators are conserved among pathogens possessing a T3SS, while the effectors are more distinct. The effectors are the proteins that affect the host cell, facilitating colonization by the invading bacterium.

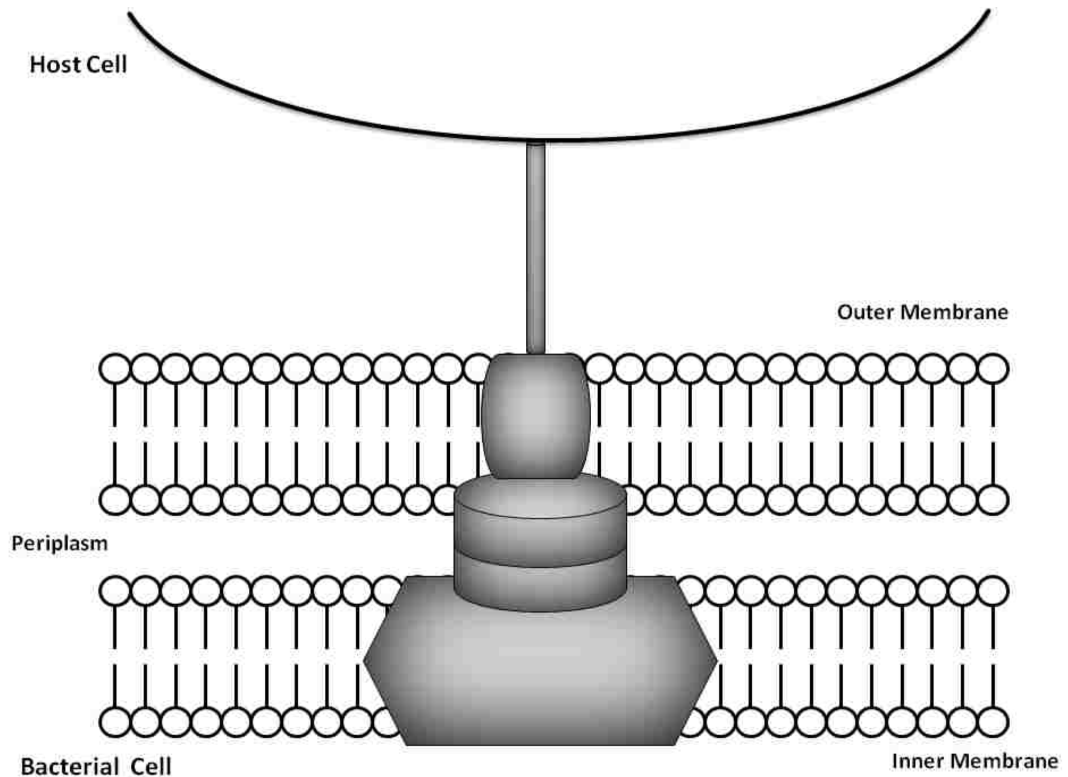

FIGURE 18: Schematic depicting the type III secretion system in a gram negative bacterium.

An important distinction between effectors and toxins is critical to understanding their role and their benefit to bacteria. Although both are secreted proteins, the functions, and resultant outcomes are different. Toxins can exert effects on host cells without the presence of the bacterial source. Furthermore, toxins are more specific, displaying singular biochemical activity and targets. Effectors, on the other hand, are not effective in the absence of the bacterial source. Proteins delivered to host cells via T3SSs often exert effects in concert with other effectors and have a more modular effect on host cell processes, rather than radically altering cellular homeostasis (Galan, 2009).

3.1.2 Type III Secretion Effector Proteins

Effector proteins and their roles are of particular interest as they are typically encoded and secreted by pathogenic bacteria such as *Shigella* spp., *Yersinia* spp., *Chlamydia* spp., *Pseudomonas* spp., *Vibrio* spp., *Bordetella* spp., and pathogenic strains of *E. coli* (Galan & Wolf-Watz, 2006). *Escherichia coli* and *Shigella flexneri* are enteric bacterial pathogens that cause diarrheal disease. Both of these bacteria possess T3SSs. Enteropathogenic *E. coli* (EPEC) delivers effector proteins to host intestinal epithelium that mediate attaching and effacing lesion (A/E) lesion formation. A/E lesions are characterized by bacterial attachment, effacement of the brush border microvilli and actin pedestal formation (Frankel & Phillips, 2008). S. *flexneri* delivers effector proteins that play many roles in virulence including invasion, intracellular survival and the inhibition of innate immune responses by targeting host signaling pathways (Coburn et al., 2007).

3.1.3 Nle and OspZ Effector Proteins

Although both Enteropathogenic *E. coli* and *S. flexneri* are pathogenic and have similar disease consequences, they have evolved different strategies to invade and colonize the human gut. EPEC is an extracellular pathogen, while *S. flexneri* is an intracellular pathogen that has the ability to invade the host cell cytoplasm and spread to neighboring intestinal epithelial cells (Newton et al., 2010). *Shigella* is distinct in that it occupies the host cell cytoplasm differing from intracellular bacteria that remain inside the phagolysosomal compartment. Both encode a type III secretion system that allows for

"injection" of effector proteins into host cells. EPEC and *Shigella* both encode homologous effector molecules, NleE and OspZ respectively.

NleE is a highly conserved 27 kDa T3SS effector protein of A/E pathogens such as EPEC (Newton et al., 2010). It has been shown that NleE was required for EPEC-induced polymorphonuclear (PMN) migration (Zurawski et al., 2008). Additionally, infection of mice with an *nleE* mutant of *C. rodentium* results in a reduction of both bacterial load and colonic hyperplasia, suggesting that *nleE* plays a role in bacterial colonization and the resulting disease (Wickham et al., 2007).

OspZ of *Shigella* is a homologue of NleE. There are two forms of the OspZ protein. The full length gene product encoded by *S. boydii* and *S. flexneri* serogroup 6, and the truncated form encoded by *S. flexneri* serotype 2a. The latter carries a 36 amino acid truncation at the C-terminus (Newton et al., 2010). Mutant strains lacking *ospZ* display no discernable difference in their ability to invade, replicate and spread cell-to-cell in invasion assays. Despite the inflammatory response being equally robust in wild-type and *ospZ* mutant strains, there was a marked decrease in the migration of PMNs to the site of infection of polarized epithelium, providing evidence for the role of *ospZ* in pathogenicity. It was concluded that OspZ has a role in virulence and more than likely, along with effectors OspF and OspC1, has a role in mediating the PMN migration phenotype (Zurawski et al., 2006).

As NleE and full-length OspZ localize to the host nucleus during infection, it is possible that their function is in subverting host immune signaling. To determine NleE and OspZ function, Hartland et al. investigated the effect of NleE on NF-kB activation

during EPEC infection and the effect of OspZ on NF-kB activation during *Shigella* infection.

3.1.4 Role of NleE and OspZ in the NF-kB Pathway

NF-κB tightly regulates activation of gene expression during inflammation. The most abundant form of NF-κB in mammalian tissues is a p65/p50 dimer that activates the expression of multiple cytokine genes in response to inflammatory signals (Perkins, 2007). Studies have shown that NleE from EPEC prevented nuclear translocation of the p65 NF-κB subunit, leading to diminished IL8 expression and a compromised IL-8 response. Additional data indicates that NleE obstructs nuclear translocation of Rel family transcriptional activators and allows nuclear import of transcriptional repressors (Newton et al., 2010). The full length OspZ from *Shigella flexneri* 6 and *Shigella boydi* were also able to inhibit NF-κB activation and p65 nuclear import. The truncated OspZ homologue from *S. flexneri* 2a and a C-terminal 36 amino acid deletion mutant of NleE were inactive. Inactivity suggests that the C-terminus is critical to immunosuppressive functions; however this sole region was not sufficient for inhibition of p65 nuclear translocation or prevention of NF-kB activation (Newton et al., 2010). The absence of a functional OspZ protein in *S. flexneri* 2a is interesting and indicates that OspZ from *S. flexneri* 2a potentially enhances inflammation by inducing polymorphonuclear migration across a polarized epithelium via a different mechanism.

3.1.5 Transcriptional Regulation of *ospZ*

While the activity of the OspZ protein has been characterized (Newton et al., 2010; Zurawski et al., 2008), the *ospZ* promoter region remains largely uncharacterized. Interestingly, it lies within an unusually long intergenic region on the large *Shigella* virulence plasmid that also contains the promoter of the divergent gene *icsP* (Fig. 19).

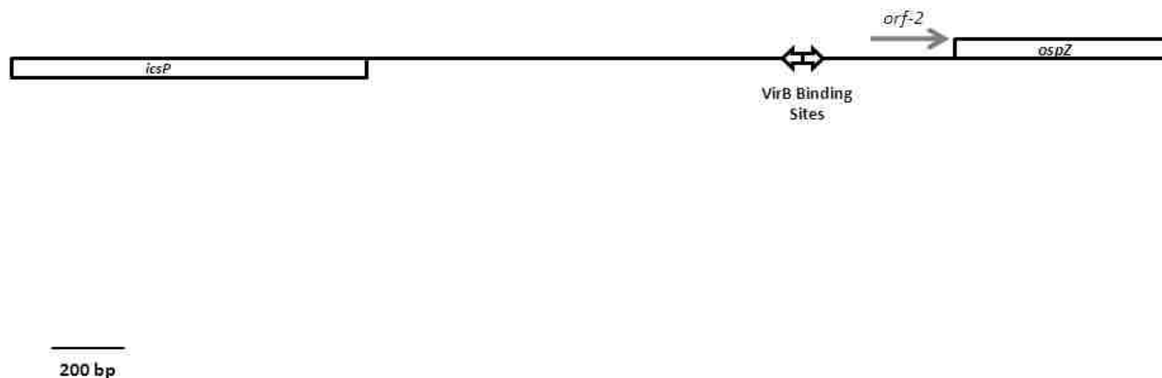

FIGURE 19: Schematic diagramming the location of *ospZ* and *icsP* on the *S. flexneri* 2a virulence plasmid. The VirB binding sites are annotated as open arrows. The gray arrow represents ORF-2. The line denotes the shared promoter region.

IcsP is an outer membrane protease which cleaves the actin-tail assembly protein IcsA from the bacterial surface (Egile, d'Hauteville, Parsot, & Sansonetti, 1997; Shere et al.,1997). This proteolytic activity has been demonstrated to modulate actin-based motility in the host cell cytoplasm and to facilitate efficient intercellular spread of *Shigella* (Egile et al., 1997; Shere et al., 1997; Steinhauer, Agha, Pham, Varga, & Goldberg, 1999). Within the intergenic region, shared by *icsP* and *ospZ,* lies binding sites for the virulence cascade regulator VirB. VirB has been shown to regulate *icsP* promoter activity from these remote sites, which are located over 1 kb upstream of the *icsP* transcription start site. Mutation of these sites by site-directed mutagenesis reduces

promoter activity to basal levels. In the presence of VirB, expression levels of a low-copy plasmid-borne P*icsP-lacZ* fusion increase 17 fold, demonstrating that *icsP* expression is VirB-dependent. With respect to *ospZ*, the VirB binding sites that are required for VirB-dependent regulation of *icsP* are located 425 bp upstream of the *ospZ* open reading frame. Considering that these sites are closer in proximity to *ospZ*, we sought to determine if VirB has a role in the regulation of *ospZ.* In addition to investigating the regulation of *ospZ*, we also chose to identify the *ospZ* transcription start site and its promoter elements. This will be critical to continued studies of *ospZ* and its transcriptional regulation.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Media

S. flexneri serotype 2a strain 2457T was isolated by Lt. Col. Oscar Felsenfeld in Tokyo, Japan, and is both serologically and biochemically a typical culture of *S. flexneri* (Formal et al., 1958). The isogenic *virB* mutant strain AWY3 was created by moving the kanamycin-resistant locus from YSH6000 *virB*::Tn5 into the *S. flexneri* wild-type strain 2457T by P1 transduction (Adler et al., 1989; Wing, Yan, Goldman, & Goldberg, 2004). Strain BS103 is a derivative of 2457T lacking the virulence-associated plasmid, pSf2a140 (Maurelli, Blackmon, & Curtiss, 1984).

S. flexneri 2a strains were routinely grown at 37 ˚C in Trypticase Soy Broth (TSB) with aeration or on Trypticase Soy Agar (TSA) (TSB containing 1.5% [wt/vol] agar). To verify virulence phenotype, *Shigella* strains were streaked on TSA plates containing 0.01% (wt/vol) Congo red (Sigma Chemical Co., St. Louis, Mo.) *E. coli*

strains were grown at 37 ˚C in Luria-Bertani broth with aeration or on LB agar (LB broth containing 1.5% [wt/vol] agar). Where appropriate, antibiotics were used in the following concentrations in *Shigella* and *E. coli* strains, chloramphenicol 25 µg ml^{-1}, ampicillin 100 µg ml^{-1}, tetracycline 20 µg ml^{-1}.

3.2.2 Plasmid Constructions

The starting point for this work was pHJW20, which carries the *icsP* promoter region transcriptionally fused to *lacZ* in pACYC184. This construct carries 1,232 bp of wild-type sequence upstream of the *icsP* transcription start site with a unique *XbaI* site upstream of the *lacZ* gene.

pMIC21 is a pHJW20 derivative lacking all *icsP* promoter sequences, resulting in a promoterless *lacZ* reporter plasmid (Castellanos et al., 2009).

pDB05 is a pHJW20 derivative that lacks the *icsP* promoter region and carries the *ospZ* promoter region. The *ospZ* promoter region was PCR amplified from the virulence plasmid of *S. flexneri* strain 2457T 2a, using oligonucleotides W152 and W153. The resulting product was digested with restriction enzymes *Sal*I and *Xba*I. pDB05 carries sequences 1,613 bp upstream to 32 bp downstream of the *ospZ* translation start site.

pDB02 is pDB05 derivative that carries a 3' truncation of the *ospZ* promoter region. The 3' promoter insert was PCR amplified using oligonucleotides W152 and W154, the resulting product was digested with *Sal*I and *Xba*I, and ligated into *Sal*I and *Xba*I digested pDB05.

The following P*ospZ-lacZ* transcription fusion constructs are 3' truncations of *ospZ* with 21 bp, 45 bp, and 82 bp removed with respect to the full length *ospZ* promoter region present in pDB05.

pKLP17 is pDB05 derivative that carries a 3' truncation of the *ospZ* promoter region. The 3' promoter truncation insert was PCR amplified using oligonucleotides W152 and W201, the resulting product was digested with *Sal*I and *Xba*I, and ligated into *Sal*I and *Xba*I digested pDB05.

pKLP18 is pDB05 derivative that carries a 3' truncation of the *ospZ* promoter region. The 3' promoter truncation insert was PCR amplified using oligonucleotides W152 and W202, the resulting product was digested with *Sal*I and *Xba*I, and ligated into *Sal*I and *Xba*I digested pDB05.

pKLP19 is pDB05 derivative that carries a 3' truncation of the *ospZ* promoter region. The 3' promoter truncation insert was PCR amplified using oligonucleotides W152 and W203, the resulting product was digested with *Sal*I and *Xba*I, and ligated into *Sal*I and *Xba*I digested pDB05.

Sequences of oligonucleotides used in plasmid constructions are listed in Table. 4.

All plasmids were sequenced at the Nevada Genomics Center using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1, and plasmids with correct sequence were used.

Oligonucleotide	Sequence
Wing 152	5'- CCGAGTCGACCAAGTACAAAGAATTTTAATTTCATCG -3'
Wing 153	5'- CCGATCTAGAACGTTCTTAATATTCTTGATGGGAC -3'
Wing 154	5'- CCGATCTAGAAAACCAGAACCTCGCTTAGGCC -3'
Wing 160	5'- TTTGCGCTCCTTCAACTGGGCA -3'
Wing 201	5'- TTACTTCTAGACCTAAGTGGAATGTCTCCACGG -3'
Wing 202	5'- TTACTTCTAGACTCAAATATAAACATTACCATGAAC -3'
Wing 203	5'- TTACTTCTAGAGGACTAATCATTTTAATCTCTATACTC -3'
M13	5'-GAGCGGATAACAATTTCACACAGG-3'

TABLE 4: Table of oligonucelotides used to create P*ospZ-lacZ* transcriptional fusion plasmids and in primer extension.

3.2.3 Promoter Activity of P*ospZ-lacZ* Transcriptional Fusions

Activity of the *ospZ* promoters were determined by measuring β-galactosidase activity using the Miller protocol in strains carrying pDB05 or derivatives. Routinely, transcription was analyzed in three independent transformants in early stationary phase cultures. Cells were routinely back-diluted 1:100 from 5ml overnight cultures grown with shaking at 30 °C. Freshly inoculated cultures were subsequently grown for 5 h in TSB at 37 °C.

3.2.4 *In silico* Analysis of Sequences Upstream of *ospZ* Open Reading Frame to Determine Transcription Start Site

The BPROM program was used to predict the *ospZ* transcription start site (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). The BPROM algorithm predicts potential transcription start positions regulated by σ^{70} promoters (major *E. coli* promoter class). BPROM has 80% accuracy for *E. coli* σ^{70}-dependent promoter recognition.

3.2.5 Transcription Start Site Mapping of *ospZ*

The *ospZ* gene transcription start site was identified through RNA extraction as described recently (Hensley et al., 2011) using a protocol adapted from (Aiba, Adhya, & de Crombrugghe, 1981). Total cellular RNA was extracted using the hot-phenol method from 10^9 cells harvested from early stationary phase cultures (Aiba et al., 1981). Samples were digested with DNase I (Invitrogen) at 37 ˚C for 1 h in DNase I buffer (Ambion) and total RNA integrity was verified by formaldehyde gel electrophoresis and ethidium bromide staining. Oligonucleotides W153 and W160 were 5'-end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Promega). One picomole of ^{32}P-labeled primer and 5 µg of total RNA were dissolved in 30 µl of hybridization buffer (Aiba et al., 1981). The annealing reaction was heated at 50 °C for 5 min, incubated at 75 ˚C for 15 min, and maintained at 45 °C for a total of 3 h. Samples were ethanol precipitated and cDNA generated using Superscript II reverse transcriptase (Invitrogen) for 50 min at 37 °C. Reactions were aborted by heating samples to 70 °C for 10 min and RNA was removed by digesting with 10 mg/ml RNase A (Sigma) for 30 min at 37 ˚C. Samples were ethanol precipitated and finally dissoleved in 5 µl of loading dye [95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene, Sequenase 2.0 kit (Affymetrix)] prior to separation by electrophoreseis on a 6% glycerol tolerant polyacrylamide gel (PAGE) containing 7M urea. PAGE gels were transferred to Whatmann paper and vacuum dried. Dried gels were exposed to a phosphor screen overnight and visualized using a Typhoon 9410 variable mode imager (Amersham). A sequencing ladder generated from pBluescript KSII+ (Stratagene) and a M13 reverse primer with the *Sequenase 2.0* DNA

Sequencing Kit (Affymetrix) was routinely used to determine the size of primer extension products.

3.3 Results and Discussion

3.3.1 *In silico* analysis of *ospZ* transcription start site

The BPROM software program was used to determine the transcription start site of the *ospZ* gene. Sequences between the *Xba*I and *Sal*I restriction sites upstream of the *ospZ* translation start site were analyzed by BPROM. A promoter was predicted to be located -110 upstream of the *ospZ* translation start site. This transcription start site lies within *orf-2*. (Fig. 20). An additional transcription start site was predicted at +10 bp downstream of the *ospZ* translation start site.

3.3.2 3' Truncations to map *ospZ* transcription start site

In order to test the BROM predicted transcription start site located 110 base pairs upstream of the *ospZ* translation start site, a series of truncations were constructed (Fig. 17). 3' truncations were created in order to serially remove BPROM predicted promoter elements of the predicted promoter located at 10 bp downstream of the *ospZ* translation start site. The first construct is truncated to the BPROM predicted TSS that lies within the *ospZ* open reading frame. The second construct is truncated 13 bp upstream of the *ospZ* translation start site, removing the BPROM predicted TSS and -10 sequences. The third construct is truncated 50 bp with respect to the *ospZ* translation start site, removing all BPROM predicted promoter elements (Fig. 20).

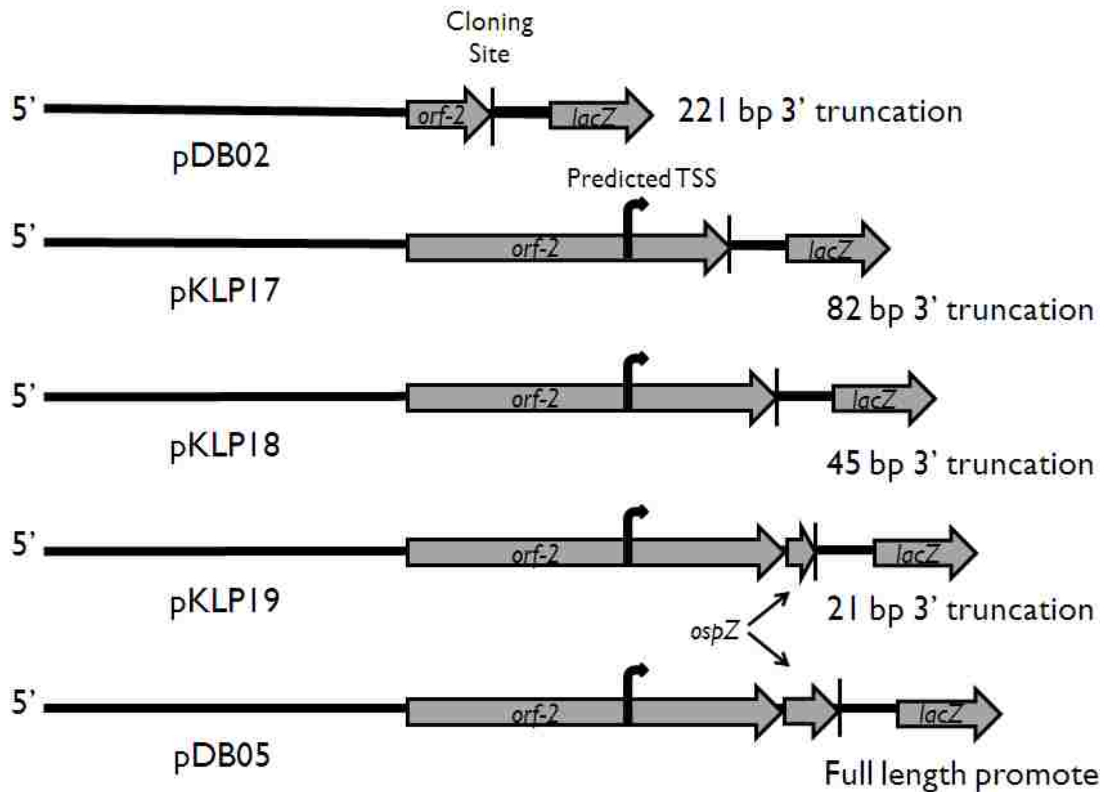

FIGURE 20: Schematic depicting the 3' truncations of the *ospZ* promoter used to identify the transcription start site of *ospZ.*

The 3' truncations of the *ospZ* promoter were fused to the reporter *lacZ* gene. Constructs carrying *ospZ* promoter fragments were introduced into wild-type *Shigella flexneri* strain 2457T to determine promoter activity using the β-galactosidase assay. Our data show (Fig. 21) that constructs pDB05, pKLP19 and pKLP18 display β-galactosidase activity. From these data we can conclude that these constructs contain an active promoter. The increased activity of pKLP18 relative to pKLP19 and pDB05 is likely due to an alteration of the 5' end of the mRNA transcript in this construct, which could lead to enhanced mRNA stability or translation. Constructs pKLP17 and pDB02 have a lack of activity, indicating that there is not an active promoter in these constructs. Furthermore, these data indicate that the BPROM prediction was incorrect, and that the

ospZ promoter lies downstream of this truncation within 50 bp upstream of the translation start site.

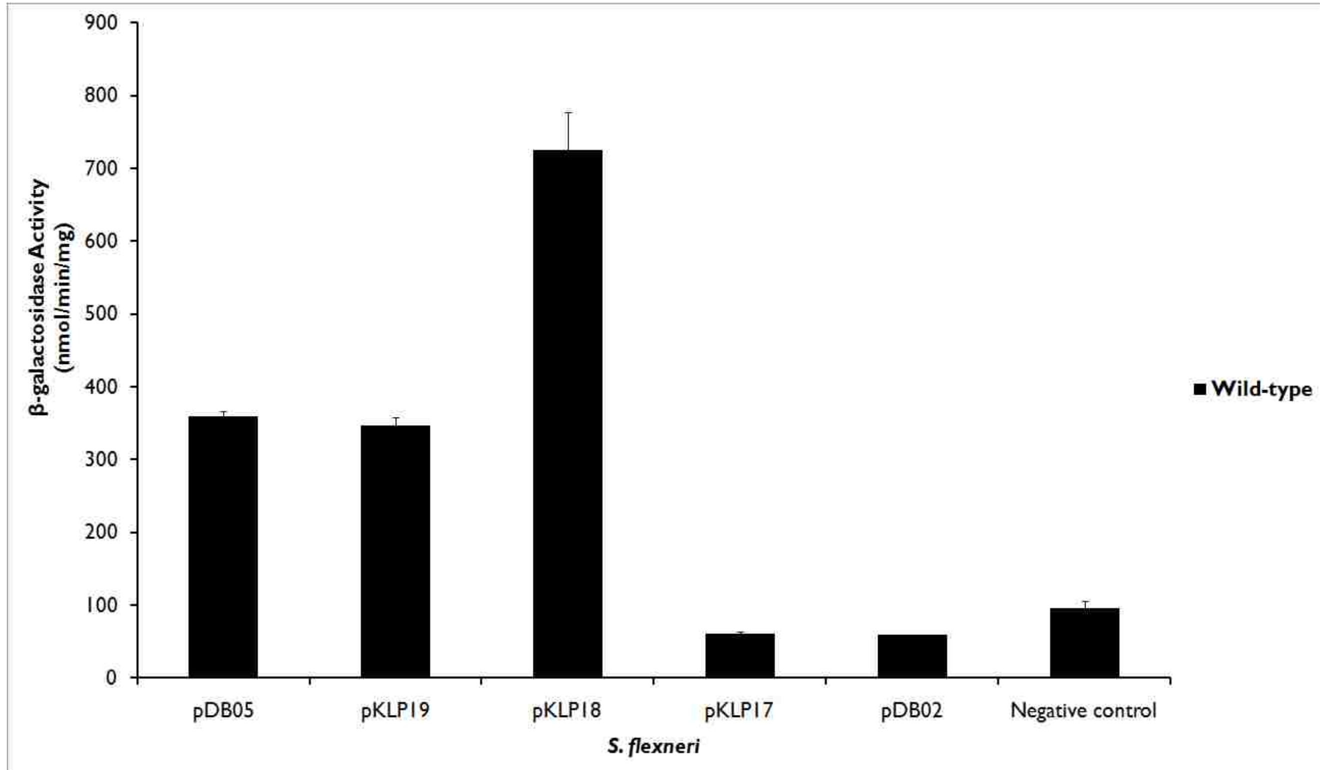

FIGURE 21: Activities of wild-type *ospZ* promoter, 3' truncations of *ospZ*, or the promoter-less control in wild-type *S. flexneri* 2a (2457T) and the *virB* mutant (AWY3). Assays were run in triplicate and the means and deviations are shown.

3.3.3 Transcription Start Site Mapping of *ospZ* using Primer Extension

As a method to specifically locate the single nucleotide transcription start site of the *ospZ* promoter, and further experimentally test the BPROM predicted promoter, we used primer extension analysis. mRNA isolated from the wild-type *S. flexneri* 2a strain, and the same strain carrying the P*ospZ-lacZ* fusion plasmid, pDB05. To reverse transcribe mRNA, one of two primers was used. W153 is complementary to the

beginning of the *ospZ* transcript and W160 is complementary to the beginning of the *lacZ* transcript. By selecting two different primers, we could determine if the P*ospZ*-*lacZ* transcriptional fusion affects the point of transcript initiation.

Using the primer internal to the *ospZ* open reading frame, W153, a single 66 bp product was obtained whether the *ospZ* promoter was carried by the virulence plasmid or the low copy *lacZ* reporter, indicating that the same TSS is used regardless of the sequence background. Using W160, the primer internal to *lacZ* that binds downstream of W153, a single 138 bp fragment was obtained, mapping exactly to the position of the TSS identified by primer W153: 28 nt upstream of the *ospZ* translation start site (Fig. 22).

These data are consistent with the 3' truncation analyses of the *ospZ* promoter (Fig. 16), which indicate that the *ospZ* TSS lies within 50 bp upstream of the *ospZ* gene. In addition, we were able to identify the -10 and -35 sequences (Fig. 19) as a result of these experiments. These data indicate that the BPROM predictions were incorrect as neither the promoter was predicted to be located -110 upstream of the *ospZ* translation start site or the additional promoter predicted at +10 bp downstream of the translation start site were identified by primer extension analysis.

2457T

- + + Plasmid
W153 W153 W160 Primer

G A T C

+1

+1

+1

-35 -10

5′-G**GAGACA**TTC CACTTAGGAA CAAT**TATATT** GTTCATGGTA **A**TGTTTATAT -3′

FIGURE 22: Primer extension analysis of the *ospZ* promoter. The plasmid pDB05 was used in conjunction with primers W153 which binds within the *ospZ* open reading frame or Wing 160, which binds within the *lacZ* gene. Arrows indicate identified transcription start site (+1). The sequence identifies the +1 transcription start site of *ospZ* and associated -10 and -35 sequences in bold typeface and underlined.

3.3.4 P*ospZ-lacZ* Expression is VirB-dependent

Considering the VirB binding sites are in closer proximity to the *ospZ* gene than to the *icsP* gene, we sought to determine if *ospZ* expression was VirB regulated as well. Additionally, we sought to establish if there was promoter activity from *orf-2*. pDB02 is a 3' truncation of the *ospZ* promoter region that truncates to the predicted translation start site of *orf-2*.

Constructs pDB02 and pDB05 carrying *ospZ* promoter fragments were introduced into wild-type *Shigella flexneri* strain 2457T or *virB* mutant *S. flexneri* strain AWY3 to determine promoter activity using the β-galactosidase assay. The wild-type promoter present in pDB05 displayed VirB-dependent expression (Fig. 23). In the presence of VirB there is a 2-fold increase in the *ospZ* promoter activity. In pDB02, there is minimal promoter activity, as levels are below those of our promoter-less control. These data indicate that *ospZ* expression is VirB-dependent.

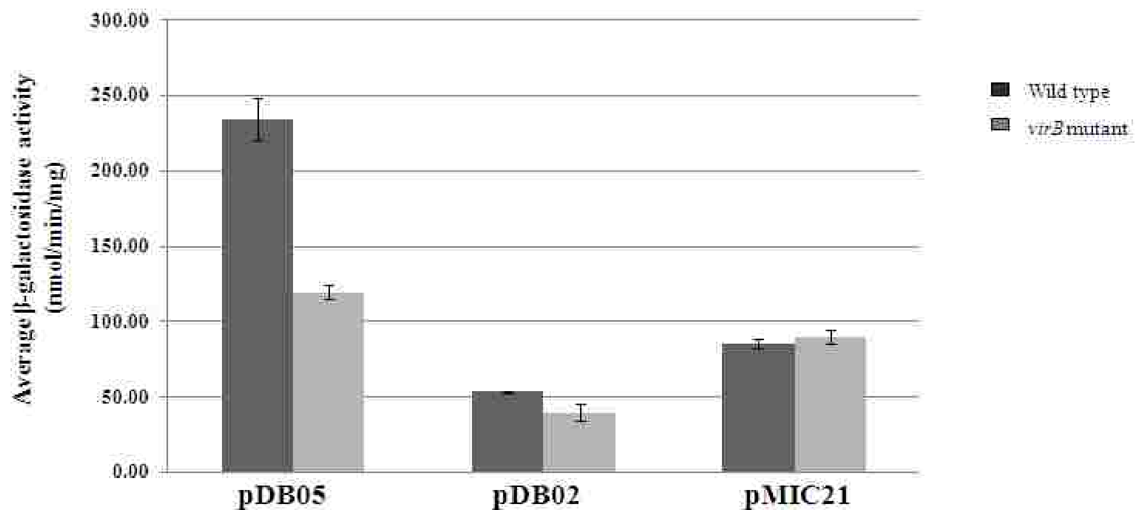

FIGURE 23: Activities of wild-type *ospZ* promoter, 3' truncation to *orf-2*, or the promoter-less control in wild-type *S. flexneri* 2a (2457T) and the *virB* mutant (AWY3). Assays were run in triplicate and the means and deviations are shown.

3.4 Conclusions

In summary, our data have identified the transcription start site of the *ospZ* gene of *Shigella flexneri.* We achieved this via several experimental approaches. *In silico* analysis was used to locate potential transcription start sites and their associated -10 and -35 using the BPROM software which identified two potential promoters. Via 3' truncation of the *ospZ* promoter region, we were able to narrow down the region that possibly contained the transcription start site. Using the β-galactosidase assay, P*ospZ-lac*Z fusions were used to assay 3' truncations of *ospZ*, which indicated that the predicted promoters by BPROM were in fact incorrect. Primer extension was used to identify the transcription start site of *ospZ*. The identified transcript maps to 28 nt upstream of the translation start site of *ospZ*. We have determined that an *orf-2* transcriptional fusion does not display associated promoter activity, suggesting that *orf-2* is not a gene. Furthermore, *ospZ* expression is VirB-dependent.

Overall, this work has characterized the *ospZ* promoter, which is critical to further studies into its transcriptional regulation. It has also demonstrated that *ospZ* is regulated by VirB. Although this study has only addressed promoter activity and not analyzed effects of VirB on mRNA or protein levels of *ospZ*, it can be speculated that the difference in VirB-dependent regulation of the *icsP* and *ospZ* promoters attests to the different roles of these proteins in *Shigella* pathogenesis. IcsP is a protease that modulates the location of outer membrane protein IcsA in the bacterial membrane, forming a polar cap. The cleavage of IcsA by IcsP facilitates directed actin-tail formation and cell-to-cell spread. In contrast, OspZ of the strain studied (*S. flexneri* 2a) has been implicated in the

recruitment of PMNs during *Shigella* infection of the colonic epithelium. Other isoforms of OspZ modulate host cell processes, having been shown to be immunosuppressive by inhibiting NF-kB activation and p65 nuclear import.

Furthermore, it is interesting that although the VirB binding sites are in closer proximity to the *ospZ* promoter in comparison to the *icsP* promoter, VirB does not have as profound an effect on the expression of *ospZ*. Whereas in the presence of VirB expression levels of a P*icsP-lacZ* fusion increase 17 fold, there is only a 2-fold increase in the promoter activity of *ospZ*. These observations are a reminder that the canonical promoter is not associated with all bacterial promoters, and that the location of transcription factor binding sites does not necessarily have a negative correlation with its influence on the expression of the targeted gene.

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CHAPTER 4

CONCLUSION

4.1 Conclusions

Shigellosis is a world-wide health burden that affects millions of people annually. Endemic to many parts of the world, *Shigella* outbreaks have plagued populations affected by natural disasters, political upheaval, and recurrent poverty. Considering the large monetary and intangible costs of *Shigella* infections, better insight is needed into the mechanisms of *Shigella* infection and virulence. Studies such as this that address the transcriptional regulation of important *Shigella* virulence factors not only progress the overall body of knowledge regarding *Shigella* pathogenesis and the resulting disease, shigellosis, but also furthers understanding of virulence gene regulation. Furthermore, such work relates to gene regulation in other enteric bacteria which possess similar regulatory systems.

4.1.1 Transcriptional Regulation of *icsP*: Repression by H-NS and De-Repression by VirB-dependent

Transcriptional regulation is one of the key means that bacterial pathogens use to respond to environmental stimuli and adapt to host niches. The aim of this work was to further characterize the unusual mechanism of VirB-dependent regulation of *icsP* promoter activity from sites over 1 kb upstream of *icsP*. We sought to better understand how *icsP* expression is controlled by VirB and understand this regulation in the context

of H-NS mediated repression. Our studies have demonstrated that there is moderate elasticity in the spacing and helical requirements for VirB-dependent regulation from remote VirB binding sites. Deletions of 5 or 10 bp did not alter *icsP* promoter activity, however, there was a decrease in *icsP* promoter activity with the introduction of a 50 bp deletion. These VirB binding sites are organized as an inverted repeat, and our work shows that there is a natural 1 bp requirement between these sites, as additional base pair insertions between the two lead to a loss of VirB-dependent regulation.

Moreover, our studies addressing the role of intrinsic curvature in the regulation of *icsP* indicate intrinsic curvature does not have a role in VirB-dependent regulation or H-NS mediated repression. Base pair substitutions introduced into predicted regions of curvature do not abolish VirB-dependent regulation or H-NS mediated repression of *icsP* promoter activity. Furthermore, rotating the H-NS binding sites with respect to each other does not reduce H-NS mediated repression.

Our findings reported here add to our understanding of transcriptional regulation of *icsP* by VirB, contributing to the knowledge of VirB-dependent regulation of virulence genes in *Shigella* species. Furthermore, the characterization of this remote VirB-dependent regulation improves our understanding of mechanisms transcription factors use to regulation gene expression.

4.1.4 Identification of the *ospZ* promoter and Characterization of *ospZ* Transcriptional Regulation

OspZ is a recently characterized effector protein secreted by the *Shigella* type II secretion system. Studies have identified a role of *ospZ* in the recruitment of

polymorphonuclear cells to the site of *Shigella* infection in the colonic epithelium and in the modulation of the host cell inflammatory response (Newton et al., 2010; Zurawski et al., 2008). Our studies were aimed at characterizing the *ospZ* promoter and understanding the transcriptional regulation of the *ospZ* gene. We have identified the transcription start and the associated -10 and -35 sequences. Preliminary work into the transcriptional regulation of *ospZ* has shown that promoter activity of a P*ospZ-lac*Z fusion increases 2-fold in the presence of VirB. Additional work with collaborators analyzed the *ospZ* promoter region in other *Shigella* species. 57 DNA sequences were analyzed with 25 sequences were highly conserved at approximately the 97% level. The promoter architecture reported here is represented by all *Shigella* species in sequences analyzed: *S. flexneri*, *S. boydii*, *S. dysenteriae*, and *S. sonnei*. This has provided a foundation for future studies of the transcriptional regulation of *ospZ* and contributed to the overall understanding of *Shigella* virulence gene regulation.

4.1.5 Future Directions

Here we have provided insight into the VirB-dependent regulation of the *icsP* promoter. To continue this work, future studies should further address the importance of the VirB binding site organization to regulation of *icsP* promoter activity. We have established that there is a small spacing requirement for VirB-dependent regulation from these sites, but is the organization of the sites suffice to mediate this effect? By placing VirB binding sites in closer proximity to the *icsP* transcription start site, we can begin to understand the contribution of binding site organization, in the context of location within

the promoter, to transcriptional regulation. Furthermore, the contribution, if any, of other VirB binding sites within the promoter should be explored.

Similarly, additional experiments to address the mechanism of H-NS mediated repression should aim to further characterize the interactions of the two H-NS binding sites. We see that placing H-NS binding sites in opposite helical orientations has a positive effect on *icsP* promoter activity, are there spacing requirements or other limitations? Does altering the H-NS binding sites affect the thermoregulation of *icsP* promoter activity? Additionally, more specific experiments are needed to determine which, if any, of the previously described mechanism of H-NS-mediated repression are represented at the *icsP* promoter.

This work has provided a solid foundation for further studies into the transcriptional regulation of *icsP* by VirB and the transcriptional regulation of *ospZ*. Our work has raised several questions regarding the mechanism of VirB-dependent regulation of other *Shigella* virulence genes and whether transcriptional regulation of bacterial promoters from remote distances is found in other systems.

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Minority Health and Health Disparities International Research Training
Genetics at Cavanilles Institute
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Project examined the quasi-species of viruses present in patients infected with Hepatis C virus to determine if the viruses present are the result of random events during replication or represent distinct genotypic subgroups.

University of California-Irvine, Molecular Biology and Biochemistry
Irvine, CA (June 2008 – June 2009)
Lab Assistant I
Project invesitagted the mechanism of the protein L22 (expressed in mammalian cells) interaction with Epstein-Barr virus EBERs (Epstein –Barr Virus Encoded Rnas) and human telomerase in order to determine a potential role with EBV contribution to tumorgenicity.

Hampton University, Biochemistry Laboratory
Hampton, VA (June 2007- May 2008)
Undergraduate Researcher
Investigated the role of the putative transcriptional regulator rv0894 plays in the regulation of septum formation in *Mycobacterium tuberculosis*. Specific aim is to clone the putative transcriptional regulator, which is encoded by the gene rv0894 of *Mycobacterium tuberculosis* into the bacterial vector pSKB2.

Hampton University/ Norfolk State University Breast Cancer Research Program
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Conducted research investigating the development of cancerous lesions in the mammary gland tissue of mice. Presentation of various topics and filming of tutorial videos regarding the stage development of cancer.

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- **Graduate Assistant, University of Nevada, Las Vegas** Las Vegas, NV (Sept. 2009- Present)
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