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Transcriptional regulation of *Shigella* virulence plasmid-encoded genes by VirB and CRP

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TRANSCRIPTIONAL REGULATION OF *SHIGELLA* VIRULENCE PLASMID-
ENCODED GENES BY VIRB AND CRP

by

Christopher Thomas Hensley

Bachelor of Science
University of Nevada, Las Vegas
2008

A thesis submitted in partial fulfillment of
the requirements for the

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School of Life Sciences
College of Science

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THE GRADUATE COLLEGE

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Christopher Thomas Hensley

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ABSTRACT

Transcriptional Regulation of *Shigella* Virulence Plasmid-Encoded Genes by VirB and CRP

by

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Shigella flexneri is a species of Gram-negative intracellular pathogens that causes bacillary dysentery in humans. *Shigella* relies on the precise transcriptional regulation of virulence genes, encoded by a large virulence plasmid, for invasion and infection of human colonic epithelial cells. The transcription of most identified virulence genes are regulated through a cascade controlled by the primary regulator of virulence genes, VirF, and the global transcriptional regulator, VirB. Currently, few studies have addressed how individual *Shigella* virulence genes are precisely regulated for optimal expression during specific stages of pathogenesis and within the constraints of the regulatory cascade. This work addresses how individual virulence genes are regulated through the study of transcriptional regulation in four *Shigella* virulence genes, *icsP*, *ipaJ*, *phoN1*, and *ipaH7.8*. Analysis of the *icsP* gene has identified multiple promoters contributing to *icsP* transcription and to the regulation of IcsP protein production through the use of two different translation start sites. In addition, analyses of the *ipaJ*, *phoN1*, and *ipaH7.8* genes has identified that the *phoN1* gene is transcriptionally regulated by the CRP, suggesting that catabolite repression is involved with the regulation of some virulence genes in *Shigella*. Together, these data suggest that the transcriptional regulation of virulence genes in *Shigella flexneri* is more complex than previously observed.

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CHAPTER 1

INTRODUCTION

1.1 Transcription Initiation in Bacteria

Initiation of transcription is a key step regulating the transfer of information from genes encoded by DNA to enzymatically active proteins required for all cellular functions in bacteria. Much of what we know about transcriptional regulation is the result of extensive study of gene expression in the Gram negative bacterium *Escherichia coli*. (reviewed in Browning and Busby 2004, Haugen et al. 2008). Five protein subunits, $\beta\beta'\alpha_2\omega$, comprise the core RNA polymerase enzyme which transcribes the DNA code into an RNA message which may or may not later be translated into a protein product. The β and β' subunits form the catalytic center of this multi-subunit structure while the two α subunits contribute to assembly of the β and β' subunits. The ω subunit appears to function as a β' chaperone assisting in the large subunits proper folding but is not required for successful transcription. The crucial step in transcription initiation is the docking of the core enzyme to the double-stranded DNA. This process requires the addition of a sixth subunit, σ , which confers binding specificity of the polymerase to regions of DNA upstream of an encoded gene, designated the promoter. *E. coli* actually contains seven of these σ factors and each of them can be used for recognition of specific promoters, although σ^{70} is considered to be the sigma factor controlling expression of housekeeping genes. Together, the core enzyme and the σ subunit constitute the holoenzyme, a structure which is both necessary and sufficient for transcription initiation. Holoenzyme recruitment to the promoter of a gene is a competitive process considering promoters outnumber the population of available RNA polymerases within the cell. The

strength of a promoter and overall ability of a promoter to attract available RNA polymerases determines whether or not the gene is expressed. Therefore, multiple elements work cooperatively to ensure a gene is transcribed at the right time and in the right abundance.

Many of these elements are already present within the promoter DNA itself. The two primary promoter elements involved in recognition and binding by the σ subunit and subsequently, the polymerase, are the -10 (5'-TATAAT-3' consensus for σ^{70}) and -35 (5'-TTGACA-3' consensus for σ^{70}) hexamers, which are named according to their proximity to the transcription start site. These specific sequences are recognized and bound by domains within the σ subunit and together with the TSS make up the classic bacterial promoter. While the -10 and -35 consensus sequences are sufficient for σ subunit recruitment, other sequences can enhance RNA polymerase binding and therefore, transcription initiation. The extended -10 element, which consists of 3-4 bp (5'-TG TG-3' consensus) immediately upstream of the -10 element, is also recognized by a domain of the σ^{70} subunit. Additionally, UP elements consisting of approximately 20 bp (5'-AAAWWTWTTTTNNNAAANN-3' consensus; W = A or T and N = any base) and located upstream of the -35 element assists RNA polymerase binding through recognition by the C-terminal domains of the α subunits. Variations in these sequence elements contribute to differential binding of the RNA polymerase holoenzyme to the approximately 5,000 genes present in the *Escherichia coli* genome. Deviations from the consensus sequence in one element may be compensated for through the presence of a closely matched consensus sequence in another element or the addition of another element such as in the case of the extended -10 element and the UP element. Complete

control over polymerase recruitment is not just restricted to sequences proximal to the gene of interest, but may also be influenced by trans-acting regulatory elements.

Transcription factors, proteins involved in the upregulation or downregulation of transcription, play a large role in regulating which genes are expressed or not. These transcription factors also allow for fine tuning of gene expression in response to environmental stimuli by giving the “edge” to one promoter when the conditions are appropriate. Most transcription factors exert their influence through sequence-specific DNA binding. Once bound to the promoter region, the transcription factors can assist in RNA polymerase recruitment through binding of the α or σ subunits. Some of these proteins can even contort the DNA curvature into a conformation that is more favorable for promoter recognition and RNA polymerase binding. Alternatively, repressive effects are generated through steric hindrance of polymerase binding, induction of DNA loops by multiple bound repressors which effectively mask the -10 and -35 promoter elements, and competitive binding of an activator by another protein blocking the positive effects of the activator. Sometimes the regulatory effect on gene transcription can change for a single transcription factor depending on where the protein binds with reference to important promoter elements. Some regulatory elements and their contributions to the regulation of transcription initiation are not as quantifiable as the promoter elements and transcription factors already mentioned.

The inherent curvature of the DNA itself can greatly influence gene expression through enhancement or repression of polymerase binding. Supercoiling of the DNA within the bacterial cell is controlled, in part, by nucleoid proteins which mostly bind DNA nonspecifically and contribute to DNA packaging. Even weak interactions between

these nucleoid proteins and a specific region of DNA could lead to inhibition through increased folding and prevention of polymerase binding similar to the looping form of repression by transcription factors. Alternatively, some nucleoid proteins relax DNA curvature and effectively act as activators of transcription. DNA curvature is also subject to modulation through simple changes in environmental conditions such as temperature, pH, and osmolarity which can influence the chemical structure of the molecule. Together these regulatory mechanisms combine to create a vast array of switches and potentiometers for the fine tuning of transcription initiation and gene expression throughout the life of the bacterium. These regulatory elements and their effects on initiation of transcription are universal throughout the bacterial domain.

1.2 *Shigella flexneri*

Shigella was first described as the etiologic agent responsible for bacillary dysentery in humans in 1898 and the genus subsequently named so in honor of its discoverer, Dr. Kiyoshi Shiga (Trofa et al. 1999). The genus currently consists of four species, *S. flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii*, of Gram-negative, non-motile, non-spore forming and non-lactose fermenting obligate pathogens which are over 90% similar to *E. coli* in DNA-DNA hybridization studies of the chromosomes (Brenner et al. 1969). Together, the four species account for over 165 million annual cases of bacillary dysentery, commonly referred to as Shigellosis (Kotloff et al. 1999). These infections lead to over 1 million deaths every year and 61% of those deaths are attributable to children under the age of five (Kotloff et al. 1999). *S. flexneri* is most often the bacterium responsible for cases of Shigellosis and as few as 100 bacterial cells have been shown to

be sufficient for infection, making *Shigella* one of the most infectious bacterial agents (DuPont et al. 1989).

Following ingestion, *Shigella* cells are passively transported through the digestive system to the colon where highly endocytic microfold (M) cells, as part of the gastrointestinal-associated lymphoid tissue (GALT), transport the bacterium across the mucous membrane to the basolateral surface. Typically, awaiting macrophages, which along with the M cells make up the Peyer's patches, phagocytose the bacterium but quickly succumb to bacterial induced apoptosis resulting in rerelease of the bacterium at the basolateral surface of the colonic epithelium (Chen et al. 1996). The bacterial induced membrane ruffling of the colonic epithelial cell leads to uptake of the bacterium by the host cell (Nhieu and Sansonetti 1999). Following host cell invasion, the bacterium is able to replicate and spread intercellularly through polymerization of the host cell actin into a tail-like structure (Goldberg et al. 1993).

Successful invasion and infection requires the expression of many effector molecules, including a host of invasive protein antigens (IPAs) which are secreted into the host cell by a type III secretion needle also encoded by the virulence plasmid (Blocker et al. 1999). Throughout the infection, *Shigella* generates many proteins which promote an increased immunological response outside of the invaded host cell while other proteins serve to maintain the integrity of this infected cell (Ogawa et al. 2005, Sansonetti et al. 1995). This strategy increases the disruption of the colonic epithelium by the host innate immune response allowing bacterial access to the basolateral surface by more *Shigella* cells present in the colon while also providing a relatively safe home for the infecting bacterial cell. Safe entry of the bacterium into the host cell would benefit from

appropriate timing of the bacterial-mediated immunological response. Furthermore, the various stages of pathogenesis require the expression of specific effector molecules by *Shigella* suggesting that precise control over virulence gene expression is required for the infectious process.

1.3 Virulence Gene Regulation in *Shigella flexneri*

The transcriptional regulation of virulence genes in *Shigella flexneri* involves a regulatory cascade in which environmental signals and competing transcription factors modulate gene expression at multiple levels (Fig. 1). Molecular studies of the intracellular pathogen and the genetic determinants required for host cell invasion have been ongoing for nearly four decades (Formal et al. 1971). Genes required for invasion of host cells are encoded by a large (approximately 230 kb) virulence plasmid present in all *Shigella* species (Sansonetti et al. 1982). Initiation of this transcriptional cascade begins with transcription of the *virF* gene, which encodes the primary regulator of virulence genes (Adler et al. 1989). The VirF protein then positively regulates the *virB* gene encoding a global regulator which is largely responsible for transcription of the structural virulence genes encoding the invasive protein antigens (IPAs), the type III secretion system, and other effector molecules. (Adler et al. 1989, Wing et al. 2004). The *virF* gene is optimally transcribed at 37 °C and with moderate pH and osmolarity (Porter and Dorman 1997b). Interestingly, these environmental signals appear to influence the transcriptional regulation of *virF* through modifications of DNA superhelicity (Porter and Dorman 1997a, Tobe et al. 1995). In fact, DNA topology is central to gene expression at all levels of this regulatory cascade. Furthermore, the transcription factors involved in

virulence gene regulation, Factor for Inversion Stimulation (FIS), Integration Host Factor (IHF), and Histone-like Nucleoid Structuring protein (H-NS), are nucleoid-associated proteins which typically modulate DNA curvature. Of these, FIS appears to specifically regulate the *virF* gene positively through restructuring of the DNA and through competition for binding with the global repressor H-NS (Falconi et al. 2001). H-NS negatively regulates many *Shigella* virulence genes, including *virB*, through increased DNA supercoiling which most likely prevents RNA polymerase binding (Falconi et al. 1998, Tobe et al. 1995, Castellanos et al. 2009). Presumably, the repression of virulence gene expression is important for the success of the bacterium, which also maintains two H-NS backups, StpA and Sfh (Beloin et al. 2003). Globally, the repressive effects of H-NS are antagonized by the IHF protein (Porter and Dorman 1997c). Additionally, the VirF protein appears to antagonize H-NS at the *virB* transcriptional level and in turn VirB antagonizes the H-NS repression of other virulence genes (Castellanos et al. 2009, Tobe et al. 1995). Interestingly, experimental evidence of direct binding of the DNA by VirF or VirB during H-NS antagonism is lacking (Dorman and Porter 1998). During optimal environmental conditions, gene expression increases 10-fold at every level of the regulatory cascade (Porter and Dorman 1997b). Together, these observations suggest that the transcriptional control of virulence genes in *S. flexneri* is regulated by a few global actors and this regulatory scheme results in either an all on or all off pattern of virulence gene expression under the proper environmental conditions. This regulatory structure appears to be counterintuitive and inefficient when considering the many steps involved in invasion and infection of human colonic epithelial cells. It is therefore likely that many *Shigella* genes require unique signals for initiation of transcription.

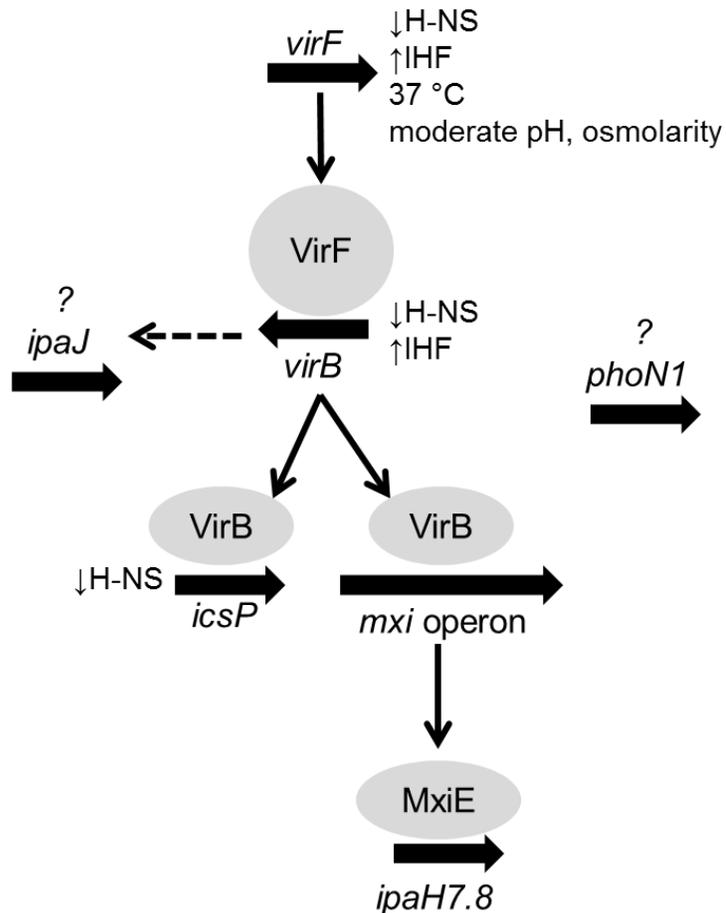
Recently, the regulatory cascade has been shown to be iron responsive through transcriptional repression of the *virB* gene by the small RNA RyhB in iron limiting conditions (Murphy and Payne 2007). While this mechanism does increase the complexity of virulence gene regulation at the transcriptional level, any increase or decrease of *virB* transcription will most likely be mirrored downstream in the regulatory cascade and will still not provide a branch point from the cascade or an instance of a unique expression profile for any one virulence gene.

One well studied example of differential regulation within the regulatory cascade involves positive regulation of a small subset of genes encoding secreted proteins by the transcription factor MxiE, which is encoded within the *mxi-spa* region required for assembly of the type III secretion apparatus (Mavris et al. 2002). This transcription factor appears to preferentially activate type III effectors following entry into the host cytosol, although the link between the transcriptional activation of these particular genes and *Shigella* pathogenesis is unclear (Kane et al. 2002).

Currently, our understanding of virulence gene expression through transcription initiation in *Shigella* creates a contradiction. The global repression of virulence gene transcription exerted by H-NS and the presence of two backup repressors encoded by the *Shigella* genome suggests that the transcriptional load present upon induction of the virulence gene regulatory cascade is unhealthy for the bacterial cell. From what we know, transcription initiation as controlled by the regulatory cascade provides the bacterial cell with an all or nothing transcriptional “choice”. Transcriptional regulatory mechanisms, similar to the activation of only certain genes by MxiE during a specific stage of pathogenesis, would allow for the precise regulation of virulence genes at appropriate

points during invasion and infection and therefore reduce any unnecessary transcription which would allow for more efficient use of cellular energy.

FIGURE 1. Summary of the *Shigella flexneri* virulence gene regulatory cascade for the *icsp*, *ipaJ*, *phoN1*, and *ipaH7.8* genes examined in this thesis. Known transcription factor/gene interactions are shown. Positive and negative regulators of transcription are listed next to the *virF*, *virB*, and *icsP* genes along with their effect indicated by an arrow. Transcriptional regulation of the *phoN1* gene is unclear while the *ipaJ* gene appears to be regulated by the regulatory cascade through VirB, but this has not been confirmed, which is indicated by a dashed arrow.



CHAPTER 2

TWO PROMOTERS AND TWO TRANSLATION START SITES CONTROL THE EXPRESSION OF THE *SHIGELLA FLEXNERI* OUTER MEMBRANE PROTEASE

ICSP

2.1 Introduction

Shigella species are gram-negative intracellular pathogens that cause bacillary dysentery in humans by invading cells of the colonic epithelium (Labrec et al. 1964, Sansonetti 1998). Once inside host cells *Shigella* move through the cytoplasm and into adjacent cells using actin-based motility. This process is mediated by the *Shigella* outer membrane protein IcsA (VirG), which polymerizes eukaryotic actin monomers into a tail of tightly bundled filaments on one pole of the bacterium (Bernardini et al. 1989, Goldberg et al. 1993). *Shigella flexneri* mutants lacking *icsA* are avirulent in animal models (Makino et al. 1986), demonstrating that actin-based motility is essential for *Shigella* pathogenicity.

The outer membrane protease IcsP modulates the amount and distribution of IcsA associated with *Shigella*. The activity of this protease was originally observed when growth medium was found to contain a 95 KDa polypeptide of IcsA after it had supported *Shigella* growth (Goldberg et al. 1993). Two groups identified IcsP (SopA) as the protease responsible for the cleavage of IcsA (Egile et al. 1997, Shere et al. 1997). Data collected by these two groups throughout five studies demonstrated that IcsP plays a role in the modulation of IcsA and the actin-based motility of *Shigella* (d'Hauteville et al. 1996, Egile et al. 1997, Shere et al. 1997, Steinhauer et al. 1999, Wing et al. 2005). Although IcsA is localized to the old pole of the bacterium in wild type *Shigella*

(Goldberg et al. 1993), expression of a non-cleavable form of IcsA in *Shigella* was found to lead to an increase in the circumferential localization of IcsA (d'Hauteville et al. 1996). Similar phenotypes were reported for *icsP* mutants *in vitro* (Egile et al. 1997, Shere et al. 1997, Steinhauer et al. 1999). When the intra- and intercellular phenotypes of *icsP* mutants were analyzed, abnormal actin-based motility and cell-to-cell spread were observed regardless of the serotype examined (Egile et al. 1997, Shere et al. 1997). Furthermore, *Shigella* cells expressing plasmid-borne *icsP* were found to lack detectable IcsA on their surfaces and the effects of *icsP* mutation on intercellular movement and plaque formation were also serotype dependent (Steinhauer et al. 1999, Wing et al. 2005). Since dysregulation of the *icsP* gene generates *Shigella* phenotypes that are consistent with attenuation of virulence, these studies strongly suggest that the *icsP* gene and its protein product will be tightly regulated.

Like many of the genes required for virulence of *S. flexneri*, *icsA* and *icsP* are encoded by the large ~230 kb virulence plasmid of *S. flexneri*. (Jin et al. 2002). This virulence plasmid encodes the transcription factor VirB which positively regulates many genes on the plasmid including *icsP* (Dorman and Porter 1998, Wing et al. 2004). The VirB-dependent regulation of the *icsP* promoter requires two distal VirB sites located between positions -1144 and -1130 relative to the annotated transcription start site (TSS) (Castellanos et al. 2009). These binding sites are located within an unusually large (~1.2 kb) intergenic region, which separates the *icsP* gene and the divergently transcribed *ospZ* gene.

In *Shigella flexneri* serotype 2a, coding sequences account for 76.24% of the virulence plasmid (Blattner et al. 1997, Jin et al. 2002). Although the coding density of

the *Shigella* virulence plasmid is lower than the *Escherichia coli* K12 chromosome (87.8%; 4), the intergenic region upstream of the *icsP* gene is still abnormally large when compared to the average size of an *E. coli* K-12 intergenic region (1.2 kb vs. 246 bp, respectively; (Pupo et al. 2000). Furthermore, the remote location of the VirB binding sites that influence *icsP* expression already implicates this large intergenic region in the transcriptional regulation of the *icsP* gene.

Based on the role that IcsP plays in maintaining the surface distribution of IcsA and how this ultimately regulates *Shigella* actin-based motility, we hypothesize that the *icsP* gene and /or its protein product will be tightly regulated. The aim of this study was to further characterize the regulation of IcsP at both the transcriptional and translational level. To do this, we chose to examine the entire upstream intergenic region for sequence elements involved in the regulation of IcsP.

2.2 Results

2.2.1 In Silico Analyses of the Intergenic Region Upstream of *icsP*

Due to the unusual length of the intergenic region and its involvement in the regulation of *icsP*, we wanted to further analyze this region for elements contributing to the transcriptional regulation of the *icsP* gene. To do this, our initial approach was to analyze the entire 1232 bp sequence using *in silico* tools. To identify putative promoter sequences the intergenic region upstream of *icsP* was entered into the BPR0M program for prediction of promoters regulated by the σ^{70} subunit of RNA polymerase (<http://linux1.softberry.com>). The BPR0M software identified four putative transcription start sites (TSSs) with associated -10 and -35 sequences at positions -84, -422, -769, and -

1106 relative to the previously annotated TSS (Fig. 2a). Interestingly, the BPROM program did not identify the originally annotated promoter. Analysis of the divergent strand predicted four additional promoters approximately 20 bp upstream of each TSS found on the complementary strand. This is not surprising considering the adenine and thymine composition of the -10 sequence. To examine whether any of the predicted promoters were aligned with potential open reading frames (ORFs), the entire intergenic region was analyzed with the microbial gene finding system Glimmer (NCBI). Only one ORF, identified on the *icsP* coding strand, lay within 150 bp of a putative TSS found by BPROM. This was the TSS at the -84 position. This ORF begins 33 bp upstream of the annotated *icsP* gene (Fig. 2b). This would allow for the production of a polypeptide exactly 11 amino acids longer than and yet still in frame with the previously described *icsP* gene. A common problem with ORF prediction software is that they identify the longest ORFs and frequently miss internally coded start codons (Delcher et al. 1999). This may explain why the Glimmer program did not identify the beginning of the originally annotated *icsP* gene (Egile et al, 1997).

2.2.2 Identification of Two Promoters Responsible for Regulation of the *icsP* Gene

Given the discrepancy between the predicted and annotated TSSs, we wanted to experimentally determine the position of all TSSs using primer extension analysis (Fig. 3). Primer extension analyses were performed on RNA isolated from the wild type *S. flexneri* 2a strain (2457T), the isogenic *virB* mutant (AWY3) and from wild type *Shigella* carrying either the *icsP* promoter and gene (pHJW6) or a *PicsP-lacZ* promoter fusion (2457T) or a low copy cloning vector (pHJW6 & pHJW20), two products were observed, indicating that the *icsP* gene is transcribed from two promoters. The sizes of these two

signal in all wild type strains. The increased signal intensity of products generated from strains carrying plasmids of the pHJW series was attributed to the copy number of these plasmids. In summary, these analyses identify two promoters involved in the transcriptional regulation of the *icsP* gene. We next wanted to examine how these two promoters are regulated.

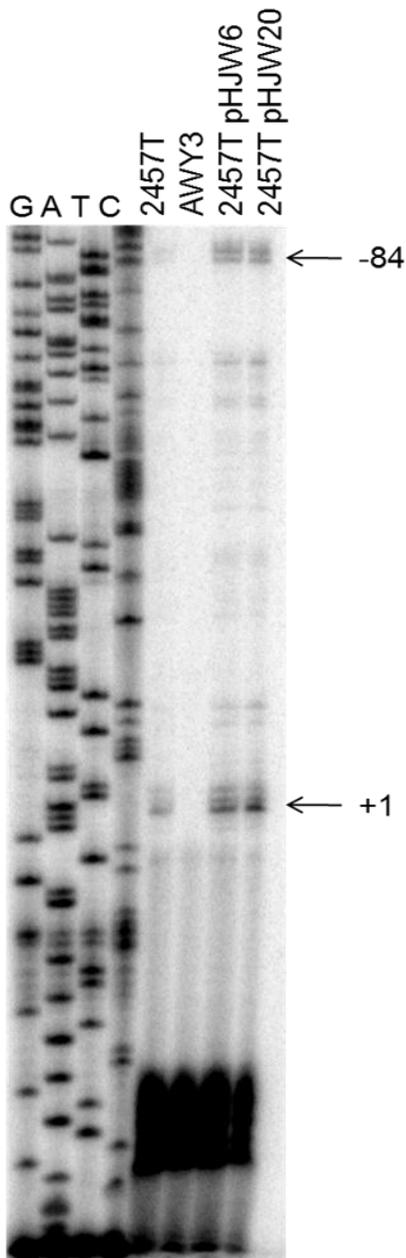


FIGURE 3. Primer extension analysis of the virulence plasmid-encoded *icsP* gene (2457T), the *virB* mutant (AWY3), the *icsP* inducible plasmid (pHJW6), and the *icsP-lacZ* promoter fusion (pHJW20). The new TSS, P2, is identified with its relative location to the annotated TSS, P1. A sequencing reaction (first four lanes) was used to calibrate the gel. The experiment was repeated three times and representative data are shown.

2.2.3 Both *icsP* Promoters Are Dependent Upon the Transcription Factor VirB and VirB Binding Sites Located Over 1 Kb Upstream of Both TSSs

Previous work has demonstrated that *icsP* is regulated by the *Shigella* transcriptional regulator VirB (Wing et al. 2004). Two VirB binding sites located over 1 kb upstream of the originally annotated TSS was shown to be required for this VirB-dependent regulation (Castellanos et al. 2009). To determine the effect of VirB-dependent regulation on P1 and P2, β -galactosidase assays were conducted with varying promoter constructs containing either P1 and P2 (pHJW20), P2 only (pKML03), or no promoter (pCTH03), in wild type *Shigella* or a *virB* mutant background.

Our data show that P2 alone contributes approximately 70% of the total promoter activity in wild type cells. In contrast, in the *virB* mutant background the activity of both promoters is significantly decreased (Fig. 4), indicating the activity of both promoters is dependent upon VirB. To further test the role of the distal VirB binding sites on P1 and P2 regulation, base pair substitutions that completely abolish the previously annotated VirB binding sites (Castellanos et al. 2009) were introduced into each of the reporter plasmids and promoter activity was measured in a wild type and *virB* mutant background. In wild type cells both constructs carrying the mutated binding sites exhibited a significant reduction in *icsP* promoter activity, and this activity was similar to that observed in the *virB* mutant background (Fig. 4). Furthermore, our primer extension analysis revealed no detectable primer extension products in the *Shigella virB* mutant lane (Fig. 3, AWY3). Taken together, these data indicate that VirB acts as a transcriptional regulator for both *icsP* promoters and that both promoters require the presence of the two distal VirB binding sites to mediate this effect.

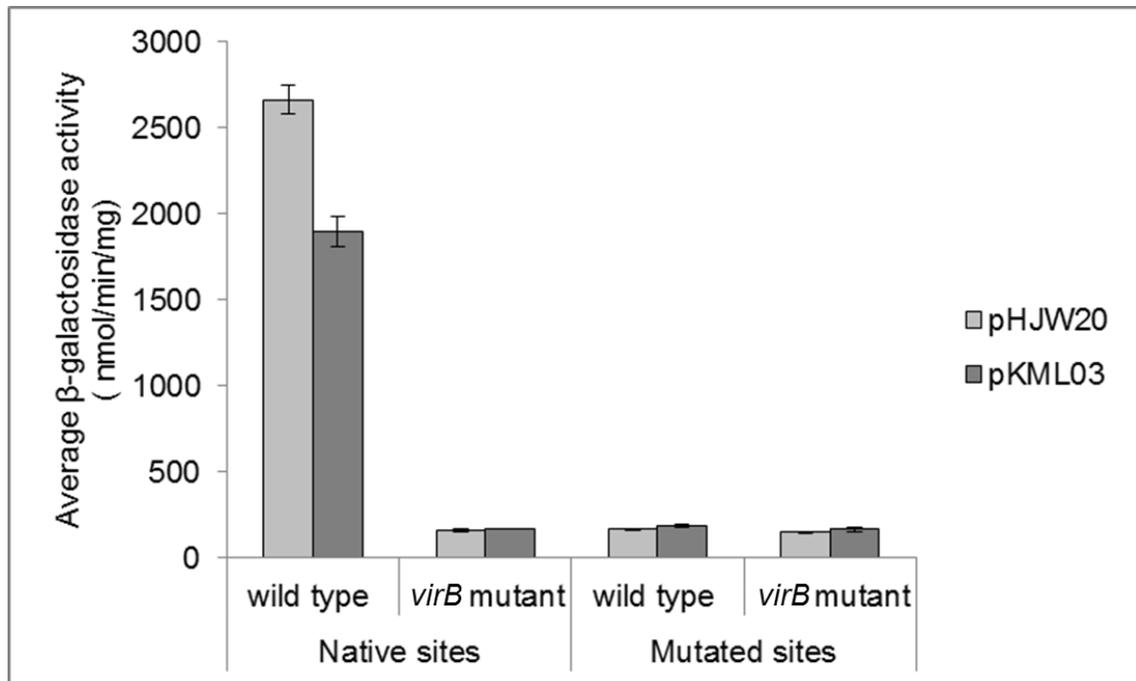


FIGURE 4. Activities of the wild type *icsP* promoter or a promoter carrying substitutions in the distal VirB binding sites (centered at -1137, with respect to P1) in wild type *S. flexneri* (2457T) and the *virB* mutant (AWY3). pHJW20 is a construct carrying both promoters and pKML03 carries the P2 promoter alone. Assays were run in triplicate and the means and standard deviations are shown.

2.2.4 Both Promoters Respond Similarly to Changes in Phase of Growth, Iron Concentration, PH, and Osmotic Pressure

Multiple promoters often allow for the differential regulation of a single gene product in response to bacterial growth or environmental stimuli (Erickson et al. 1987, Raina et al. 1995). In *Shigella*, many virulence genes are regulated at the level of transcription by changes in temperature, pH, osmolarity, and iron concentration (Mitobe et al. 2009, Murphy and Payne 2007, Porter and Dorman 1997a). The presence of two promoters upstream of the *icsP* gene raises the possibility that these promoters respond

differently to growth phase and/or environmental cues. Although expression of VirB is known to be regulated by these environmental signals and VirB is required for transcription from both promoters as demonstrated here, we wanted to investigate whether changes in environmental conditions experienced by *Shigella* during colonization would allow for more refined control of *icsP* transcription through capitalization of the two-promoter architecture. Therefore, we examined the change in activities of the two promoters following a decrease in either, pH, osmolarity, or iron concentration using the pHJW20 and pKML03 constructs. A decrease in iron concentration, pH, or osmotic pressure all seemed to affect each promoter similarly, with P2 contributing between approximately 60-75% of the combined promoter activity (Table 1). Since *icsA* expression is regulated in a growth phase-dependent manner (Goldberg et al. 1994), we also conducted a time course assay to determine whether the relative activity of the two promoters varies with phase of growth. Our data show that both *icsP* promoters are maximally active during stationary phase and that P2, represented by pKML03, contributes approximately 59% (2 h) to 76% (10 h) of the combined promoter activity, represented by pHJW20 (Fig. 5). These data indicate that the relative contribution of P1 and P2 to overall activity of the *icsP* promoters remains constant under a variety of conditions, suggesting that the two *icsP* promoters do not appear to be differentially regulated, at least under the conditions tested here.

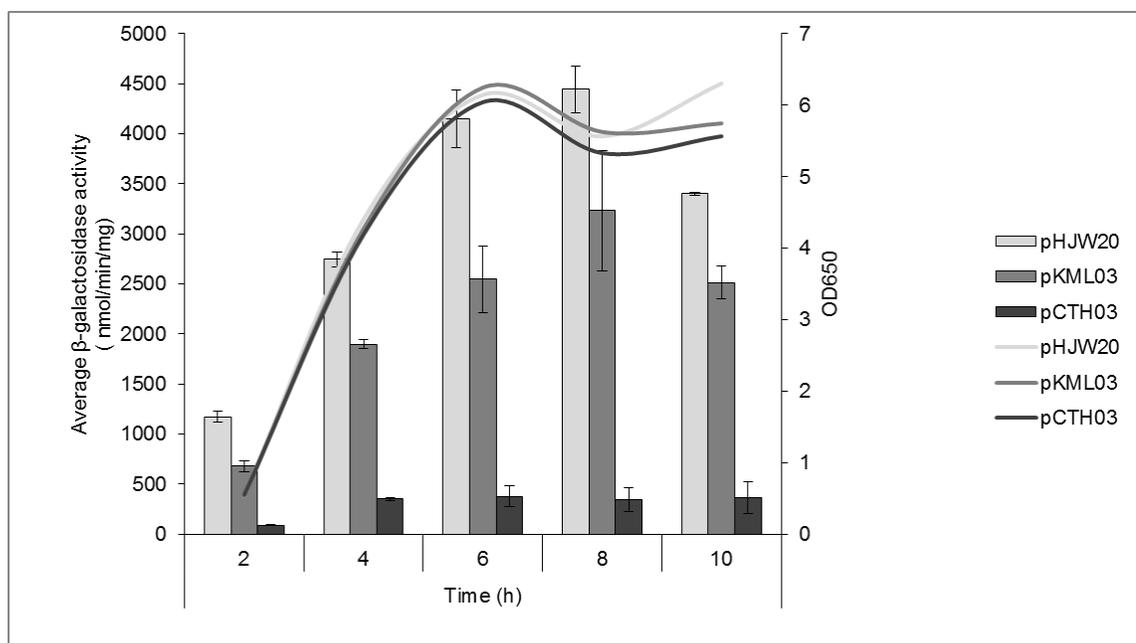


FIGURE 5. Activity of the *icsP* promoter constructs in response to growth phase in wild type *Shigella* (2457T). pHJW20 is a construct carrying both promoters, pKML03 carries the P2 promoter alone, and pCTH03 carries neither *icsP* promoter. Promoter activities were measured throughout growth (0-10 h) and optical densities of the cultures carrying each promoter construct are shown. The data are normalized using pMIC21, the promoterless *lacZ* gene. Assays were run in triplicate and the means and standard

TABLE 1. Contribution of P2 promoter activity to total *icsP* promoter activity

Environmental parameter	Specific condition in Luria-Bertani medium	Percent contribution ^a
pH	7.4	75.54 ± 6.96
	5.5	61.88 ± 19.55
Iron concentration	Normal (0 µg ml ⁻¹ EDDA)	69.86 ± 1.12
	Reduced (15 µg ml ⁻¹ EDDA)	74.81 ± 5.48
Osmotic pressure	Normal NaCl concentration (LB)	72.32 ± 0.07
	Half NaCl concentration (LO)	67.20 ± 3.96

^a Average percent contributions are based upon the promoter activity of P2 (pKML03) compared to the total promoter activity (pHJW20). Assays were run in triplicate and the means and standard deviations are shown.

2.2.5 Transcription from the Two *icsP* Promoters Allows for Translation Initiation from Two Sites

Our *in silico* analysis revealed the potential for an additional translation start site upstream and yet still in frame with the annotated translation start site (Fig. 2b). This second translation start site lies downstream of P2, raising the possibility that it is unique to P2 regulated transcripts. To examine whether this translation start site is used in the production of the mature, secreted IcsP protein, IcsP levels were measured in an *icsP* mutant carrying a low copy plasmid containing either both translation start sites (pHJW6), a single translation start site (pCTH16/downstream only or pCTH17/upstream only) or no translation start sites (pCTH18). Western blot analysis of whole cell protein preparations harvested from cells carrying each of these constructs, show that levels of the mature form of IcsP decrease (10-20% of wild type production) when either the upstream or downstream translation start sites are eliminated (pCTH16 and pCTH17 respectively) and are undetectable when translation from both sites is prevented (pCTH18). These data indicate that both translation start sites can be used to produce the mature form of IcsP (Fig. 6a). Furthermore, since IcsP is an outer membrane protease, which is secreted across the inner membrane via the general secretion pathway, our detection of mature IcsP in cells which lack either one translation start site or the other, strongly suggests that two isoforms of nascent IcsP are made and that each form is rapidly processed to a single mature form during secretion. This idea is supported by the signal peptide prediction program SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP>) which predicts a single signal peptide cleavage site for each of the predicted, nascent IcsP isoforms (data not shown).

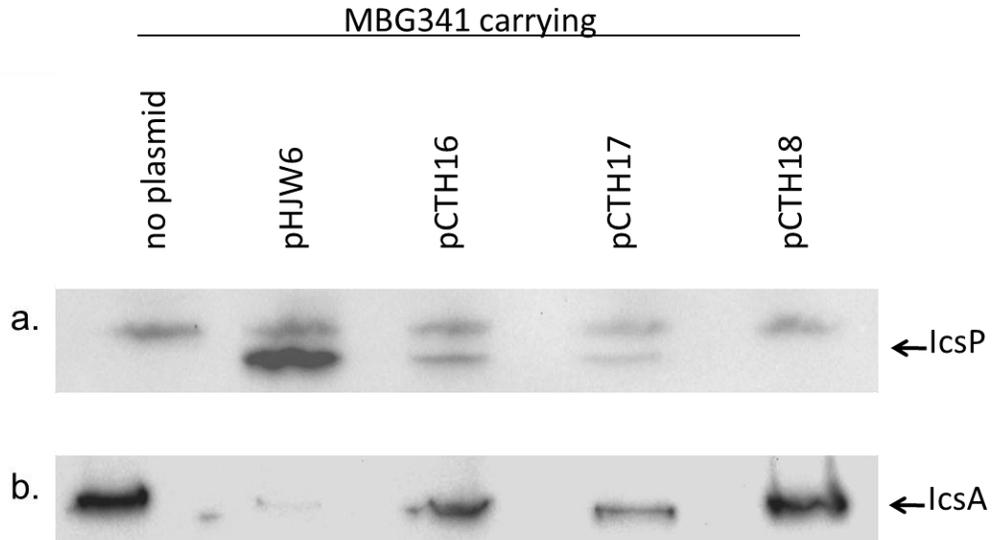


FIGURE 6. Amount and activity of IcsP produced from either the upstream or the downstream translation start site. IcsP levels were measured in the *icsP* mutant (MBG341) carrying either pHJW6 (both translation start sites), pCTH16 (the downstream translation start site), pCTH17 (the upstream translation start site), or pCTH18 (no translation start site) (a). Proteolytic activity of IcsP generated from either the upstream or the downstream translation start site, as judged by loss of IcsA, was measured in the *icsP* mutant (MBG341) carrying either pHJW6, pCTH16, pCTH17, or pCTH18 (b). The same whole cell protein extracts were used for both the IcsP and IcsA blots. The experiment was repeated three times and representative data are shown.

2.2.6 Translation Initiation from Either Start Codon Produces IcsP Capable of Proteolytically Cleaving IcsA

Having established that two translation start sites are used in the production of IcsP, we next wanted to test whether the resulting IcsP proteins differed in their proteolytic activity, as judged by their cleavage of IcsA. To do this we probed the same protein cell extracts utilized for the IcsP analysis with an IcsA antibody. Our data show a

reduction of the IcsA signal is seen in all lanes except those containing cell extracts from the *icsP* mutant (MBG341) and 2457T pCTH18, a construct which lacks both translation start sites (Fig. 6b). This observed decrease in full length IcsA protein levels is consistent with cleavage of IcsA by IcsP. These data therefore suggest that regardless of which translation start site is used to make the IcsP protein, the resulting protease is capable of cleaving IcsA.

2.2.7 Putative Translation Start Sites Are Commonly Found Around Start Codons of Annotated Open Reading Frames of the *Shigella flexneri* Chromosome and Virulence Plasmid

Having established that two methionine codons located at the start of the *icsP* gene can act as translation start sites for the production of IcsP, we wanted to examine how frequently additional translation start sites are found around annotated translation start sites within genome of *S. flexneri*. We chose to address this question via genome-wide scan for putative translation start sites. We assumed that translation start sites consist of two main elements: the start codon and Shine-Dalgarno sequence or ribosome binding site (RBS). First we examined fifty codons of genomic sequence up and downstream of start codons of all annotated ORFs of *S. flexneri* 2a strain 301 encoded by the virulence plasmid pCP301 and the chromosome for presence of potential start codons (Jin et al. 2002, Wei et al. 2006). Second, we determined whether the identified potential alternative start codons are associated with RBSs by applying a position-specific scoring matrix (PSSM) specific for the RBS in *S. flexneri* 2a strain 301 (Supplementary Fig. 1), to 20 nucleotide stretches of sequence upstream of every potential start codon.

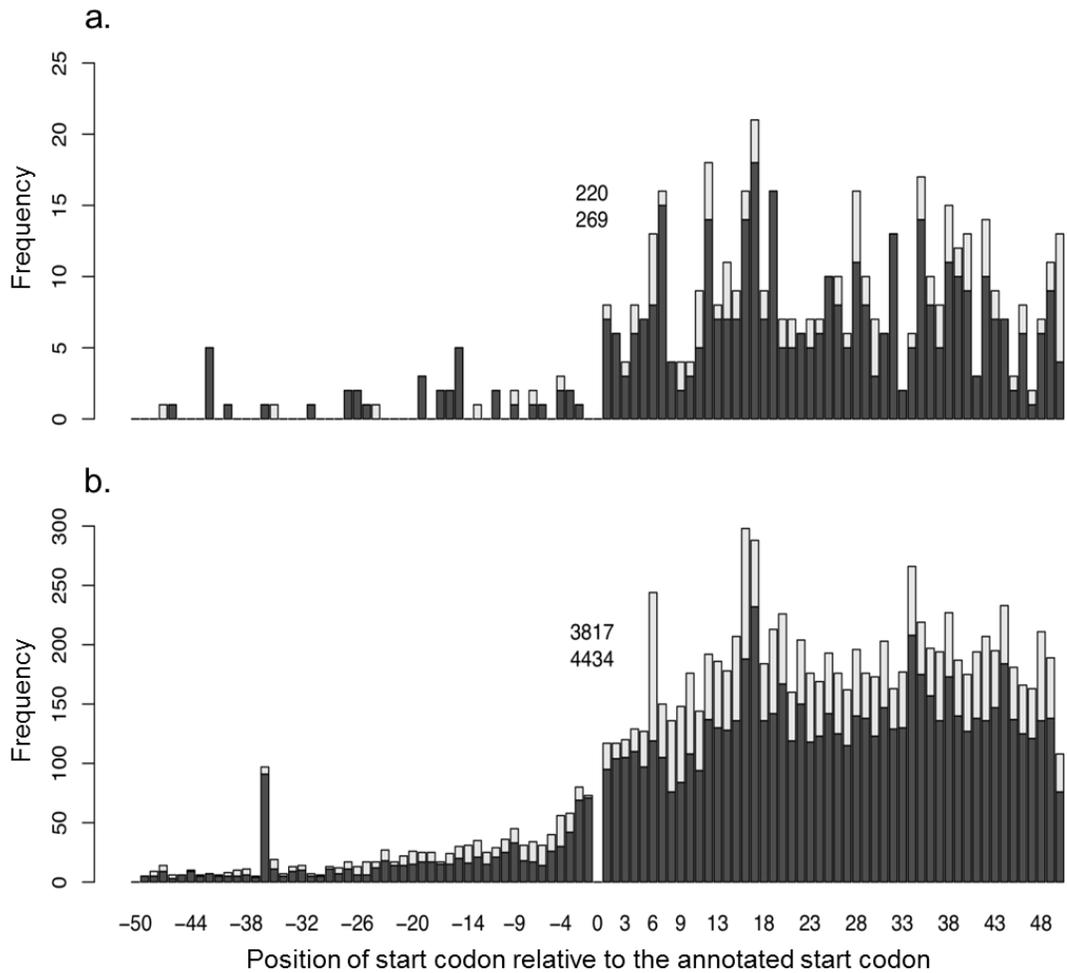


FIGURE 7. Distribution of putative start codons positioned up and downstream of the annotated start codon (0) in the *S. flexneri* virulence plasmid (a) and chromosome (b). The total number of predicted start codons are shown for each coordinate (light grey) along with the total number of predicted start codons associated with predicted Shine-Dalgarno sequence (dark grey). The inset values indicate the total number of annotated translation start sites (bottom) and annotated translation start sites associated with a predicted Shine-Dalgarno sequence (top). Translation start site analysis scripts were designed and run by Olga Kamneva, University of Wyoming.

We revealed that out of the 4705 analyzed ORFs, 4165 have at least one additional putative start codon within a fifty codon range up and downstream of annotated start codons. For 2070 of the ORFs at least one alternative start codon is associated with a putative RBS. Figure 7a and b shows the distribution of alternative start codons and alternative translation start sites within the examined range of genomic DNA. The trend within the distribution is better defined among ORFs on the chromosome, because of the larger number of ORFs examined. The number of detected start codons quickly declines within intergenic spaces compared to those within the ORFs, due to present of in-frame stop codons, however local maxima of alternative translation start sites are located at codon 10, and between 35 and 40 codons upstream of the annotated translation start site. Within the coding regions, local maxima are found between codon 18-20 and at codon 35. The distribution of p-values for RBSs associated with annotated and alternative start codons are shown on (Supplementary Fig. 2). It is clear from these values that some alternative translation start sites are associated with much stronger RBSs than the annotated translation starts.

2.3 Discussion

This work has identified a second TSS for the *Shigella icsP* gene. Transcription from this site ultimately allows translation to proceed from a newly identified translation start site located 33 bp upstream of the originally annotated translation start site. Our work demonstrates that regardless of which translation start site is used, a mature form of IcsP is made that is capable of proteolytically cleaving the *Shigella* actin based motility protein IcsA. It remains unclear whether the complex organization of the *icsP* regulatory

region simply allows for transcriptional and translational redundancy or whether this organization allows for the exquisite control over *icsP* transcription and subsequent protein production in response to cellular and/or environmental cues.

Regulation from multiple promoters has been well documented in other bacterial species and is usually found to contribute to the differential regulation of a single gene. While our experiments did not allow us to identify conditions which lead to the differential regulation of the two *icsP* promoters, we can now eliminate decreases in pH, osmolarity and iron concentration from other environmental conditions encountered in the human host, which have the potential to differentially regulate the two *icsP* promoters. Our data show that both *icsP* promoters are most active during stationary phase cultures. This pattern of expression is in agreement with the clearance of IcsA from the bacterial cell surface in stationary phase cultures (Goldberg et al. 1994) and the model for IcsP activity during pathogenesis, which proposes a build-up of IcsA on one pole of the bacterial outer membrane, maintenance of a tight polar cap of IcsA by removing IcsA that diffuse away from the pole through the activity of IcsP, and finally clearance of IcsA from the bacterial surface by IcsP (Goldberg et al. 1994, Steinhauer et al. 1999).

Another way multiple promoters can be differentially regulated is by the use of different transcription factors. Many virulence genes in *Shigella* are commonly regulated by the transcription regulator, VirB. Previous work, by us and others, has demonstrated that transcription of *icsP* is positively regulated by VirB (Castellanos et al. 2009, Le Gall et al. 2005b, Wing et al. 2004). The work presented here reveals that both *icsP* promoters are positively regulated by VirB and that this regulation is mediated by a VirB binding site located over 1 kb upstream of the originally annotated TSS, because site directed

mutagenesis of these binding sites reduce promoter activity of both P1 and P2 to level observed in *virB* mutant. VirB functions at *Shigella* promoters, including the *icsP* promoter, by alleviating transcriptional repression mediated by the nucleoid structuring protein H-NS, rather than by activating the promoter per se. Since the activity of P1 and P2 increases in the presence of VirB, both promoters are likely to be repressed by H-NS. Whether or not other DNA binding proteins interact with the long intergenic region upstream of the *icsP* promoter, and what role this has in the regulation of the two *icsP* promoters remains unclear at this stage, but this is an avenue of research investigation in our laboratory.

In bacterial genomes, alternative sigma factors sometimes allow the differential regulation of multiple promoters associated with a single gene. In our study the BPROM software failed to identify the previously annotated TSS in our analysis. This might indicate the possible use of an alternative sigma factor, even though both *icsP* promoters contain -35 sequences closely resembling the consensus for σ^{70} -dependent regulation. Since the activity of both *icsP* promoters is maximal in stationary phase cultures, the most likely alternative sigma factor to be used to control *icsP* expression is the stationary phase sigma factor, σ^S . Despite maximal activity of the two *icsP* promoters in stationary phase cultures, the sequences surrounding P1 and P2 contain no consensus sequences known to bind σ^S or other alternative sigma factors.

In all of our β -galactosidase assays the relative contribution of each *icsP* promoter to total *icsP* promoter activity remained similar, regardless of the conditions used; P2 promoter activity contributed approximately 60-75% of the total promoter activity. Nevertheless, in our primer extension analysis, the P2 generated transcripts contribute to

approximately 33% of the total signal intensity from both P1 and P2. This apparent inconsistency may be caused by an inherent bias for shorter transcripts within the primer extension analysis. Alternatively, it is possible that the P2 transcripts are less stable than the P1 transcripts. Interestingly, the reduced level of P2 versus P1 transcripts in our primer extension analyses (Fig. 3) is consistent with the decreased amount of IcsP protein detected by western blot analyses when only the upstream translation start site is used (Fig. 6a, pCTH17 lane).

The organization of the *icsP* promoter region means that if transcription occurs from P1, translation can only occur from the downstream translation start site, whereas if transcription occurs from P2 then translation has the potential to start from either the upstream or downstream translation start site. This raises the question whether both translation start sites are used in a P2 transcript. Recent studies on polysome organization (Brandt et al. 2009) indicate that the distance between the two translation start sites in a P2 transcript (11 codons) is unlikely to allow simultaneous ribosome binding. This, along with the fact that our bioinformatics studies reveal no good match to the consensus Shine-Dalgarno sequence associated with the downstream translation start site, strongly suggests that the upstream translation start site would be favored in P2 transcripts, at least in the absence of accessory translation initiation factors.

Our work demonstrates that two translation start sites can be used to generate the mature and active form of IcsP. Since IcsP is secreted using the general secretion pathway, the two nascent isoforms must include an amino terminal signal sequence consisting of positively charged amino acids involved in protein targeting to the inner membrane for secretion (Fekkes and Driessen 1999). Interestingly, analyses of the two

nascent isoforms of IcsP using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP>), predicts that each isoform is likely to be cleaved at the same position, to release two different signal peptides, but the same mature IcsP protein. The extended positively charged amino terminus of the longer nascent IcsP protein could allow for enhanced protein processing and translocation similar to the enhanced secretion of outer membrane proteins observed in *E. coli* (Akita et al. 1990). More efficient processing and translocation of the longer IcsP product may explain why the shorter IcsP protein product is expressed at a higher level but contributes to less IcsA cleavage as indicated by densitometry analyses of our IcsP and IcsA western blots.

While this study adds to our understanding of the *icsP* intergenic region and the role of this region plays in the regulation of the *Shigella* outer membrane protease, further investigation is needed to understand whether additional regulatory elements exist within this intergenic region and how these elements affect the production of IcsP and ultimately *Shigella* actin-based motility. Although the purpose of the second promoter and second translation start site remains unclear, our results suggest that production of the outer membrane protease IcsP may be more intricately regulated than previously thought.

The genome-wide screen for alternative translation start sites conducted within the present study, along with our observations at made at the *icsP* promoter, provides the first evidence that functional alternative in-frame translation start sites in the genome of *S. flexneri* 2a strain 301 is a general phenomenon rather than something specific for *icsP* gene. Whether our observation is restricted to the genome of *S. flexneri* 2a strain 301 or is the general property of microbial genomes will require additional studies. It should be noted that recent work by Tucker and Escalante-Semerena (Tucker and Escalante-

Semerena 2010), demonstrates that two isoforms of CobB are made from a single gene in *Salmonella enterica* and that these two isoforms have different biological activities. Our findings, in conjunction with these studies, imply that the use of alternative translation start sites may increase the size of the proteome and, in some instances, lead to a larger range of physiological functions being encoded by the bacterial genome than was previously acknowledged.

2.4 Materials and Methods

2.4.1 Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in the present study are listed in Table 2. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) broth with aeration or on LB agar (LB broth containing 1.5% [wt/vol] agar). *S. flexneri* were grown at 37 °C in Trypticase Soy Broth (TSB) with aeration or on Trypticase Soy Agar (TSA) (TSB containing 1.5% [wt/vol] agar). Where appropriate, chloramphenicol was added at a final concentration of 25 µg ml⁻¹. To ensure that *Shigella* strains had maintained the large virulence plasmid during manipulation, Congo red binding was tested on TSA plates containing 0.01% (wt/vol) Congo red (Sigma Chemical Co., St. Louis, Mo.).

2.4.2 Plasmid Construction

The starting point for this work was the *PicsP-lacZ* reporter plasmids pHJW20 and pMIC18 (described in (Castellanos et al. 2009); Table 2). pHJW20 carries 1232 bps upstream of the TSS of the *icsP* promoter annotated by Egile *et al.* (Egile et al. 1997), the first 48 bp of the *icsP* coding region cloned upstream of a translation stop site, and a unique *XbaI* site upstream of a promoterless *lacZ* gene, so the expression of *lacZ* is

directly regulated by the *icsP* promoter. pMIC18 is identical to pHJW20, but carries a 14 bp substitution that destroys the two upstream VirB-binding sites that are required for the VirB-dependent regulation of *icsP* (Castellanos et al. 2009).

To create pKML03, a truncated *icsP* promoter fragment was amplified from pHJW20 using oligonucleotides W93 (5'-TGGGTTGAAGGCTCTCAAGGGC-3') and W123 (5'-TATTTTGCTCTAGATTTTAATTAATATTTGTTTATGTTACC-3'). The PCR fragment was digested with *Pst*I and *Xba*I, and the resulting DNA fragment was ligated into pHJW20 previously digested with *Pst*I and *Xba*I. The resulting construct lacked the P1 TSS, and its -10 and -35 promoter elements, due to a 48 bp truncation at the 3' end of the *icsP* promoter region. To create pCTH02, mutated VirB binding sites from pMIC18 were isolated on a *Pst*I and *Pac*I restriction fragment and introduced into pKML03 previously digested with *Pst*I and *Pac*I. The resulting construct therefore carried mutated, instead of wild-type VirB binding sites. To create pCTH03, a truncated *icsP* promoter fragment was amplified from pHJW20 using oligonucleotides W93 and W167 (5'-TATTTTGCTCTAGACCTCATTGTGCGAATAAAGTAACGG-3'). The PCR fragment was digested with *Bgl*II and *Xba*I, and the resulting DNA fragment was ligated into pHJW20 previously digested with *Bgl*II and *Xba*I. The resulting construct therefore lacked both P1 and P2, due to a 132 bp truncation at the 3' end of the *icsP* promoter region.

To measure IcsP production and IcsP protease activity, the plasmid pHJW6 and its derivatives were used (described in (Wing et al. 2004); Table 2). pHJW6 is identical to pHJW20, but instead of carrying a *PicsP-lacZ* fusion this plasmid carries the full *icsP* coding region downstream of the *icsP* promoter region.

To create pCTH16, the sequence encoding the *icsP* gene was isolated from pHJW6 using *PacI* and *BamHI* restriction enzymes and used to replace the *lacZ* gene in pHJW36. pHJW36 lacks the -35 and part of the -10 promoter elements for P2 and this has been demonstrated to result in an inactive P2, as evidenced by i) primer extension analysis (unpublished data) and ii) the drop in total *icsP* promoter activity (Castellanos et al. 2009), consequently the newly formed construct pCTH16 could be used to measure IcsP protein production generated from P1 specific transcripts and hence the downstream translation start site. To create pCTH17, regions encoding a portion of the downstream Shine-Dalgarno sequence and the downstream methionine were mutated by introducing base pair substitutions in both sites using a QuikChange Lightning site-directed mutagenesis kit from Agilent Technologies and oligonucleotides W259 (5'-GTGCAAGTACAAAGAATTTTAATTTGAGCGAGAACTCGACTTTTTTGGTTGAAATGTCCATGA-3') and W260 (5'-TCATGGACATTTCAACCAAAAAAGTCGAGTTCTCGCTCAAATTA AAAATTCTTTGTACTTGCAC-3'). The substitutions used to disrupt the downstream translation start site were chosen to minimize the effect on the upstream translated protein product. Specifically the Shine-Dalgarno sequence was mutated from AAGTAG to AAGTCG, this resulted in the substitution of a valine codon for another valine codon, and the methionine codon ATG was mutated to a leucine codon CTC. The resulting amino acids have similar biochemical properties. To create pCTH18, pCTH17 was digested with *PacI* and *BamHI* to obtain the mutated sequence eliminating the downstream translation start site, and the resulting DNA fragment was ligated into pCTH16 previously digested with

PacI and *BamHI*. The resulting construct consequently lacked the upstream translation start site and carried a mutated downstream Shine-Dalgarno and translation start site.

2.4.3 Quantification of *icsP* Promoter Activity Using *PicsP-lacZ* Reporters

Activity of the *icsP* promoters were determined by measuring β -galactosidase activity (as described previously (Castellanos et al. 2009) using the Miller protocol (Miller 1972)) 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 and grown for 5 h in TSB, to ensure *icsP* expression. To measure the effects of growth phase on promoter activity, cells were grown for 2 to 10 h in 2 h intervals. To measure the effects of pH on promoter activity, cells were grown in LB with a pH of 5.5 buffered with a final concentration of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) or a pH of 7.4 buffered with 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS). To measure the effects of osmotic pressure on promoter activity, cells were grown in either LB or LO (Porter and Dorman 1994). To measure the effects of iron concentration on promoter activity, cells were grown in either LB or LB supplemented with 15 $\mu\text{g ml}^{-1}$ EDDA to chelate iron. Optical densities were measured using a DU 520 general purpose UV/Vis spectrophotometer (Beckman Coulter). Promoter activity was normalized using pMIC21, the promoterless *lacZ* reporter construct.

2.4.4 Transcription Start Site Mapping of the *icsP* Gene

Transcription start sites of the *icsP* gene were identified through RNA extraction and primer extension analysis procedures as described previously (Wing *et al.*, 1995) using a protocol adapted from Aiba (Aiba et al. 1981, Wing et al. 1995). Total cellular

RNA was extracted using the hot-phenol method from 10^9 cells harvested from early stationary phase cultures (Aiba et al. 1981). Residual DNA within samples was digested with DNase I (Qiagen) at 37°C for 1 h in DNase I buffer according to Ambion instructions (Ambion 2001). Integrity of total RNA was checked by formaldehyde gel electrophoresis and ethidium bromide staining as described by Sambrook (Sambrook and Russell 2001). The oligonucleotide primer W183 (5'-AAAGTGCAAGTACAAAG-3') was 5'-end-labeled with [γ - 32 P]ATP by using T4 polynucleotide kinase (Promega). One picomole of 32 P-labeled primer and 5 μ g of total RNA were lyophilized and redissolved in 30 μ l of hybridization buffer (Aiba et al. 1981). The reaction was incubated at 75°C for 15 min followed by a cooling and incubation at 37°C for a total of 3 h. Following an ethanol precipitation, reverse transcription was completed using Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Remaining RNA was degraded with 10 mg/ml RNase A (Sigma) for 30 min at 37°C and the reaction terminated by ethanol precipitation. The precipitate was dissolved in 5 μ l of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and electrophoresed on a 6% glycerol tolerant polyacrylamide gel containing 7 M urea. Following electrophoresis, the gel was transferred to Whatman paper and then vacuum dried before overnight exposure to a phosphorescent screen. The screen was visualized the following morning using a Typhoon 9410 variable mode imager (Amersham). The sequencing ladder generated from pBluescript KSII+ (Stratagene) and a M13 reverse primer (5'-GAGCGGATAACAATTTACACAGG-3') with the Sequenase 2.0 kit (usb) according to manufacturer's instructions, was used to size the primer extension products.

Densitometry analysis was conducted using VisionWorksLS image acquisition and analysis software (UVP).

2.4.5 Quantification of IcsP Production and IcsA Cleavage in *Shigella*

IcsP production and activity (IcsA cleavage) was measured by western blot analysis. Cells from early stationary phase cultures were harvested and whole-cell protein extracts were prepared as described previously (Steinhauer et al. 1999). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 12.5% SDS-PAGE gel. Equivalent amounts of protein were loaded by normalizing the volume to cell density. Western blot analyses were performed with an affinity-purified IcsP or IcsA rabbit antiserum. The IcsP and IcsA antisera were raised against peptides sequences predicted to fall in surface exposed regions of the two proteins, the L3 loop of IcsP (based on the model of OmpT; (Vandeputte-Rutten et al. 2001) and the α -domain of IcsA, which is proteolytically cleaved from the surface of *Shigella* by IcsP. Each antibody was ultimately detected by chemiluminescence using a UVP BioSpectrum imaging system and accompanying software. Densitometry analysis was conducted as previously described.

2.4.6 *In Silico* Analyses of the *icsP* Gene, Its Protein Product and the Position of Translation Start Sites in *Shigella* Open Reading Frames

Throughout our work sequences files were accessed and analyzed using the software program “Clone Manager 9 Basic Edition” (Scientific and Educational Software). Transcription start site predictions were performed using the BPROM program

(<http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfin>)

db). This algorithm predicts potential transcription start positions regulated by σ^{70} promoters (major *E. coli* promoter class). The linear discriminant function combines characteristics describing functional motifs and oligonucleotide composition of these sites. BPROM has 80% accuracy for *E. coli* σ^{70} -dependent promoter recognition. Open reading frame predictions were performed using Glimmer (NCBI), which is a system for finding genes in microbial DNA using interpolated Markov models (IMMs) to identify the coding regions and distinguish them from noncoding DNA (Delcher et al. 1999). To predict the presence and location of proteolytic cleavage sites within nascent IcsP the SignalP 3.0 Server program (<http://www.cbs.dtu.dk/services/SignalP/>) was used (Emanuelsson et al. 2007). This algorithm predicts potential signal peptides and cleavage sites based on a combination of several artificial neural networks and hidden Markov models.

2.4.7 Computational Analysis of Translation Start Sites in *S. flexneri* 2a Str. 301*

Full genome sequence of *S. flexneri* 2a str. 301 (chromosome and plasmid) as well as the most recent annotation was downloaded from GenBank FTP site (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/Shigella_flexneri_2a/). Translation start sites were assumed to consist of two main components: start codon and Shine-Dalgarno sequence (Ribosome Binding Site, RBS). We used three variants of start codon confirmed for *E. coli* (ATG and GTG) (Jacques and Dreyfus 1990).

To identify position-specific scoring matrix (PSSM) for Shine-Dalgarno sequence specific for *S. flexneri* 2a str. 301, 25 nucleotides of sequence data upstream of the start codon of each annotated open reading frame (ORF) were extracted from the genome (chromosome and plasmid). Then retrieved regions of DNA were searched for

overrepresented motifs using locally installed MEME program (Multiple Em for Motif Elicitation). Identified RBS motif was truncated to include only highly conserved positions (Supplementary Fig. 1). A motif sequence logo was created using an online program (<http://weblogo.berkeley.edu/logo.cgi>).

To identify presence of alternative translation start sites in genome of *S. flexneri* 2a str. 301 fifty codons upstream and downstream of start codon of every annotated ORF was tested for presence of possible in-frame start codon, if in-frame stop codon (TAG, TAA or TGA) was detected further search around this particular annotated start codon was terminated. 20 nucleotide sequences upstream every possible alternative start codon were collected, and then searched for presence of RBS using the program MAST (Motif Alignment & Search Tool) and determined before PSSM for RBS. Distance from annotated start site, p-value for motif presence (if RBS was found) and exact sequence of start codon were extracted. Data were manipulated, managed and graphically represented using custom R and Perl scripts. * Translation start site analysis scripts were designed and run by Olga Kamneva, University of Wyoming.

TABLE 2. Bacterial strains and plasmids

Strain or plasmid	Description ^a	Source or reference
Strains		
<i>S. flexneri</i>		
2457T	<i>S. flexneri</i> serotype 2a	(Labrec et al. 1964)
AWY3	2457T <i>virB</i> ::Tn5; Kn ^r	(Wing et al. 2004)
MBG341	2457T <i>icsP</i> ::Amp ^r	(Shere et al. 1997)
Plasmids		
<i>PicsP-lacZ</i> reporters		
pHJW20	<i>icsP</i> promoter region transcriptionally fused to <i>lacZ</i> in pACYC184 Cm ^r ; carries 1232 bp of wild-type sequence upstream of the <i>icsP</i> transcription start site and unique <i>XbaI</i> site upstream of <i>lacZ</i> gene	(Castellanos et al. 2009)
pHJW36	pHJW20 lacking P2 promoter elements	(Castellanos et al. 2009)
pMIC18	pHJW20 carrying 14 bp substitutions in the two upstream VirB-binding sites	(Castellanos et al. 2009)
pMIC21	pHJW20 lacking all <i>icsP</i> promoter sequences	(Castellanos et al. 2009)
pKML03	pHJW20 lacking previously annotated promoter elements	This work
pCTH02	pKML03 carrying 14 bp substitutions in the two upstream VirB-binding sites	This work
pCTH03	pHJW20 lacking P1 and P2 sequences	This work
<i>PicsP-icsP</i> reporters		
pHJW6	<i>icsP</i> promoter and gene cloned into pACYC184	(Wing et al. 2004)
pCTH16	pHJW6 lacking P2 specific promoter elements	This work
pCTH17	pHJW6 with 4 bp substitutions in the downstream translation start site	This work
pCTH18	pCTH17 lacking P2 specific promoter elements	This work

^a Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Kn^r, kanamycin resistance.

CHAPTER 3

CRP-DEPENDENT REGULATION OF *SHIGELLA* VIRULENCE GENES.

3.1 Introduction

Changes in environmental conditions often serve as signals for transcription of virulence genes in *Shigella flexneri*. In fact, transcription of many virulence genes is dependent upon a regulatory cascade which begins with the transcription of the primary regulator VirF (Dorman and Porter 1998). Maximal transcription of the *virF* gene occurs at 37 °C when accompanied by moderate osmolarity and moderate pH (Porter and Dorman 1997a) . These conditions would be encountered by the bacterium as it transitions from outside of the host to the inside of the host. Additional environmental cues may also be sensed as *Shigella* enters the colon or invades cells of the colonic epithelium, which would provide a signal necessary for initiation of transcription when virulence factor production is necessary.

Metabolites are an example of one class of environmental factors known to regulate gene transcription in bacterial cells. Catabolite repression is one of the most widely studied mechanisms for “sensing” metabolite presence and transforming that information into a regulatory response (reviewed in (Kolb et al. 1993)). Catabolite repression occurs when the presence of a preferred carbon source leads to the transcriptional repression of genes required for catabolism of a non-preferred carbon source. This mechanism ensures catabolism of the preferred carbon source prior to catabolism of one or several other carbon sources. This effect is sometimes referred to as the “glucose effect” due to the repressive effects glucose was observed to have on the transcription of genes encoding catabolic enzymes specific for other carbon sources in

Escherichia coli (Epps and Gale 1942). In fact, transcription of the *lac* operon, which encodes three genes involved in the catabolism of lactose, is not truly repressed but rather transcriptional activation is prevented due to the presence of glucose. The cyclic AMP (cAMP) receptor protein (CRP) acts as a transcriptional activator for the *lac* operon but requires cAMP as a cofactor for binding (de Crombrughe et al. 1984, Emmer et al. 1970). In the absence of glucose, the adenylate cyclase enzyme, encoded by the *cyaA* gene, catalyzes the conversion of ATP to cAMP and pyrophosphate (Harwood and Peterkofsky 1975). Therefore, the absence of glucose allows for cAMP production which can then be used as a cofactor for transcriptional activation of the *lac* operon by CRP. Transcriptional activation of the *lac* operon leads to production of enzymes necessary for lactose catabolism.

Pathogenic bacteria are also known to use this carbon “sensing” mechanism in order to regulate the transcription of virulence genes. The transcription of two, alternately expressed toxin encoding genes in enterotoxigenic *E. coli* (ETEC) has been shown to be dependent upon CRP binding (Bodero and Munson 2009). ETEC expresses two toxins, a soluble heat-labile toxin (LT-1) typically expressed in the duodenum and a heat-stable toxin (STa) typically expressed in the ileum. Interestingly, CRP binding prevents transcription of *eltAB*, the two genes encoding LT-1 while CRP is required for transcriptional activation of the STa gene, *eltAp* (Bodero and Munson 2009). This regulatory network allows for continued toxin production while exposed to a decreasing glucose gradient as the bacterium moves through the duodenum and into the ileum.

Catabolite repression has not yet been shown to transcriptionally regulate any *S. flexneri* virulence genes, although little work has been done in this area. Reports of CRP-

dependent transcriptional regulation of virulence genes in other enteric pathogens, such as ETEC, however, raises the possibility that some *Shigella* virulence genes may also be regulated by catabolite repression. Analysis of the *Shigella* virulence plasmid has revealed that three *Shigella* virulence genes are associated with promoters that encode putative CRP binding sites (Table 3) (G. P. Munson, personal communication). The CRP binding site consensus sequence consists of a palindromic TGTGA separated by a six-base-pair spacer (Kolb et al. 1993). These three genes, *ipaJ*, *phoN1*, and *ipaH7.8* encode a substrate of the type III secretion system with unknown function (Buysse et al. 1997), a periplasmic non-specific phosphatase (Uchiya et al. 1996), and a secreted E3 ubiquitin ligase (Rohde et al. 2007, Singer et al. 2008, Zhu et al. 2008), respectively.

TABLE 3. Predicted CRP binding sites

Gene	Promoter sequence with predicted CRP binding site
<i>ipaJ</i>	5'-tgg <u>tg</u> gac <u>ctgtatcaca</u> ata-3'
<i>phoN1</i>	5'-ttat <u>gtgatagcacacaca</u> att-3'
<i>ipaH7.8</i>	5'-attat <u>tgactgcagccaca</u> ata-3'

^a Underlined sequences signify the predicted CRP binding sites

The *ipaJ* gene appears to be transcriptionally regulated in a temperature-dependent fashion and previous data also suggests *ipaJ* is also regulated through the regulatory activities of both VirF and VirB (Buysse et al. 1997, Le Gall et al. 2005a). Although, *ipaJ* mutants were Sereny positive, attenuation of keratoconjunctivitis was observed (Buysse et al. 1997). The secreted protein is a *Shigella* immunogen and does react with convalescent sera in western blot analyses (Buysse et al. 1997, Slagowski et al. 2008). An ORF encoding a protein with 49% homology to IpaJ was also found on a small

plasmid present in *Salmonella choleraesuis*, an important swine pathogen (Liu et al. 2002).

The protein encoded by the *phoN1* gene exhibits a non-specific phosphatase activity mostly within the periplasm and this activity is optimally expressed at a pH of 6.6 and a temperature of 37 °C (Uchiya et al. 1996). Mutation of *phoN1* does not appear to affect invasiveness or intercellular spread as demonstrated by infections of epithelial cell monolayers (Uchiya et al. 1996). Transcription of *phoN1* does increase following a drop in pH but does not appear to be affected by the same regulatory elements involved with expression of other *Shigella* virulence genes such as temperature or VirB (Cheng et al. 2007, Le Gall et al. 2005a). The *phoN1* gene has an 83% sequence similarity with another virulence plasmid-encoded phosphatase, *phoN2* (*apy*), which encodes an apyrase that is dependent upon the VirF and VirB regulatory cascade for transcription and does appear necessary for efficient intercellular spread (Berlutti et al. 1998, Santapaola et al. 2006).

The *ipaH7.8* encoded E3 ubiquitin ligase marks host cell proteins for degradation by the eukaryotic proteasomes within the cytoplasm. This activity of IpaH7.8 has been demonstrated to be important in bacterial escape from the endocytic vacuole of the macrophages and may be involved in suppression of the host inflammatory response (Fernandez-Prada et al. 2000). Transcription of *ipaH7.8* has already been shown to require both the transcriptional activator MxiE and the chaperone IpgC which acts as a coactivator for MxiE (Mavris et al. 2002). Four other *ipaH* family members (*ipaH 9.8*, *4.5*, *2.5*, and *1.4*) possessing the same E3 ubiquitin ligase activity but involved in different stages of infection are also found on the virulence plasmid (Hartman et al. 1990).

Since little is known about the regulation of *ipaJ*, *phoN1*, and *ipaH7.8* or the roles of the encoded proteins in *Shigella* virulence, this study examines the role CRP plays in the transcriptional repression of these three virulence plasmid-encoded genes.

3.2 Results and Discussion

3.2.1 The Promoter Activity of *phoN1* Is Adenylate Cyclase and CRP Dependent

To assess whether the three genes are dependent upon CRP for promoter activation, the promoter regions of *ipaJ*, *phoN1*, and *ipaH7.8* were transcriptionally fused to the *lacZ* gene and the resulting constructs (pCTH05, pCTH06, and pCTH07, respectively) were cloned into a parent *E. coli* strain (BW25113), an adenylate cyclase (*cyaA*) mutant (JW3778-3), and a *crp* mutant (JW5702-2). β -galactosidase assays were then conducted with exponential phase cultures to see if the promoters of either of the three genes is dependent upon CRP and the cofactor cAMP for transcriptional activation.

The *phoN1* transcriptional fusion exhibited strong promoter activity in the wild type background, yet this activity was greatly reduced when the construct was in a strain missing either CRP or the enzyme responsible for the production of the CRP cofactor (Fig. 8). This indicates that the promoter for the *phoN1* gene is dependent on both adenylate cyclase and CRP for retention of wild type promoter activity (Fig. 8). This suggests that *phoN1* may be transcriptionally regulated by catabolite repression through activation by CRP. Since glucose concentrations are reduced in the digestive tract as the bacterium moves towards the colon (Bodero and Munson 2009), the catabolite repression signaling mechanism may allow for additional phosphate scavenging by PhoN1 in a phosphate limited environment such as the colon. Alternatively, increased transcription of

phoN1 and resulting PhoN1 production could result in the increased concentration of phosphates which are known to increase adenylate cyclase activity (Lieberman et al. 1985). Increased adenylate cyclase activity would lead to more cAMP production resulting in more transcriptional activation leading to a positive feedback loop. More information is required about the cellular implications of increased PhoN1 activity before any conclusions can be drawn about the purpose for CRP-dependent regulation of the *phoN1* gene.

The *ipaJ* and *ipaH* constructs exhibited no significant promoter activity in the wild type strain or in any of the mutants. It should be remembered that these assays were conducted in an *E. coli* background which lack the *Shigella*-specific transcriptional regulator VirB. Since previous studies suggest *ipaJ* may be positively regulated by the virulence gene regulator VirB, future experiments should test the activity of the *ipaJ* promoter in a wild type *Shigella* strain that expresses VirB, or in an *E. coli* strain expressing VirB from an inducible plasmid. Once the appropriate conditions for *ipaJ* expression have been found, the CRP dependence of the promoter can then be tested using the *crp* and *cyaA* mutants under those conditions to measure the effect CRP plays in *ipaJ* expression. Furthermore, the *ipaJ* promoter encodes an exact match of the palindromic consensus sequence required for CRP binding, suggesting that CRP does play some role in *ipaJ* transcriptional regulation. Similar work is also needed before CRP dependence of the *ipaH7.8* gene can be tested. Unlike the *ipaJ* promoter, the predicted CRP binding site of the *ipaH7.8* promoter contains several base pair variations from the consensus sequence which may suggest this site is less than optimal for CRP binding. Currently, attempts to activate the *ipaH7.8* promoters through expression of

plasmid-based *mxIE* and *ipgC* genes originally similar to the methods used by Mavris *et al.* (Mavris *et al.* 2002) did not significantly enhance promoter activity and no difference was observed between the wild type strain and the mutant strains (data not shown) .

While further testing is required to truly understand the transcriptional regulation of all of the genes examined in this study, the CRP-dependent regulation of *phoN1* indicates that catabolite repression is involved in the transcriptional regulation of at least one gene encoded by the *S. flexneri* virulence plasmid, *phoN1*.

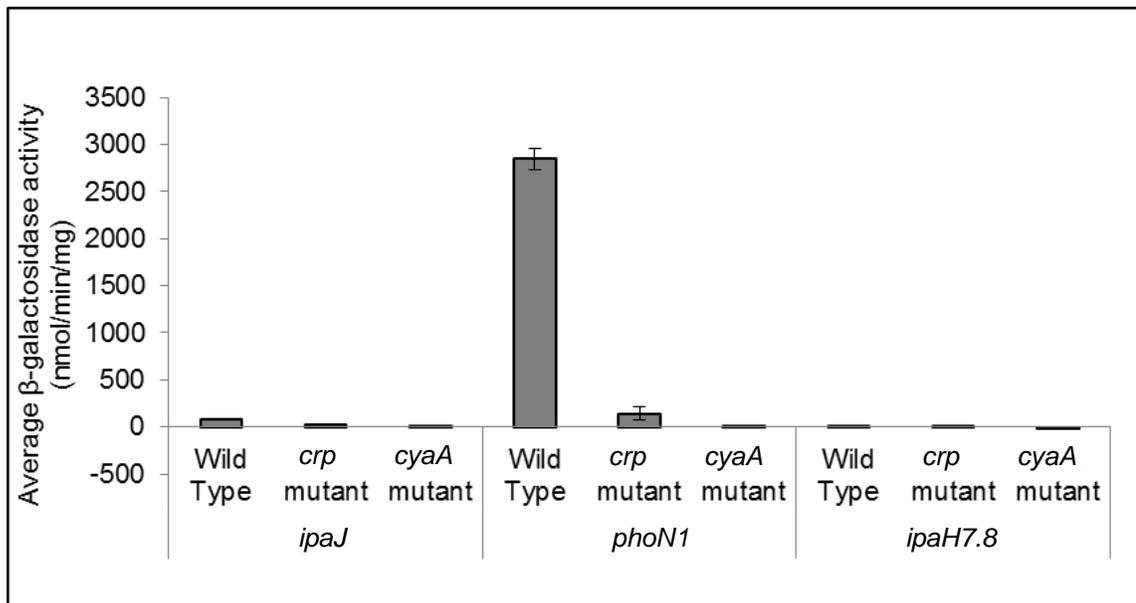


FIGURE 8. Activities of the *ipaJ*, *phoN1*, and *ipaH7.8* promoters in wild type *E. coli* (BW25113), the CRP mutant (JW5702-2), and the adenylate cyclase mutant (JW3778-3). Assays were run in triplicate and the means and standard deviations are shown.

3.3 Materials and Methods

3.3.1 Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in the present study are listed in Table 4. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) broth with aeration or on LB agar (LB broth containing 1.5% [wt/vol] agar). When required, chloramphenicol was added at a final concentration of 25 µg ml⁻¹.

3.3.2 Construction of the *PicsP-lacZ* Reporter Plasmids

The starting point for this work was the *PicsP-lacZ* reporter plasmid pHJW20 (described in (Castellanos et al. 2009); Table 1). All *PicsP-lacZ* reporter plasmids were constructed through PCR amplification of the gene promoter regions from a *S. flexneri* 2457T strain using oligonucleotide primers with *SalI* and *XbaI* restriction sites. The PCR fragments were digested with *SalI* and *XbaI*, and the resulting DNA fragments were ligated into pHJW20 previously digested with *SalI* and *XbaI*. To create pCTH05, the *ipaJ* promoter region was PCR amplified using oligonucleotides W195 (5'-TGAGGTCGACCTGCATATATCATTACTGC-3') and W196 (5'-TGAGTCTAGATTCTCTTGGTATAGCCC-3'). To create pCTH06, the *phoN1* promoter region was PCR amplified using oligonucleotides W197 (5'-TGAGGTCGACTCCGTAAACTCAGGCTACC-3') and W198 (5'-TGAGTCTAGATTCCCGGAGGGAATGATG-3'). To create pCTH05, the *ipaH7.8* promoter region was PCR amplified using oligonucleotides W199 (5'-TGAGGTCGACTGCATTCCAGTGATTCAGGATAT-3') and W200 (5'-TGAGTCTAGAGAAAGCACTGGGAGAGTC-3').

3.3.3 Quantification of *icsP* Promoter Activity Using the *PicsP-lacZ* Reporter and Derivatives

Activities of the examined promoters were determined by measuring β -galactosidase activity (as described previously (Castellanos et al. 2009) by using the Miller protocol (Miller 1972)) in strains carrying pCTH05, pCTH06, or pCTH07. Routinely, transcription was analyzed in three independent transformants in exponential phase cultures. Cells were routinely back-diluted 1:100 and grown for 2 h in LB, to ensure gene expression. Optical densities were measured using a DU 520 general purpose UV/Vis spectrophotometer (Beckman Coulter).

TABLE 4. Bacterial strains and plasmids

Strain or plasmid	Description ^a	Source or reference
Strains		
<i>E. coli</i>		
BW25113	$\Delta(\text{araD-araB})567$, $\Delta\text{lacZ}4787(::\text{rrnB-4})$, $\text{lacIp-4000}(\text{lacIQ})$, λ -, $\text{rpoS}396(\text{Am})$ (but made $\text{rpoS}+$), rph-1 , $\Delta(\text{rhaD-rhaB})568$, hsd514	(Baba et al. 2006, Datsenko and Wanner 2000)
JW5702-2	BW25113 $\text{crp}::\text{Kan}^r$ 100	(Baba et al. 2006)
JW3778-3	BW25114 $\text{cyaA}::\text{Kan}^r$ 751	(Baba et al. 2006)
Plasmids		
pHJW20	Cloning vector; pACYC derivative Cm^r	(Castellanos et al. 2009)
pCTH05	<i>PipaJ-lacZ</i> promoter fusion Cm^r	This work
pCTH06	<i>PphoN1-lacZ</i> promoter fusion Cm^r	This work
pCTH07	<i>PipaH7.8-lacZ</i> promoter fusion Cm^r	This work

^a Cm^r , chloramphenicol resistance; Kn^r , kanamycin resistance.

CHAPTER 4

DISCUSSION

The results of these investigations demonstrate that some *Shigella* virulence genes are transcriptionally regulated through more gene-specific mechanisms in addition to the seemingly *en bloc* regulation mediated by the *Shigella* virulence gene regulatory cascade, that classically involves VirF and VirB. In Chapter 2, I discuss my identification of a second *icsP* promoter, which allows for translation of an additional IcsP protein product. Although I was unable to identify conditions which would allow for differential regulation of the *icsP* gene, the mere presence of this complex regulatory structure suggests the two *icsP* promoters are poised to respond differently to some modulation in the intercellular environment. In Chapter 3, I present evidence of *phoNI* transcriptional regulation by CRP, which indicates a more precise and distinct pattern of gene expression does exist for at least one virulence gene. The regulation of *phoNI* by CRP also suggests the expression of some virulence plasmid-encoded genes may be influenced by environmental conditions such as carbon availability and not just by temperature, pH, and osmolarity. While the study of individual virulence genes, both by others and in support of this thesis, has provided insight into the specific transcriptional regulation of these genes, recent developments have allowed for a more global view of virulence gene expression. From these studies, we can identify particular patterns of gene expression and possibly identify any anomalies.

One recent example of a genome-wide study of virulence gene expression in *Shigella* was completed by Le Gall and others. This work utilized microarray analysis to simultaneously study the gene expression of 71 virulence plasmid-encoded genes (Le

Gall et al. 2005a). Their data suggest three general and distinct patterns of expression exist for virulence genes. The first expression pattern appears to be constitutive and is not dependent on temperature or VirF. The second pattern includes those genes which are known to be temperature dependent and rely on VirF-dependent regulation for gene transcription. Many of these genes, although not all, are actually regulated by VirF indirectly through the VirF-mediated production of VirB. The final subset of genes rely on VirF and VirB production, but are further regulated through the transcription factor, MxiE. What is striking from the results of this broad study of virulence gene expression by Le Gall, is the fact that the expression levels of similarly regulated genes are highly disparate suggesting other regulatory elements could be influencing expression rates of these genes. The genes examined in my work appear to fall into all three patterns of expression. Expression of *icsP* is clearly temperature dependent in the Le Gall study and shares a similar expression pattern with *ipaJ*. Maximal transcription of the *ipaH7.8* gene requires the transcription factor, MxiE. As mentioned previously, *phoN1* regulation appears to be independent of the VirF/VirB-dependent regulatory cascade even though the very similar *phoN2* gene does appear to be regulated via this regulatory cascade and most likely requires additional input from MxiE.

Production of enzymatically active proteins is the result of many regulatory steps throughout transcription and translation, which collectively determine protein expression levels. In Chapter 2, my data show that while initiation of *icsP* transcription, as measured by promoter activity, appears to be mostly dependent on the newly identified promoter (P2), the mRNA message, as measured by primer extension analysis, appears to be mostly from the originally annotated promoter (P1) (Fig. 3). This contradiction may be

due to the inherent bias of primer extension analysis in favor of the shorter transcript or it could be an indication that the IcsP transcripts may be differentially regulated post-transcriptionally possibly through increased degradation of the longer transcript. The idea of post-transcriptional regulation appears to also be supported by my western blot analysis. The western blot data indicates more IcsP is produced from the downstream translation start site and not the P2-specific upstream translation start site, although the difference is small (Fig. 6a). Consequently, the agreement between the primer extension analysis and the western blot analysis suggests that P2-generated transcripts are subject to negative regulation following initiation of transcription. It is possible that the longer leader sequence present on the P2-generated transcript contains sequence elements which may negatively influence mRNA half-life. The post-transcriptional modulation of virulence gene expression has been observed for the gene encoding the global transcription factor, VirB. The stability of VirB mRNA was shown to markedly decrease upon a reduction in temperature from 37 to 30 °C and was due to increased binding of the transcripts by the RNA-binding protein Hfq (Mitobe et al. 2009). Similar post-transcriptional modifications may be affecting the ultimate production of other virulence factors throughout all stages of *Shigella* pathogenesis.

Chapter 3 focused on the CRP-dependent activation of *phoNI* suggesting a link exists between carbon metabolism and virulence gene expression. Other evidence to support the importance of carbon metabolism in virulence gene expression includes the attenuation of virulence accompanied by the production of end products following lysine decarboxylation and the metabolism of glycerol 3-phosphate in *Shigella*. In both of these examples, the metabolic pathways and their end products contribute to an attenuation in

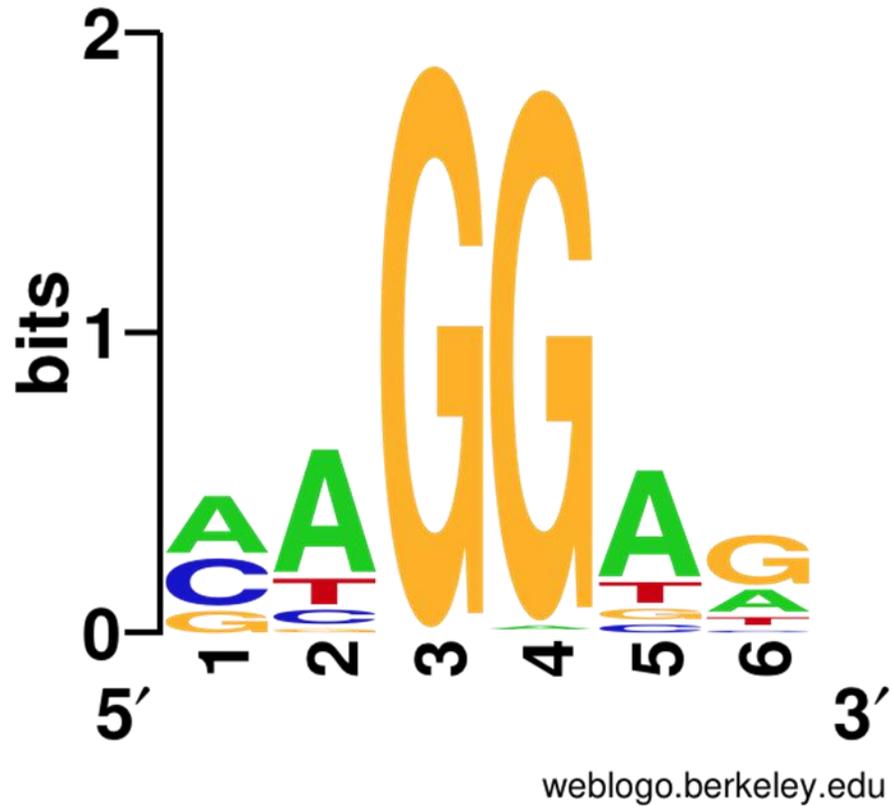
virulence, although the mechanism of this attenuation is not completely understood. Decarboxylation of lysine by lysine decarboxylase (LDC) leads to cadaverine production, which inhibits enterotoxic activity of *Shigella* following invasion (Maurelli et al. 1998). While over 90% of *E. coli* isolates express LDC, all *Shigella* strains share a large deletion, termed a “blackhole”, in the region containing the *cadA* gene which encodes LDC in *E. coli* (Maurelli et al. 1998). More recently, the presence of the virulence plasmid has been shown to decrease expression of genes within the *glp* regulon, which are important in degradation of glycerol 3-phosphate and its precursors (Zhu et al. 2010). These metabolic processes allow for utilization of glycerol and glycerol phosphates following breakdown of phospholipids. Another observation related to the seemingly antagonistic nature of some metabolic processes to pathogenesis, is that *Shigella* is a nicotinamide adenine dinucleotide (NAD) auxotroph due to gene alterations preventing the conversion of L-aspartate to quinolinate, a NAD precursor. Quinolinate was found to actually inhibit multiple stages of *Shigella* pathogenesis resulting in attenuation of virulence (Prunier et al. 2007). The fact that multiple independent origins of *Shigella* species all share similar phenotypes with respect to metabolism suggests that these metabolic processes are detrimental to the virulent lifestyle of this obligate pathogen (Escobar-Paramo et al. 2003). The CRP-dependent regulation of virulence genes may help uncover other metabolism/pathogenesis relationships or provide insight into the effects of metabolism on pathogenesis through studies of gene expression patterns.

Many of the variations noted in *Shigella* virulence gene expression levels may be due to the strength of promoter elements in attracting the RNA polymerase or the strength of VirB binding sites in recruiting the transcription factor. This simplified mechanism for

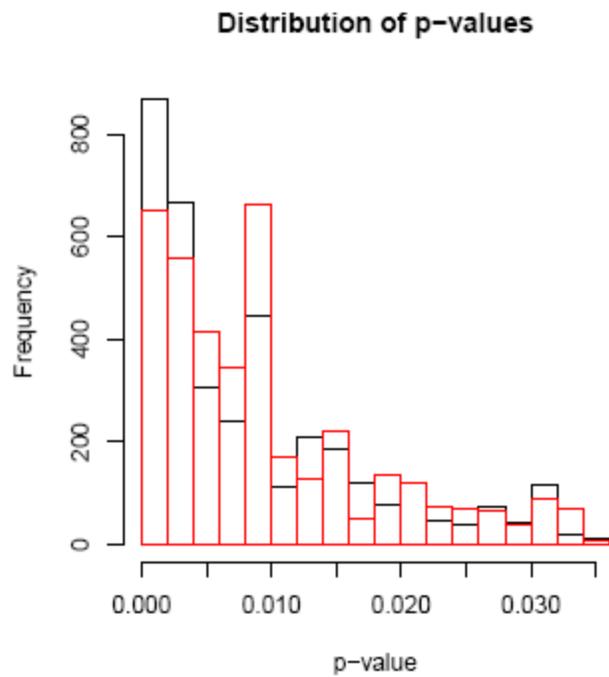
differentially regulating gene transcription could also include the use of alternative σ factors. While Chapter 2 suggests that, according to sequence analysis, alternative σ factors do not appear to be involved in differentially regulating *icsP*, alternative σ factors could provide a unique mechanism for differentially regulating genes with respect to the regulatory cascade since transcription of both the *virF* and *virB* gene are not dependent upon the most commonly used alternative σ factor, σ^S (Porter and Dorman 1997d).

This work raises the possibility that virulence gene regulation in *S. flexneri* is not just simply controlled through activation of a regulatory cascade. The observations reported in Chapter 2 suggest that some genes possess unique mechanisms for fine tuning their own expression through multiple promoters, as exhibited by the *icsP* gene, and that the expression of these genes may also be affected by post-transcriptional mechanisms. Also, the expression of some virulence genes may be independent of the regulatory cascade and may involve unique transcription factors as seen in, as exhibited by the CRP-dependent regulation of *phoNI* shown in Chapter 3. Ultimately, further study is required to truly understand the transcriptional regulation of virulence genes and how virulence gene expression is modulated throughout different phases of pathogenesis in the obligate pathogen, *S. flexneri*. A better understanding of virulence gene expression and how it relates to Shigellosis in the host is the key to identifying a possible molecular weakness of the pathogen, which may lead to new treatments or preventative techniques.

APPENDIX 1
SUPPLEMENTARY DATA



SUPPLEMENTARY FIGURE 1. Position-specific scoring matrix for ribosome binding sites associated with the *S. flexneri* 2a strain 301 virulence plasmid pCP301 (NC_004851.1) and chromosome (NC_004337.1). Six nucleotide-long motif overrepresented in immediate upstream regions of annotated start codons identified by MEME (<http://meme.sdsc.edu>) and used to search for Shine-Dalgarno sequences upstream of predicted additional start codons. Data collected by Olga Kamneva, University of Wyoming.



SUPPLEMENTARY FIGURE 2. Distribution of p-values for predicted ribosome binding sites with respect to both the annotated translation start sites (black) and the predicted additional translation start sites (red). Data collected by Olga Kamneva, University of Wyoming.

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