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DOMAIN 8 PATHOGENESIS

Virulence Gene Regulation in *Shigella*

CHARLES J. DORMAN
Department of Microbiology, Moyne Institute of Preventive Medicine, University of Dublin, Trinity College, Dublin 2, Ireland

ABSTRACT *Shigella* species are the causative agents of bacillary dysentery in humans, an invasive disease in which the bacteria enter the cells of the epithelial layer of the large intestine, causing extensive tissue damage and inflammation. They rely on a plasmid-encoded type III secretion system (TTSS) to cause disease; this system and its regulation have been investigated intensively at the molecular level for decades. The lessons learned have not only deepened our knowledge of *Shigella* biology but also informed in important ways our understanding of the mechanisms used by other pathogenic bacteria to cause disease and to control virulence gene expression. In addition, the *Shigella* story has played a central role in the development of our appreciation of the contribution of horizontal DNA transfer to pathogen evolution. A 30-kilobase-pair "Entry Region" of the 230-kb virulence plasmid lies at the heart of the *Shigella* pathogenesis system. Here are located the *virB* and *mxiE* regulatory genes and most of the structural genes involved in the expression of the TTSS and its effector proteins. Expression of the virulence genes occurs in response to an array of environmental signals, including temperature, osmolarity, and pH. At the top of the regulatory hierarchy and lying on the plasmid outside the Entry Region is *virF*, encoding an AraC-like transcription factor. Virulence gene expression is also controlled by chromosomal genes, such as those encoding the nucleoid-associated proteins H-NS, IHF, and Fis, the two-component regulators OmpR/EnvZ and CpxR/CpxA, the anaerobic regulator Fnr, the iron-responsive regulator Fur, and the topoisomerases of the cell that modulate DNA supercoiling. Small regulatory RNAs, the RNA chaperone Hfq, and translational modulation also affect the expression of the virulence phenotype transcriptionally and/or posttranscriptionally.

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Correspondence: Charles J. Dorman: cjdorman@tcd.ie

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INTRODUCTION

An outline sketch of the *Shigella* pathogenic system is given here to place in context the description of the regulatory processes that follows. Readers are referred to the cited research papers and review articles for a more detailed treatment of the cell biology of *Shigella* infection.

Shigellosis is a disease of humans that is caused by the four *Shigella* spp.: *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*. It is a particular problem in developing countries and children are especially at risk (1). The bacteria are highly infectious, with only 10 to 100 microbial cells being required to initiate

the disease (2). Transmission is via the oral route and infection occurs in the colon where the *Shigella* first invade and then spread between the cells of the epithelium (3). The bacteria are unable to invade the colonic epithelial cells through the apical surface (4). Instead they exploit the antigen-sampling M cells to gain access to their basolateral surfaces (5, 6). Macrophage located beneath the epithelial layer engulf the bacteria but the microbes in turn escape the endocytic vacuole (7) and induce the macrophage to initiate programmed cell death. The trigger is IpaB, a secreted *Shigella* virulence protein that binds and activates mammalian caspase-1 (8, 9). Cytokine release by the dying macrophage recruits polymorphonuclear cells and these begin to destabilize the epithelium (10, 11, 12, 13). This facilitates further bacterial access to the basolateral surfaces of the epithelial cells. The infected epithelia in turn release further cytokines, recruiting more host-defense cells and enhancing the inflammation (14, 15). Symptoms associated with shigellosis are fever, abdominal cramp, and a characteristic bloody diarrhea (16).

At the core of the virulence machinery is the Mxi-Spa type III secretion system and its associated effector proteins (17, 18, 19, 20, 21, 22). Four Ipa proteins, IpaA, B, C, and D together with IpgD are required for host cell invasion (23, 24, 25, 26, 27, 28, 29, 30). These are produced and stored in the bacterium and then released through the Mxi-Spa system after contact with host cells (31, 32, 33). The IpgC cytoplasmic chaperone serves to stabilize IpaB and IpaC (32), and the IpgE cytoplasmic chaperone stabilizes IpgD (26). A complex composed of IpaB and IpaD regulates the rate of release (25). IpaB and IpaC combine once outside the bacterium and form a pore in the host cell membrane through which other *Shigella* proteins can enter the cytoplasm (34). IpaA and IpaC induce host cytoskeletal rearrangements that cause the bacterium to be engulfed (35, 36). The internalized bacterium then escapes from its vacuole by IpaB-mediated lysis of the vacuole membrane (37). The microbes acquire mobility by recruiting and polymerizing host actin through a process that depends on the IcsA (VirG) protein (38, 39, 40, 41). This is a bacterial outer membrane protein that is also encoded by the virulence plasmid (42, 43, 44). IcsA is localized at the old pole of the bacterium, and actin assembly there confers directionality on the movement of the bacterium. This mobility not only permits the bacterium to traverse the cell it has just entered but also to penetrate into adjoining cells (42, 45). This spreads the infection and associated tissue damage through the epithelium. The activity of IcsA is modulated by the outer membrane protease, IcsP (46). This virulence plasmid-encoded protein cleaves IcsA to release the alpha domain, containing the actin assembly region, from the beta domain that is embedded in the bacterial cell surface. This results in inactivation of IcsA (46, 47, 48, 49, 50).

THE PLASMID-ENCODED VIRULENCE SYSTEM

The principal virulence genes are located on a high-molecular-weight plasmid (51, 52, 53) where they are found within a 31-kb segment known as the entry region (54, 55, 56, 57, 58, 59) (Fig. 1). There are 37 open reading frames in this region extending from *ipaJ* to *orf131b* and most are arranged in divergently transcribed operons (Fig. 1). The genes all have a similar G + C content (almost all in the range 30 to 35% compared with approximately 50% for the *Shigella* chromosome) and the entry region is flanked by truncated insertion sequences. These features are consistent with a common origin, probably outside the *Enterobacteriaceae* (54, 59). The transcription start sites and potential promoters of the entry region operons have been mapped. Promoters are located upstream of the *virB* regulatory gene (60, 61, 62, 63), the *icsB* gene (60, 64, 65), the *ipgD* gene (29, 60, 64), the *spa47* gene (60, 21), and the *ipaJ* gene (66).

Other genes contributing to the virulent phenotype lie elsewhere on the plasmid (Fig. 1). The *virF* regulatory gene is located 60 kb away from the entry region (54, 59). Scattered around the plasmid are five alleles of the *ipaH* locus. These contain constant and variable domains at the 3′ and 5′ regions, respectively, and code for proteins that are secreted when the bacterium is in an intracellular niche (67, 68, 69, 70, 71). The alleles are distinguished from one another by numbers (*ipaH2.5*, *ipaH7.8*, etc.) that reflect the size in base pairs of the *Hin*dIII restriction fragments that carry each gene. Also dispersed on the plasmid are the *osp* genes coding for other secreted proteins. These genes have a G + C content similar to the entry region and this may indicate a common origin. Similarly, the 5′ variable region of the *ipaH* genes (but not the 3′ constant portion) resembles the entry region and *osp* genes in G + C content (54). The *icsP* (or *sopA*) gene codes for an outer membrane protease that cleaves the IcsA (or VirG) protein (46, 50). IcsA is the actin tail assembly protein that is required for motility of the bacteria in infected cells (42, 43, 44, 47, 72, 73, 74). Its gene is located outside the entry region and has a higher G + C content than the majority of the virulence genes on

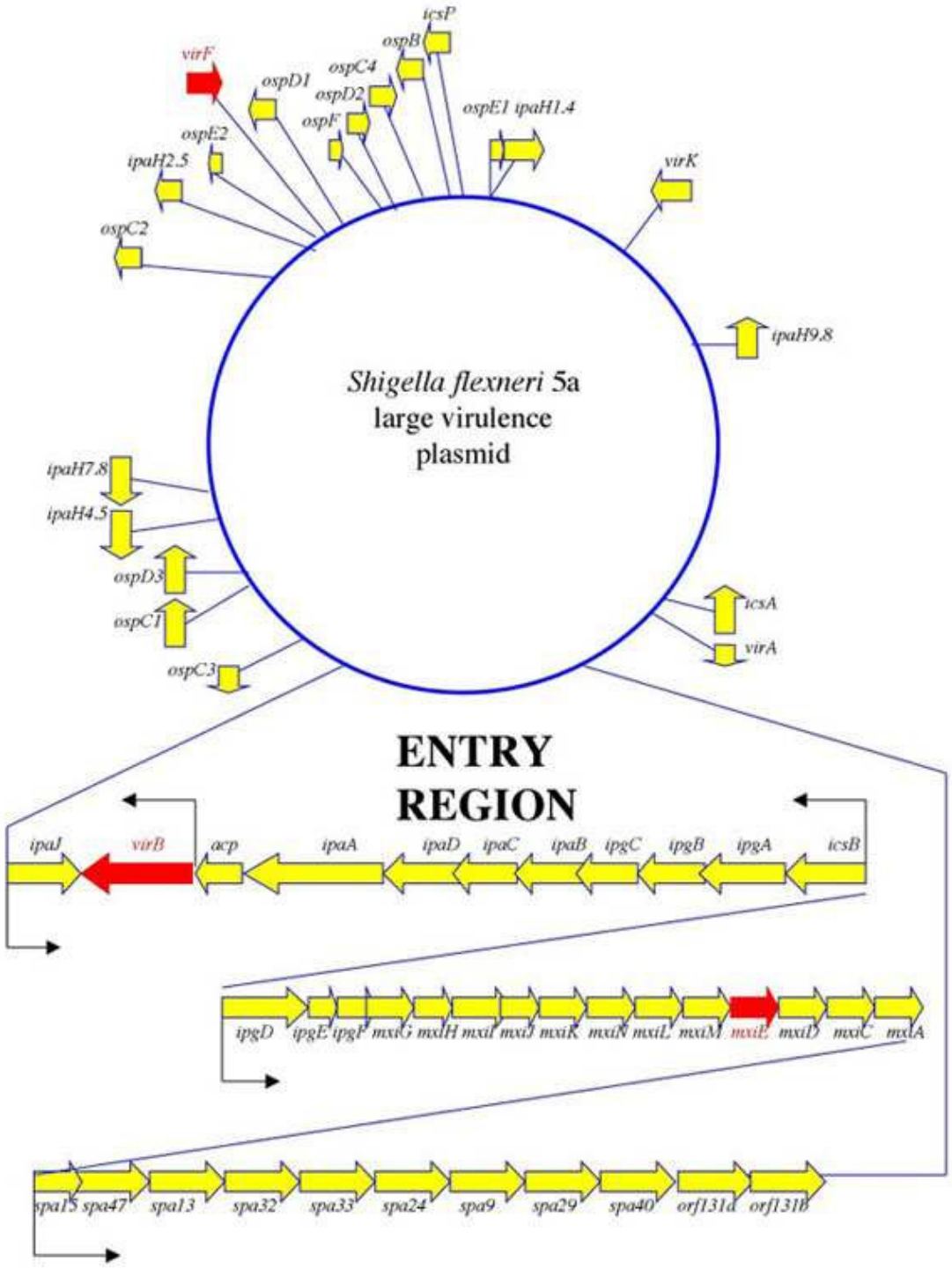

Figure 1 Genetic map of the *S. flexneri* large virulence plasmid showing the locations and direction of transcription of the major virulence genes. The entry region is shown in an expanded form below the circular map. Structural genes are yellow, and regulatory genes are red. Within the entry region, the angled arrows show the locations and orientations of major transcriptional promoters. The diagram is not drawn to scale.

the plasmid (54, 59). The *virK* gene has been described as contributing to the correct expression and localization of IcsA (75). This gene lies within a region of the plasmid that is not adjacent to *icsA*. Upstream of *icsA* and transcribed from the opposite DNA strand is the *virA* gene (54, 59) coding for a protein that is secreted via the type III secretion system (76). VirA promotes efficient internalization of *Shigella* in host cells through a mechanism that involves destabilization of microtubules with associated membrane ruffling (77, 78).

THE REGULATORY CASCADE

Virulence gene activation occurs in response to environmental signals. Optimal expression of VirF- and VirB-dependent genes under laboratory growth conditions requires a temperature of 37°C, moderate osmolarity, and pH 7.4 (79, 80, 81, 61). Presumably, these conditions signal to the bacterium that it has passed the acidic environment of the stomach and arrived in the gut of the host. A regulatory cascade involving the products of the *virF* and *virB* genes plays a central role in controlling the transcription of the virulence genes (82, 83, 84, 85) (Fig. 2). Each gene codes for a positive regulator. VirF activates the *virB* promoter and the VirB protein activates transcription of the structural genes and operons under its control. VirF activates the *icsA* (*virG*) structural gene promoter directly, without acting through VirB (86). VirB-dependent promoters are located at several sites on the virulence plasmid. In the entry region it binds to the promoters of the divergently oriented *icsB* and *ipgD* genes and at the *spa15* gene (60, 65, 87). Elsewhere on the plasmid, it binds to the *virA* promoter (87) and activates the promoter of the *icsP* gene (88).

Northern blotting data indicate that a gearing effect exists within the cascade. As one descends level by level, the stringency of transcriptional regulation is tightened. At the top of the cascade, expression of *virF* gene transcription is least tightly controlled. Expression of *virB* mRNA shows an intermediate level of regulation in response to inducing growth conditions, while the structural genes show the most stringent control (89). This is consistent with a system that is primed to respond to the correct set of inducing conditions but is configured to avoid wasteful expression of genes in an inappropriate environment.

Within the VirB-dependent operons is another regulatory gene, *mxiE*, which codes for another positive activator. The MxiE protein activates transcription of a set of genes located at dispersed sites on the virulence plasmid, outside the entry region. These genes code for secreted proteins that leave the bacterium via the Mxi-Spa type III secretion system. The MxiE protein requires a cofactor protein, IpgC, which is only available when protein secretion is underway (67, 68, 69, 90) (Fig. 2). The VirF, VirB, and MxiE regulators are discussed in more detail below.

The VirF Regulatory Protein

At the top of the transcription regulatory cascade is the VirF protein (Fig. 2). This is a member of the AraC family of DNA-binding proteins (91, 92). The prototypic member of the family is AraC, which controls transcription of the *ara* genes involved in the uptake and utilization of arabinose (93, 94, 95). VirF resembles AraC in having a carboxyl-terminal domain with two helix-turn-helix (HTH) motifs for sequence-specific DNA recognition and binding, and a linker that connects the functionally distinct domains (91, 96) (Fig. 3). All AraC family members, except CelD which is a repressor, are transcription activators and can be divided into three classes (97, 98). The first class contains proteins that regulate transcription on binding a chemical signal, usually a carbohydrate. AraC is itself a member of this class. The second class lacks the amino-terminal domain and act as monomers. These do not bind carbohydrates and include the stress-response proteins MarA and SoxS (98). VirF belongs to the third group, which is made up of proteins that regulate transcription in response to a physical signal, usually temperature. Many are involved in virulence gene regulation and show homology throughout their length. The temperature-responsive proteins do not bind ligands. Data from *trans*-dominance tests indicate that VirF is a dimeric protein (91). To some extent, the members of this subgroup are interchangeable in regulating the systems under their command (92, 99, 100, 101). For example, Rns, a regulator of adhesin gene expression in enterotoxigenic *E. coli* can substitute for VirF in controlling *S. flexneri* virulence genes, but not vice versa (101).

Expression of the *virF* gene is controlled both at the level of transcription and posttranscriptionally (Fig. 2). The nucleoid-associated protein H-NS (discussed below) represses *virF* promoter activity, whereas the FIS protein activates it (102, 103, 104, 105, 106). In vitro and in vivo footprinting studies have identified two binding sites for

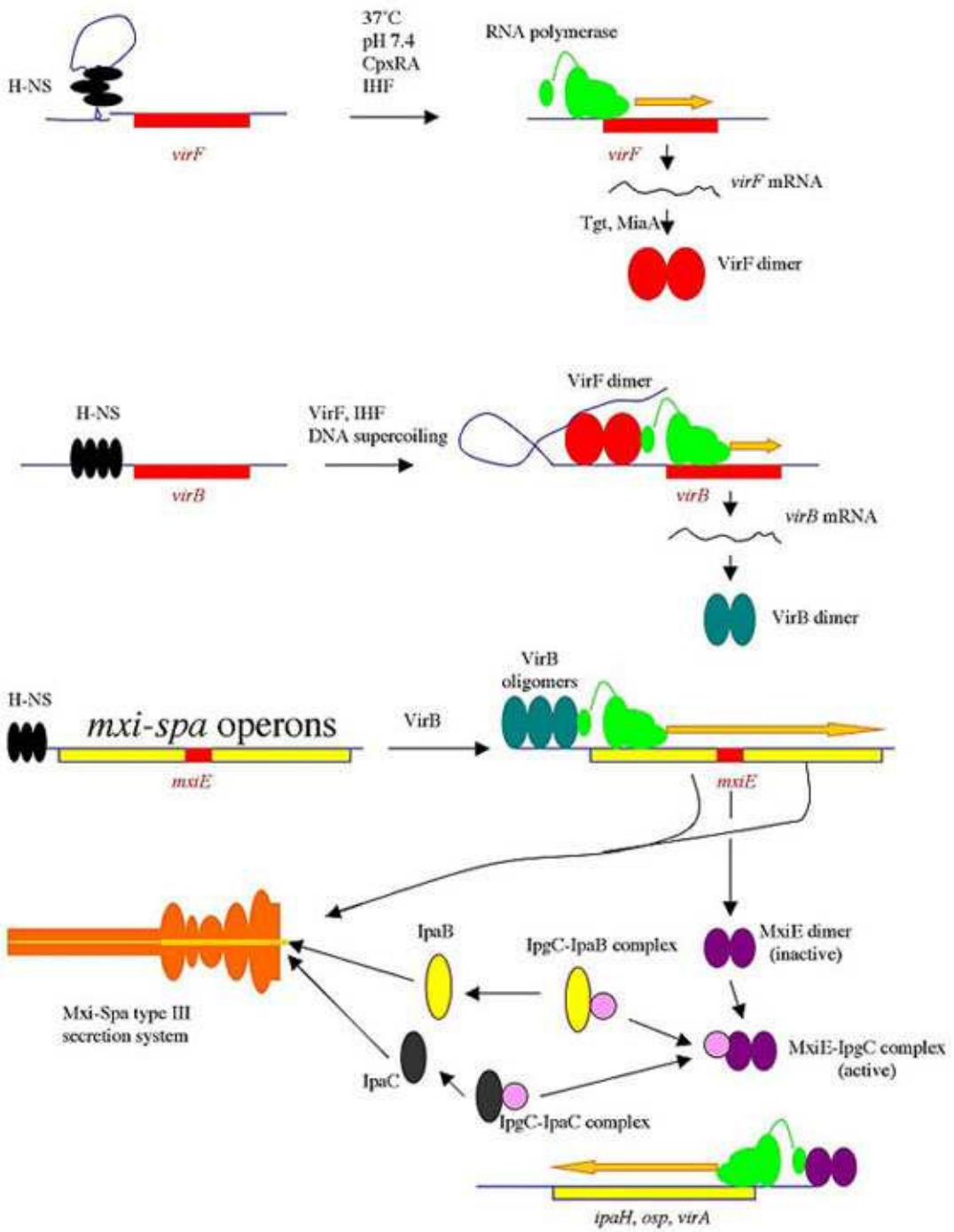

Figure 2 Virulence gene regulatory cascade of *S. flexneri*. Regulatory genes are red, and the structural genes are yellow. Colored spheres or ovals represent proteins. The horizontal red/gold arrows indicate transcriptional activity. The diagram is not drawn to scale.

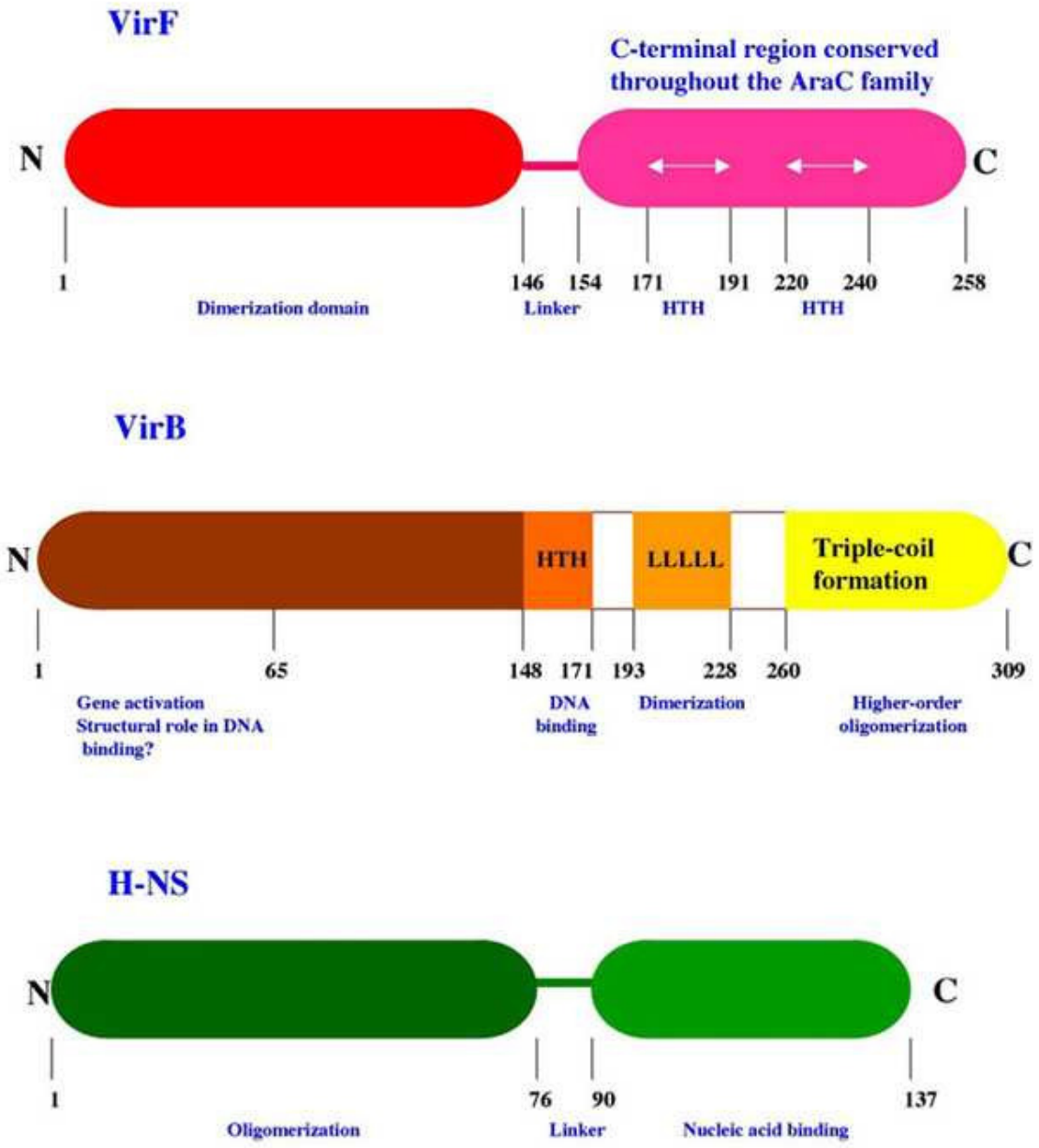

Figure 3 Summaries of the main structural features of the VirF, VirB, and H-NS regulatory proteins. The diagrams represent the domain structures of the positive transcription regulators VirF and VirB and the repressor H-NS. The numbers shown below each protein indicate amino acids at the amino-terminal and carboxyl-terminal boundaries of structural features; the amino-terminal methionine is at position 1 in each case. Abbreviations: HTH, helix-turn-helix DNA-binding motif; LLLLL, a 5-heptad leucine zipper in which every seventh amino acid is L-leucine.

H-NS, one overlapping the transcription start site and another centered upstream at position –250. The region between the sites displays intrinsic curvature, and this is thought to contribute to interaction between them. Repression involves the formation of a nucleoprotein complex in which H-NS proteins bound at these two sites are thought to act cooperatively. The complex is temperature sensitive. It forms below 32°C but is disrupted at 37°C, the permissive temperature for virulence gene expression. The DNA template senses the change in temperature, and the resulting change in DNA topology abrogates formation of the repression complex (102). This appears to involve the movement of the center of the bend down the DNA such that a binding site for the transcription activator FIS is abruptly revealed (107). Consistent with the importance of local DNA architecture at the *virF* promoter, the DNA-binding and -bending protein IHF is required for optimal expression of the *virF* gene (64).

The level of the VirF protein in the cell is influenced by the *miaA* and *tgt* loci, which contribute to tRNA modification (108). The product of the *miaA* gene is required for the production of 2-methylthio-N^6-isopentenyladenosine (ms^2I^6A37) found adjacent to and on the 3′ side of the anticodon in tRNA-reading codons beginning with U, with the exception of the serine tRNAs encoded by *serI* and *serV* (109). The *tgt* gene is needed for the biosynthesis of quenosine (or Q) at the wobble position (position 34) (110). In *Shigella,* the *tgt* gene is also known as *vacC* (111). Lack of Q34 reduces the virulence of *S. flexneri* to 50% of that of the wild type when cells are grown in a rich medium (111). The negative effect is enhanced a further fivefold when the bacteria are grown in minimal medium (112). This effect can be offset by the addition of putrescine or a combination of methionine and arginine to the medium, perhaps by influencing translational efficiency (112). These influences on virulence gene expression act through the *virF* gene at the level of translation with production of *virF* mRNA being unaffected (108, 112). The effect seems to be specific for VirF. When this protein is overexpressed in a *tgt* mutant full virulence is restored, suggesting that the translational defect targets *virF* mRNA and not any other mRNA in the virulence regulon (108). A sensitivity to tRNA modification opens the virulence regulon to influences beyond temperature, osmolarity, and pH. It could allow the system to sense iron availability, oxygen levels, and the presence and absence of certain amino acids and polyamines (84, 112, 108).

The interaction of the VirF protein with the *virB* promoter has been investigated. DNA protection assays with a MalE‘-’VirF fusion protein have identified a binding site at the *virB* promoter spanning the region –17 to –105 with reference to the transcription start site, +1 (62). This footprint intrudes somewhat into that of the region protected from DNase I digestion by H-NS, a sequence extending from –20 to +20 (62). These binding sites are consistent with the antagonistic roles of VirF (activator) and H-NS (repressor) in controlling *virB* expression. However, removal of H-NS from the cell will not permit transcription of *virB* in the absence of VirF. This shows that VirF does not act simply to antagonize the negative influence of H-NS; it is also an essential activator of the VirB promoter (62). Moreover, activation of the *virB* promoter by VirF is direct and can be driven by a MalE‘-’VirF fusion polypeptide in vitro (62).

The *virB* promoter depends on negative supercoiling for activity. Relaxed DNA templates are not transcribed in vitro or in vivo (62). The provision of a suitably supercoiled template allows the *virB* promoter to be activated even at a normally nonpermissive temperature provided VirF protein is present (63). These experiments show that even at low temperature, there is sufficient VirF in the cell to permit *virB* activation. What is lacking at the nonpermissive temperature is an appropriate DNA structure at the promoter. An attractive model that is supported by the experimental data is one in which the change to the promoter structure is brought about by the effect of temperature on DNA supercoiling. The ability of temperature to influence DNA topology in bacteria is well documented (113, 114, 115, 116, 117, 118, 119, 120). The role of altered DNA structure may be to facilitate the formation of an active configuration at the promoter involving VirF and RNA polymerase. It is also possible that the formation of this structure is accompanied by the disruption at *virB* of the H-NS-DNA nucleoprotein repression complex. In agreement with this model is an observation that *virB* loses its sensitivity to temperature and DNA supercoiling in a *rho* mutant background (121). The *rho* gene codes for a protein factor (Rho) required for transcription termination at certain terminators and mutations mapping to *rho* are known to alter the ability of *Escherichia coli* to regulate DNA supercoiling normally (122, 123). Additional evidence pointing to a role for DNA supercoiling comes from data showing that mutations in genes coding for topoisomerases result in a shift in the profile of *S. flexneri* virulence gene expression (124, 125). Furthermore, the *virB* promoter is positively regulated by

IHF (64), an architectural protein that organizes local DNA structure by introducing bends of up to 180° (126).

The VirB (InvE) Regulatory Protein

The VirB protein (called InvE in the *S. sonnei* literature) has no homology to previously described transcription activators. Small (35.4 kDa) and basic, the VirB protein closely resembles the ParB/SopB plasmid partition proteins required for stable maintenance of low-copy-number plasmids such as F and P1/P7 (127, 128, 129, 130, 131). The similarity is greatest in the amino-terminal two-thirds and includes a helix-turn-helix (HTH) motif that is also found in ParB (87) (Fig. 3).

VirB binds to a sequence in the promoter region of the *ipa* operon that resembles the BoxA-binding site of ParB. The BoxA-like sequence is centered at position −80 with respect to the transcription start site. Matches to this sequence can be found in the regulatory regions of other genes under VirB control (65). The VirB protein has been shown to bind to these promoter regions in vivo by the chromatin immunoprecipitation assay (87). It has been shown that a GST-InvE (VirB) fusion protein can bind to the *icsB* promoter, provided the ParB BoxA-like sequence is present (65). (The role of the IcsB protein in virulence is unknown, although it has been shown to be secreted via the Mxi-Spa type III system, chaperoned by IpgA [132].)

The ability to form oligomers is critical for VirB function. The protein forms dimers using a leucine-zipper motif located between amino acids 193 and 228 (Fig. 3). Deletion of this motif or the substitution of key residues within it results in a loss of oligomerization and gene activation (87, 133). The carboxyl terminus of the protein also contributes to oligomerization. It has been suggested that initial VirB-VirB protein-protein interactions are promoted by the leucine zipper, allowing dimers to form, and that higher-order oligomers are formed via interactions involving triple-coiled structures at the carboxyl terminus (87).

The purified protein will bind to target promoters in vitro. It rapidly oligomerizes on the DNA, forming a large complex that cannot be resolved by gel electrophoresis (133). This oligomerization activity is essential for its biological function. How this facilitates VirB in its role as an activator of transcription remains to be determined. Similar behavior has been described for the plasmid partition proteins, which VirB resembles (134, 135, 136).

A critical factor in VirB-mediated transcription activation is the level of the protein in the cell. If VirB is overexpressed at 30°C, the genes of the virulence regulon are transcribed despite growth at a temperature that is normally nonpermissive for expression (60). This shows that VirB does not need to undergo a temperature-dependent modification or to interact with a cofactor that is only available at higher temperatures. It simply needs to reach a threshold concentration to activate the genes under its control.

VirB acts as an antirepressor, and the repressor it opposes is the H-NS protein (see next section). At a minimum, activation of transcription by VirB involves the removal of this repressor. However, it may also involve remodeling of the promoter region to aid in the recruitment of RNA polymerase and/or help the polymerase to form an open transcription complex.

H-NS, a Repressor of Virulence Gene Expression

The gene coding for H-NS in *S. flexneri* was originally designated *virR* and a transposon Tn*10* knockout mutation in *virR* was found to derepress virulence gene expression at 30°C (137). The *virR* gene was shown subsequently to be allelic with the pleiotropic *osmZ* locus of *Escherichia coli,* and knockout mutations there were accompanied by alterations in DNA topology (138). Subsequent analysis revealed that both genes were identical with *hns* (139, 140). DNase I protection studies have shown that H-NS binds to sites that overlap the *icsB* and *virA* promoters, a situation that is reminiscent of H-NS-binding sites at the *virB* and *virF* regulatory genes (60). Significantly, those H-NS-repressible virulence gene promoters in *Shigella* that have been examined have been found to encompass regions of intrinsic curvature, a prerequisite for H-NS binding to DNA (60, 103).

The H-NS nucleoid-associated protein represses the promoters of the *virF* and *virB* regulatory genes, as described above. In addition, H-NS represses each of the promoters that is activated by VirB (60) (Fig. 2). H-NS is a small, basic DNA-binding protein of approximately 15 kDa that is present in up to 20,000 copies per genome equivalent (141, 142, 143, 144). It binds to regions of DNA with intrinsic curvature, and these are frequently associated with promoters (145, 146, 147, 148). The H-NS protein can condense DNA and can constrain supercoils in vitro (149) and in vivo (150). It has a wide-ranging influence on bacterial gene expression, and in

most cases this influence is negative (151). The protein is composed of an amino-terminal domain that contributes to oligomerization, a carboxyl-terminal domain that is required for nucleic acid binding, and a flexible linker sequence that connects these two domains (152, 153, 154, 155) (Fig. 3). The linker may contribute to higher-order protein-protein interactions that are important for long-range transactions between separate DNA sequences to which H-NS has bound (153). This description applies to the H-NS-binding sites at the *virF* promoter (103, 106).

H-NS Paralogues

The StpA protein is a paralogue of H-NS and is found in several enteric bacteria, including *Shigella*. It is 52% identical with H-NS in amino acid sequence and has a similar domain structure (156). StpA can form heteromeric complexes with H-NS and the proteins cross-regulate negatively each other's genes (157, 158). Knockout mutations in *stpA* have no effect on virulence gene expression in *Shigella*, although overexpression of the protein causes repression, as does overexpression of H-NS (159, 129). This indicates that StpA has the potential to affect expression of the virulence phenotype and may do so under certain (as yet undetermined) circumstances.

The widely studied *S. flexneri* serotype 2a strain 2457T expresses yet another paralogue of H-NS, in addition to StpA (159, 160). This protein, Sfh, is 61.9% identical with StpA and is expressed from a gene located on a cryptic IncHI1 plasmid related to the R27 episome of *Salmonella enterica* serovar Typhimurium (160, 161, 162). Sfh represses the *hns* and *stpA* genes and their products, in turn, repress *sfh*. In addition, Sfh forms homomers and can form heteromers with StpA and H-NS. Overexpression of Sfh results in repression of the virulence genes and Sfh (like StpA) can bind to the promoters of the *virF* and *virB* genes. When a knockout mutation in *sfh* is combined with one in *hns* the result is an enhancement of the transcriptional derepression of virulence genes normally associated with *hns* lesions (160). This shows that the R27-like plasmid-encoded Sfh protein can repress virulence gene expression, at least in the absence of H-NS.

MxiE and Type III Secretion

The *mxiE* gene is located within the *mxi* operon in the entry region of the virulence plasmid (Fig. 1). Its product is predicted to be a transcription regulator of the AraC-like protein family (97). A knockout mutation in *mxiE* renders *S. flexneri* avirulent, although expression and secretion of invasion proteins is unaffected (67). The MxiE protein plays an important role in controlling the expression of a subset of the virulence genes in response to protein secretion. Transcription of these genes is induced when the bacteria are treated with Congo red or when the *ipaD* gene is inactivated, both of which are known to be treatments that enhance secretion through the Mxi-Spa type III secretion system (90). Initially, these genes were identified as *virA* and four *ipaH* genes (90). Subsequently, 11 genes on the virulence plasmid and at least one *ipaH* paralogue on the chromosome were found to be coregulated in this way (69). The plasmid-linked genes lie outside the entry region and include the secreted protein genes *ipaH4.5*, *ipaH7.8*, *ipaH9.8*, *ospB*, *ospC1*, *ospE2*, *ospF*, and *virA* (67, 69) (Fig. 1). The promoter of one of them, *virA,* is also bound by VirB (65, 87). The significance of this dual control is unknown at present.

MxiE requires the IpgC protein as a cofactor for gene activation (68). IpgC is a chaperone that associates independently with IpaB and IpaC (32), but in its free form it represents a signal for MxiE-dependent activation of secreted protein genes (68). Promoters activated in this way possess a consensus sequence known as an MxiE box (69). This sequence, 5′-GTATCGTTTTTTTAnAG-3′, is located between positions –33 and –49 with respect to the transcription start sites or presumed transcription start sites of eight promoters. In this location the box overlaps the –35 box of the promoter and it is presumed that this promotes interaction between MxiE and RNA polymerase at the promoters (69).

Two-Component Systems and Virulence Gene Expression

The OmpR-EnvZ two-component regulatory system is required for full virulence in *Shigella* (163). The system consists of a cytoplasmic membrane-located histidine protein kinase (EnvZ) and a cytosolic DNA-binding protein (OmpR) that is phosphorylated by EnvZ at high osmolarity (164). A transposon insertion mutation in the *envZ* gene coding for the cytoplasmic membrane-located EnvZ histidine protein kinase impairs the ability of *S. flexneri* to invade HeLa cells. It also results in a loss of ability to form plaques on a confluent lawn of HeLa cells and a delayed and mild keratoconjunctivitis in a Sereny test. A deletion mutant from which the *ompR* and *envZ* genes have been removed has a similar phenotype. Work with *lacZ* reporter fusions to virulence genes on the plasmid suggests a role for EnvZ-OmpR in regulation of

the virulence cascade (163). However, the details have not been worked out.

The *ompC* gene coding for a major outer membrane protein in several gram-negative bacteria is expressed constitutively in *S. flexneri*, in contrast to *E. coli* where it is induced at high osmolarity and repressed under low-osmolarity conditions (165). In both species OmpC depends on EnvZ-OmpR for high-level expression. An *ompC* deletion mutant in *S. flexneri* has a virulence phenotype that is similar to that of a mutant deficient in both OmpR and EnvZ. Each mutant is impaired in its ability to spread from cell to cell and to kill epithelial cells (165). These data suggest that at least part of the effect of OmpR and EnvZ on virulence is exerted via the OmpC porin.

In *Salmonella* the two-component system composed of the PhoQ sensor kinase and the PhoP DNA-binding protein plays a well-established role in adaptation to conditions within the phagosome (166). Despite the fact that *Shigella* escapes from this compartment in both epithelial and macrophage cells, a knockout mutation in the *phoP* gene results in attenuation of *Shigella* virulence (167). The *phoP* mutant remains competent for invasion, intracellular growth, cell-to-cell spreading, and induction of macrophage apoptosis; it retains full acid resistance. However, the mutant displays enhanced sensitivity to host-defense peptides and polymorphonuclear leukocytes. The genes subject to PhoP regulation that contribute to these sensitivities are unknown, but the finding suggests that *Shigella* experiences environmental signals similar to those encountered by *Salmonella* during at least some stages of the infection process.

The CpxR-CpxA two-component system, encoded by the chromosomal genes *cpxRA*, mediates the response of the *virF* promoter to changes in pH (80, 81). CpxA shows homology to the family of histidine protein kinases while CpxR has homology to the response regulator DNA-binding proteins. Mutations in either *cpxR* or *cpxA* abolish pH regulation of *virF* transcription in *S. sonnei* and presumably have an identical effect in *S. flexneri*. The CpxR protein binds between positions –37 and –103 with respect to the *virF* transcription start site. This DNA binding is enhanced in vitro when the CpxR protein is phosphorylated. It has been suggested that under acid conditions, CpxA may act as a phosphatase to dephosphorylate CpxR, preventing it from binding to the *virF* promoter (81).

Miscellaneous Regulatory Factors

The *dsbA* gene plays an important role in virulence-factor expression. It codes for a disulfide oxidoreductase activity that is needed for the release of plasmid-encoded Ipa proteins. Specifically, the Spa32 protein contains two cysteine residues and it becomes misfolded under reducing conditions in the absence of DsbA activity. The Spa32 protein is a determinant of needle length in the secretion apparatus (168). Derivatives of Spa32 in which the Cys residues are substituted accumulate in the periplasm and this results in a reduced capacity on the part of the Mxi-Spa type III secretion system to secrete Ipa proteins (169). A *dsbA::kan* mutant of *S. flexneri* showed reduced virulence but was found to invade HeLa cells and to display a capacity for intra- and intercellular spreading. However, the mutants failed to divide normally in the host cells and lysed within the double-membrane-enclosed vacuoles that arose from actin-mediated cell-to-cell spreading (170). Thus, the *dsbA* effect apparently may be more far reaching than the Spa32 data alone suggest.

It has been reported that quorum sensing via the *luxS* locus modulates expression of virB (171). *S. flexneri* produces the autoinducer AI-2 and its levels peak in the late-log phase of growth, with a correlation being reported with *virB* expression. However, mutants deficient in AI-2 production express their virulence genes normally and are fully virulent, at least under the conditions tested. Nevertheless, this represents an interesting aspect of *Shigella* physiology and it is hoped that further work can shed more light on its significance.

The impact of genetic rearrangements on virulence gene regulation has been documented. The virulence plasmids of *S. flexneri* and enteroinvasive *E. coli* can integrate at a specific site on the bacterial chromosome, leading to strong down-regulation of virulence-gene expression (172, 173). Integration occurs at *metB*, a gene involved in methionine biosynthesis, and results in methionine auxotrophy. Excision of the plasmid, which is RecA dependent, restores prototrophy (173). Loss of virulence-gene expression in the integrated strains correlates with a loss of virulence. Expression of the *virF* gene is normal but *virB* is repressed. Provision of *virB* in trans restores virulence-gene expression, as does inactivation of the *hns* repressor gene (172). The data suggest that in the integrated state the *virB* promoter is rendered inoperative, possibly because of its adoption of a DNA topology that is unfavorable for transcription, and that H-NS

collaborates in this silencing of *virB*. The mechanism is unknown but most likely involves the formation of an H-NS-DNA nucleoprotein complex at the *virB* promoter. Another study has reported a correlation between stability of the virulence plasmid and inactivation of the *virF* or *virB* regulatory genes (174). Genetic instability is manifest in terms of plasmid curing or rearrangement (both RecA dependent and independent, including transposition of IS*1* into *virF*) and is most pronounced when the bacteria are grown under laboratory conditions that activate the virulence genes (174, 175). Given the genetic fragility of the plasmid when the virulence genes are active, integration with the chromosome with concomitant silencing of the virulence genes might provide a means of preserving plasmid integrity during growth away from the host.

Sequencing data point to the existence of a gene (S0103) on the virulence plasmid with the potential to express an AraC-like protein with homology to the HilC and PerA virulence regulators of *Salmonella* and enteropathogenic *E. coli*, respectively (59). It would thus appear that there may be further regulatory dimensions to be explored.

Regulation of Chromosomal Virulence Genes

Although it is clear that several key virulence factors are encoded by structural genes located on the chromosome (54, 56, 59, 176), studies of their regulation generally lag behind those of the genes on the large virulence plasmid. The *pic*/*set* virulence genes provide an example of a chromosomal gene regulation story where some molecular details are emerging. These genes are located within a pathogenicity island called SHI-1 (177) or SHE-1 (178). The *pic* gene codes for the 109-kDa Pic mucinase, an autotransported serine protease that degrades intestinal mucin. On the complementary DNA strand and completely encompassed by *pic* are the *setAB* genes. These encode the *Shigella* enterotoxin 1 (ShET-1) comprising one 20-kDa catalytic A subunit and five 7-kDa B subunits, the products of the *setA* and *setB* genes, respectively (177).

The *pic* and *setAB* genes are transcribed as complementary mRNA species. The main *pic* promoter is most active at 37°C in the exponential phase of growth. The *setB* promoter is located far upstream, perhaps 1.5 kb away, resulting in a long untranslated leader sequence. The DNA coding for the leader has a strong silencing effect on *setB* transcription. The *setA* gene is located 3′ to *setB* and may have its own promoter and/or may utilize the *setB* promoter. Induction of *set* gene expression requires continuous anaerobic culture in a simulated human intestinal ecosystem. Regulatory factors that interact with the transcription silencer have not been identified, but genetic studies have ruled out roles for the Fis, H-NS, or StpA proteins (177). Intriguingly, the silencer only works in its native orientation. Inversion of the sequence leads to loss of silencing activity. It can also silence the *pic* gene promoter in an orientation-dependent manner. Full activity of the ShET-1 toxin is contingent on the absence from *S. flexneri* of a region of chromosomal DNA that includes the *cadA* gene coding for lysine decarboxylase, an enzyme required for cadaverine production (179, 180). This deletion, known as a "black hole," prevents production of cadaverine, an inhibitor of ShET-1 enterotoxin activity, from lysine by decarboxylation. *Shigella* species have come under strong selective pressure to lose the *cadA* locus, presumably to enhance the potency of their pathogenic interactions with the host (179). It should be noted that a gene (*shet2*) coding for a distinct enterotoxin, ShET-2 (also called Sen or OspD3), is located on the virulence plasmid (54, 181) (Fig. 1) together with a gene that has been identified by sequence analysis that may encode a third enterotoxin, ShET2-2 (59). Details of the regulation of these genes are lacking at present.

A second pathogenicity island, SHI-2, located at the *selC* locus harbors genes that code for the aerobactin iron-uptake system, colicin V immunity, and the inflammation-attenuating ShiA protein in *S. flexneri* (182, 183, 184). The Fur repressor protein is known to regulate aerobactin and colicin V genes in response to iron starvation (185); this is a key environmental signal in *Shigella* pathogenicity (107, 186). Fur and iron starvation are also key transcriptional regulators of the gene coding for Shiga toxin in *S. dysenteriae* (187). However, the Fur-iron mechanism is clearly distinct from those governing expression of the virulence regulon on the large virulence plasmid.

REGULATORY OVERVIEW

The *Shigella* virulence system is subject to complex control and this is presumed to reflect the need to restrict its expression to appropriate niches within the host. Building in regulatory checkpoints enhances the likelihood that the virulence genes will be expressed only

in the correct ecological context. The *virB* promoter represents a critical point for the "go" or "no-go" decision process. Its dependence on the VirF regulatory protein is absolute, yet the promoter will not function even in the presence of VirF if its DNA structure is not adjusted correctly. This adjustment occurs in response to environmental cues characteristic of the niche in which expression of the virulence system is required. Provision of the VirF protein is contingent on the cell receiving the right environmental signals, not least those required to ensure that *virF* mRNA is translated efficiently into protein. In this way further checkpoints are imposed upstream of those acting directly at the *virB* promoter. Once VirB protein appears in the cell, successful activation of the genes it controls seems to depend on its concentration. In addition to the many structural genes that VirB activates, there is the regulatory gene encoding the MxiE transcription factor. With its cofactor protein IpgC, MxiE is responsible for transcriptional activation of its own regulon of virulence genes coding for secreted proteins. Since the *ipgC* and *mxiE* genes depend on VirB for activation, this regulon can only be expressed after VirB reaches its threshold concentration in the cell. However, there is a further checkpoint that concerns protein secretion itself. The IpgC protein is unavailable to MxiE before type III secretion has been triggered. This is because IpgC is in separate complexes with the IpaB and IpaC proteins. Only when these two are secreted is IpgC free to interact with MxiE to facilitate gene activation.

The foregoing is a brief summary of the main players involved in virulence gene regulation in the Shigella system. They are organized within a strict hierarchy with VirF at the apex, VirB next, and the MxiE-IpgC regulatory proteins below that. All these control factors are encoded on the virulence plasmid. The low G + C content of their genes indicates a common origin, and this origin is shared by most of the structural genes they control. The virulence plasmid presents something of a mosaic structure today, making difficult molecular archeology aimed at elucidating its evolution (54, 59, 188, 189). This is equally true of the evolution of the regulatory circuits that govern expression of the virulence genes. A key conundrum concerns the VirB protein, which seems almost certainly to have been derived from a plasmid partition factor. At some point it was recruited as a transcription regulator of major operons within the *Shigella* system. How was regulation achieved in the pre-VirB era? Did VirF simply control everything in a much less sophisticated circuit? It is certainly conceivable that a circuit governed by VirF alone would still provide many of the features we see in the modern system. However, the absence of the *virB* checkpoint might make this circuit more prone to inappropriate activation, resulting in significant competitive disadvantages for the bacterium.

The *Shigella* plasmid-borne regulatory system operates against a background of transcriptional repression imposed by the *hns* gene on the chromosome. Removal of the H-NS protein from the cell allows the virulence genes to be expressed even in the absence of the appropriate environmental signals. This shows that the positive regulatory features encoded on the plasmid exist to some extent to oppose repression imposed by H-NS. This repressive activity is not confined to one or two key promoters but is found at every virulence-gene promoter where its influence has been investigated, including those of the transcription activator genes. H-NS possesses features that make it particularly good at being a comprehensive repressor of transcription. These include the lack of a consensus DNA sequence for recognition and binding and a preference for a DNA structural feature (curvature) that is frequently associated with promoters. Consequently, many genes require an opponent of H-NS (i.e., an activator) if they are to be expressed. This generalized ability to repress transcription is likely to be useful in regulating newly imported genes such as those acquired by horizontal transfer. It is interesting to observe that the *S. flexneri* virulence genes, which are regarded as having been imported horizontally, are subject to wholesale repression by this protein and must bring to bear their own specific activators to overcome its negative influence. On the other hand, one must be mindful that the large virulence plasmid in *Shigella* cannot be regarded as newly arrived (190, 191), and it is to be assumed that ample time has elapsed for its genes to become embedded in the preexisting regulatory arrangements of the cell. Studies addressing the molecular negotiations that take place between existing or ancestral regulatory circuits and horizontally transferred newcomers are likely to shed light on the evolution of modern integrated gene control networks.

Many of the regulatory features described in this chapter are not unique to *Shigella*. As virulence gene regulatory circuits become better understood in this and other pathogens it is hoped that the details they reveal will provide new and better opportunities for intervention,

leading to more effective management and prevention of disease.

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