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Genetic and Environmental Control of *Salmonella* Invasion

Craig Altier

College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA

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An early step in the pathogenesis of non-typhoidal *Salmonella* species is the ability to penetrate the intestinal epithelial monolayer. This process of cell invasion requires the production and transport of secreted effector proteins by a type III secretion apparatus encoded in *Salmonella* pathogenicity island I (SPI-1). The control of invasion involves a number of genetic regulators and environmental stimuli in complex relationships. SPI-1 itself encodes several transcriptional regulators (HilA, HilD, HilC, and InvF) with overlapping sets of target genes. These regulators are, in turn, controlled by both positive and regulators outside SPI-1, including the two-component regulators BarA/SirA and PhoP/Q, and the csr post-transcriptional control system. Additionally, several environmental conditions are known to regulate invasion, including pH, osmolarity, oxygen tension, bile, Mg^{2+} concentration, and short chain fatty acids. This review will discuss the current understanding of invasion control, with emphasis on the interaction of environmental factors with genetic regulators that leads to productive infection.

Key words: *Salmonella* pathogenicity island 1, type III secretion, virulence, review

Infections with non-typhoidal *Salmonella* are a common cause of food-borne illness worldwide. The number of cases each year is difficult to estimate, since in many instances the disease caused by these organisms is relatively mild and self-limiting. Yet, in susceptible individuals, such as the very young, the elderly, and those with suppressed immunity, the more common gastrointestinal disease can progress to a life-threatening septicemia. Also important to the spread of salmonellosis is the ubiquitous nature of the organism. It can infect a wide variety of animal species used for food, and can also thrive on various plant products, making human infection common.

An early step on the pathogenesis of non-typhoidal *Salmonella* is the ability to penetrate the intestinal epithelium. Whether salmonellosis is confined to the intestinal form or progresses to systemic involvement, the ability of the organism to invade and penetrate intestinal epithelial cells is required. This invasion process is not merely a passive consequence of bacterial contact with epithelial cells, but instead requires the active participation of the bacterium, with the expression of numerous bacterial virulence genes. The expression of these genes is regulated by an array of transcriptional and post-transcriptional regulators that exert intricate control over invasion. In addition, a number of environmental conditions known to exist in the mammalian intestinal tract also induce invasion (Table 1). The goal of this review will be to examine control of Salmonella invasion, in an attempt to integrate what is currently known about its genetic and environmental controls.

* To whom correspondence should be addressed.
(Tel) 1-919-513-6274; (Fax) 1-919-513-6464
(E-mail) craig_altier@ncsu.edu

Invasion is encoded by Salmonella Pathogenicity Island I

Located at centisome 63 of the *Salmonella* serovar Typhimurium chromosome is an island of genes termed *Salmonella* Pathogenicity Island I (SPI-1). It encodes the structural components and secreted effector proteins of a type III secretion apparatus. Proteins encoded by SPI-1 assemble to form a "needle complex", a multi-protein structure that spans the inner and outer bacterial membranes (Kubori *et al.*, 1998). Other proteins within the island are secreted and are able to alter the cytoskeletal structure of eukaryotic cells. It is thought that, when bacteria are in close contact with the epithelial cells of the intestinal tract, the needle complex delivers these secreted proteins to the epithelial cell cytoplasm. Once within cells, the secreted proteins alter the cytoskeletal structure, inducing the rearrangement of actin filaments around the associated bacteria. This rearrangement then leads to engulfment of the bacteria, which remain within vacuoles (reviewed by Galán, 2001). Bacteria are able to survive in these cells and can reach deeper tissues, where they induce a potent inflammatory response. Intestinal salmonellosis ends here, with the organism confined to the regional lymphoid tissue of the intestinal tract. In cases of *Salmonella* septicemia, however, bacteria proliferate in the lymphoid tissue and are spread via blood and lym-

Table 1. Regulators and environmental conditions that control *Salmonella* invasion

Invasion Regulator	Genes or proteins proposed to be affected[a]	Effect on Invasion	References
HilA	*prg*, *sip*, and *inv/spa* operons	+	Bajaj *et al.*, 1995; Darwin and Miller, 1999; Eichelberg and Galán, 1999; Lostroh *et al.*, 2000; Lostroh and Lee, 2001
HilC	*hilA*, *hilC*, *hilD*	+	Johnston *et al.*, 1996; Eichelberg *et al.*, 1999; Schechter *et al.*, 1999
HilD	*hilA*, *hilC*, *hilD*	+	Schechter *et al.*, 1999
InvF	*sip* operon, *sopB*	+	Kaniga *et al.*, 1994; Darwin and Miller, 1999
BarA/SirA	*csrB*, *csrC*, *hilA*, *hilC*	+	Johnston *et al.*, 1996; Altier *et al.*, 2000b; Lawhon, *et al.*, 2002; Teplitsky *et al.*, 2003
CsrA	*rtsA* and SPI-1 genes	+/–	Altier *et al.*, 2000a
CsrBC	CsrA	+	Altier *et al.*, 2000a
RtsA	*dbsA*, *hilA*, *hilC*, *hilD*, *slrP*	+	Ellermeier and Slauch, 2003
FliZ	*hilA*	+	Lucas *et al.*, 2000; Iyoda *et al.*, 2001
FadD	*hilA*	+	Lucas *et al.*, 2000
Hu	*hilA*	+	Schechter *et al.*, 2003
Fis	*hilA*	+	Wilson *et al.*, 2001; Schechter *et al.*, 2003
CpxA	*hilA*	+	Nakayama *et al.*, 2003
H-NS	*hilA*	–	Schechter *et al.*, 2003
Hha	*hilA*	–	Fahlen *et al.*, 2001
PNPase	SPI-1 genes	–	Clements *et al.*, 2002
PhoP/PhoQ	*hilA*	–	Behlau and Miller, 1993; Bajaj *et al.*, 1996
Lon	*hilA*	–	Takaya *et al.*, 2002; Takaya *et al.*, 2003; Boddicker and Jones, 2004
HilE	HilD, *hilA*	–	Baxter *et al.*, 2003
Environmental Condition			
Low oxygen tension	*hilA*, *orgA*	+	Jones and Falkow, 1994; Bajaj *et al.*, 1996
High osmolarity	*hilA*	+	Galán and Curtiss, 1990; Bajaj *et al.*, 1996
Neutral pH	*hilA*	+	Bajaj *et al.*, 1996
Acetate	SirA	+	Durant *et al.*, 2000; Lawhon *et al.*, 2002
Propionate/Butyrate	Unknown	–	Lawhon *et al.*, 2002
Cationic peptides	PhoP/PhoQ	–	Bader *et al.*, 2003
Bile	BarA/SirA	–	Prouty and Gunn, 2000
Signaling Molecule			
ppGpp	Unknown	+	Pizarro-Cerda and Tedin, 2004; Song *et al.*, 2004
Np_nN	Unknown	–	Ismail *et al.*, 2003

[a]For regulators, such as *hilA*, additional downstream genes may be regulated but not listed.

phatic channels to the major organs. Thus, the process of invasion is required for either form of salmonellosis.

Control of invasion by regulators in SPI-1

In addition to the structural components and effector proteins encoded by the type III secretion system of SPI-1, several regulators are also present (Fig. 1). All of these are transcriptional regulators that activate (rather than repress) invasion, and they comprise a complex regulatory circuit that controls genes both within and outside the island. Central to this control is HilA. It is a member of the ToxR/OmpR family that activates the *sip* operon (also known as the *ssp* operon), which encodes secreted proteins, and the *inv/spa* and *prg* operons, encoding components of the secretion apparatus (Bajaj *et al.*, 1995; Darwin and Miller, 1999; Eichelberg and Galán, 1999; Lostroh *et al.*, 2000; Lostroh and Lee, 2001). In addition to its direct role in the regulation of SPI-1 genes, HilA is also an activator of a second transcriptional regulator, InvF. This regulator is of the AraC family and induces the expression of the secreted proteins of the *sip* operon (Kaniga *et al.*, 1994; Darwin and Miller, 1999). Thus, HilA

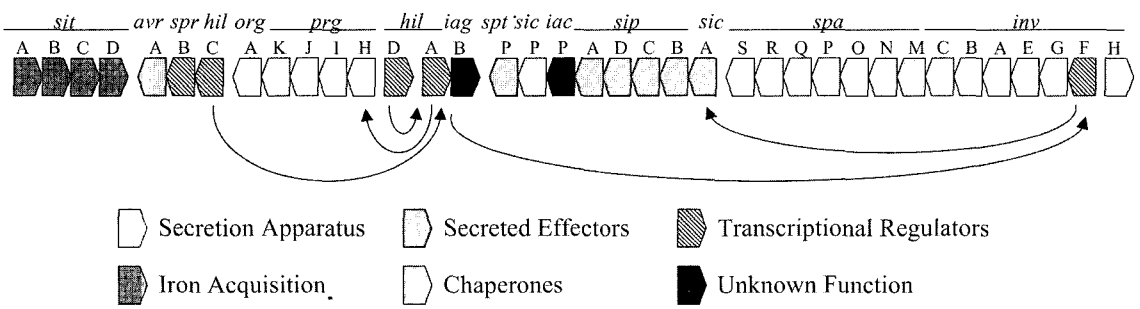

Fig. 1. Regulation of invasion gene expression by transcriptional activators within SPI-1. HilA activates the *prg*, *inv/spa*, and *sip* operons, as well as the regulator *invF*. InvF, in turn, activates the *sip* operon. HilD and HilC bind to the upstream untranslated region of *hilA*, activating it.

directly regulates the expression of secreted proteins and the secretion machinery and also indirectly controls expression of secreted proteins through its induction of *invF*. InvF, however, has functions independent of HilA as well. It controls expression of at least one secreted effector protein, SopB, that is encoded outside SPI-1 (Eichelberg and Galán, 1999). Therefore, the two regulators HilA and InvF have overlapping, but not identical sets of target genes. Why would such a complex method of control exist? One possibility is that it allows for the proper sequential expression of invasion genes in response to multiple environmental and genetic signals. As will be discussed below, the regulators of SPI-1 are themselves controlled by regulators found outside the island. In some cases, InvF is subject to the control of these regulators independent of their control of HilA (Rakeman *et al.*, 1999; Altier *et al.*, 2000a; Altier *et al.*, 2000b). It is therefore possible that the environmental cues that stimulate invasion invoke a regulatory cascade that induces elements of the type III secretion system with the proper timing to produce maximal invasion. Similarly, control by HilA and InvF could serve to amplify production of specific proteins that might be needed in larger quantities (such as secreted effectors), while maintaining a lower expression of other type III secretion system components.

Although HilA is a central regulator of invasion within SPI-1, the expression of *hilA* is itself controlled by two additional SPI-1 regulators: HilC (alternatively known as SirC and SprA) and HilD (Johnston *et al.*, 1996; Eichelberg *et al.*, 1999; Schechter *et al.*, 1999). Both function by binding to a region upstream of the *hilA* promoter that is required for both environmental and genetic control of *hilA*, thus activating *hilA* expression (Lucas and Lee, 2001; Schechter and Lee, 2001; Boddicker *et al.*, 2003; Olekhnovich and Kadner, 2004). HilD is clearly important for invasion, as a *hilD* mutant fails to invade epithelial cells and to express *hilA* (Schechter *et al.*, 1999). The role of *hilC* is less clear, with a *hilC* mutant producing little invasion defect, but inducing SPI-1 genes when overexpressed (Eichelberg *et al.*, 1999; Schechter *et al.*, 1999). In addition to directly binding to the upstream untranslated region of *hilA*, each of these two regulators also binds to the promoter region of both its own gene and that of the other (Olekhnovich and Kadner, 2004), suggesting a complex regulatory feed-back circuit. The importance of these control measures to invasion, however, remains unknown.

Induction of invasion by regulators outside SPI-1

In its distant past, *Salmonella* presumably acquired SPI-1 through horizontal gene transfer as an intact group of genes, including the regulators described above. Over its evolution, however, the invasion genes of SPI-1 have come under the control of a number of both positive and negative regulators that existed in the organism prior to the acquisition of the pathogenicity island. Some of these are global regulators that also serve to control functions other than invasion, demonstrating that the control of invasion genes has been incorporated into existing regulatory pathways. Presumably, coordinate regulation has evolved in these cases as a means to stimulate invasion in response to regulatory cascades activated when the organism reaches the site productive for invasion, the intestinal tract of an animal host.

One regulator outside SPI-1 known to control invasion is the two-component regulator BarA/SirA. BarA is a sensor kinase of the phospho-relay type, and SirA is its cognate response regulator. The loss of either of this pair greatly reduces invasion (Johnston *et al.*, 1996; Altier *et al.*, 2000b). BarA has been shown to regulate the expression of *hilA*, and to regulate other genes of SPI-1 in a HilA-independent fashion (Johnston *et al.*, 1996; Altier *et al.*, 2000b). Recently, it has become clear that at least some of the control of invasion by BarA/SirA is due to its control of a second regulatory system. The *csr* system consists of a small protein, CsrA, that acts post-transcriptionally. First identified in *E. coli* as a regulator of carbon degradation and storage pathways, it binds to target messages in the region of the ribosome binding site and alters their expression (reviewed by Romeo, 1998). Depending upon the target, CsrA can either stabilize the message, acting as a positive regulator, or conversely can reduce message half-life, thus reducing gene expression (Liu *et al.*, 1995; Wei *et al.*, 2001). In *Salmonella*, *csrA* is

required for SPI-1 gene expression, but also reduces expression of invasion genes when *csrA* is itself overexpressed, suggesting that the level of this protein must be tightly controlled *in vivo* to achieve optimal invasion (Altier *et al.*, 2000a). In addition to CsrA, the *csr* regulatory system consists of two untranslated RNA molecules, CsrB and CsrC. Each of these has a predicted structure that consists of several stem-loops with the sequence of the loop being similar to that of a ribosome-binding site (Liu *et al.*, 1997; Altier *et al.*, 2000a; Weilbacher *et al.*, 2003). In *E. coli*, it has been shown that CsrA can bind to both CsrB and CsrC (Liu *et al.*, 1997; Weilbacher *et al.*, 2003). In *Salmonella*, genetic experiments show that both CsrB and CsrC oppose the action of CsrA and that both are required for full expression of SPI-1 genes (Altier *et al.*, 2000a and published results). It is therefore speculated that the binding of CsrA to either of these RNA molecules reduces the concentration of CsrA available to bind its targets, thus reducing its activity. The *csr* system is a global regulator in *Salmonella*, as it is in *E. coli*, but the functions controlled by this system are different in the two organisms. In *Salmonella*, invasion regulation is an important function and one that does not exist in *E. coli*. In addition, *csr* regulates the degradation of ethanolamine and propanediol and the acquisition of maltose and maltodextrin, all of which would likely be required in the mammalian intestinal tract, at the site of *Salmonella* invasion (Lawhon *et al.*, 2003).

The BarA/SirA two-component regulator controls invasion, in part, by activating the expression of both CsrB and CsrC. A mutant of either *barA* or *sirA* poorly expresses these functional RNA molecules (Lawhon *et al.*, 2002 and unpublished results; Teplitski *et al.*, 2003). Thus, it is likely that invasion is inhibited by CsrA and that BarA/SirA activates invasion by inducing CsrB and CsrC, which bind CsrA, reducing the concentration of active protein and allowing SPI-1 gene expression. The control of SPI-1 genes is not, however, limited to regulation of the *csr* system. In addition, phosphorylated SirA has been shown to bind to the DNA of *hilA* and *hilC* (Teplitski *et al.*, 2003), suggesting that BarA/SirA directly, as well as indirectly, controls SPI-1 genes. Such a complex regulatory cascade provides multiple layers of control that allow both stringent regulation and rapid changes in gene expression. In addition to the transcriptional control of BarA/SirA, post-transcriptional control by the *csr* system could allow the rapid response to changing environmental signals by altering the stability of existing messages. Despite our understanding that CsrA controls SPI-1 gene expression, it is not currently known which gene or genes in the invasion pathway are the direct message targets of its action. Also unclear is whether CsrA targets genes encoded within SPI-1 or whether it directly controls regulators outside the island that, in turn, control SPI-1 genes.

Several other regulatory proteins outside SPI-1 also induce invasion genes. A newly identified gene, *rtsA*, has been shown to induce SPI-1 genes (Ellermeier and Slauch, 2003). RtsA, along with the SPI-1 regulators HilC and HilD, induces *dbsA*, which encodes a periplasmic disulfide bond isomerase that is required for the activity of the SPI-1 type III secretion system (Ellermeier and Slauch, 2004). Regulators of flagella production also regulate SPI-1. FliZ induces *hilA* (Lucas *et al.*, 2000; Iyoda *et al.*, 2001) and does so independently of BarA/SirA (Lucas *et al.*, 2000). The sensor kinase CpxA acts to induce *hilA* only at low pH, but its cognate response regulator CpxR is apparently not required for this function (Nakayama *et al.*, 2003). Two nucleoid-binding proteins, HU and Fis, are also positive regulators of SPI-1, presumably through interaction with *hilA* (Wilson *et al.*, 2001; Schechter *et al.*, 2003).

Recently, it has been shown that invasion can be controlled by small signaling molecules. The alarmone ppGpp has long been known to mediate the stringent response under conditions of amino acid starvation. It is produced by the activity of two genes, *relA* and *spoT*. Expression of this molecule reduces RNA synthesis as a means of energy conservation. ppGpp is known now to be required for invasion in *Salmonella*, as a mutant of *relA* and *spoT* fails to express invasion genes and is avirulent in mice (Pizarro-Cerda and Tedin, 2004). The effects of this molecule appear to be at the level of *hilA* induction (Song *et al.*, 2004). Such a mechanism of virulence control is not confined to *Salmonella*. In the pathogenic bacterium *Legionella pneumonophila*, ppGpp also induces virulence. The alarmome is proposed to function by relieving the repression of virulence functions caused by CsrA (reviewed by Molofsky and Swanson, 2004). It is presently unclear, however, whether this signal is used similarly by *Salmonella*.

Environmental conditions stimulate invasion

Control of invasion genes presumably leads to expression of the SPI-1 type III secretion apparatus at the point of infection most productive for virulence. The preferred site of *Salmonella* invasion is the distal small intestine, the ileum, where it associates with the M cells that overlay the gut-associated lymphoid tissue (Jones *et al.*, 1994). Therefore, it is likely that *Salmonella* has adapted to sense the local environment and to invoke the regulatory cascades described above when it reaches the ileum. Indeed, several environmental conditions likely to be found in the intestine promote invasion gene expression. The lumen of the intestine is anaerobic, while the brush border of the small intestine is considered microaerophilic. Oxygen tension has been shown to be a key regulator of invasion gene expression, with SPI-1 genes being maximally expressed through HilA under low oxygen conditions (Jones and Falkow, 1994; Bajaj *et al.*, 1996; Russell *et al.*,

2004). Osmolarity also presents a probable signal for invasion. The osmolarity of the small intestine is greater than 300 mOsm (Fordtran and Ingelfinger, 1968). This high osmolarity induces *hilA* expression and also causes changes in DNA supercoiling that affect invasion gene transcription (Galán and Curtiss, 1990; Bajaj *et al.*, 1996). Finally, the near neutral pH of the small intestine can provide a signal for invasion, as it too induces *hilA* expression (Bajaj *et al.*, 1996).

Despite the fact that these environmental cues for invasion have been identified, little is known about the way in which such signals are received and interpreted by the genetic regulators of invasion. In only a few instances has a potential environmental stimulus been associated with a bacterial regulatory system. One such example is the induction of invasion genes by acetate. Acetate is a short chain fatty acid produced by the anaerobes of the large intestine. Its concentration raises through the intestinal tract, with a concentration of approximately 15-30 mM in the distal ileum, the primary site of *Salmonella* invasion, and with much higher concentrations in the cecum and colon (Argenzio *et al.*, 1974; Argenzio and Southworth, 1975). It has been shown that acetate at the concentration found in the ileum induces invasion and the expression of SPI-1 (Durant *et al.*, 2000; Lawhon *et al.*, 2002). This effect requires SirA, but not BarA. Additionally, acetate has its effects only when bacteria are grown at an acidic pH, and fails to act in the absence of *ackA* and *pta*, two *Salmonella* genes that produce acetyl-phosphate from acetate (Lawhon *et al.*, 2002). Because acetate concentrates within the bacterial cytoplasm when the medium is acidic, these results, taken together, suggest that acetate works as a signal within the bacterium in the form of acetyl-phosphate. The requirement for SirA, but not for BarA, additionally suggests that the response regulator might be activated by phosphorylation from the acetyl-phosphate donor.

Genetic repression of invasion

Induction of invasion is tightly controlled by numerous overlapping regulatory circuits, indicating that the correct timing of gene expression is essential for virulence. Similarly, one might expect repression of invasion to use an equally complex system to ensure that SPI-1 genes are not expressed at times when invasion would be unproductive. In its passage through the intestinal tract, invasion is likely to be accomplished in only a small region. Attempts to invade the hostile environment of the proximal intestinal tract (i.e. the stomach and duodenum) would likely be unproductive, while invasion of the distal tract (the cecum and colon) appears to be equally unfavorable to the organism, as *Salmonella* rarely causes colitis. Thus, the ability to sense the environment of the distal small intestine and to invade the epithelium of this region appears paramount to success. Similarly, expression of invasion genes within epithelial cells, after invasion has occurred, would be unproductive and inefficient.

Several studies have identified negative regulators of invasion that might serve to limit SPI-1 gene expression to the optimal point of infection. Interesting among these is the two-component regulator PhoP/PhoQ. This pair is essential to the expression of genes of *Salmonella* pathogenicity island 2 (SPI-2), which encodes a second type III secretion system. SPI-2 is required for survival in macrophages, the cell type encountered by *Salmonella* immediately after the invasion of the epithelium. PhoP/PhoQ also serves to repress SPI-1 genes, a function mediated by *hilA* (Behlau and Miller, 1993; Bajaj *et al.*, 1996; Fahlen *et al.*, 2000). Thus, PhoP/PhoQ may act as a genetic switch, activating traits required for macrophage survival while repressing those no longer needed for invasion. Similarly, Lon protease is known to repress invasion (Takaya *et al.*, 2002; Takaya *et al.*, 2003; Boddicker and Jones, 2004). It does so in bacteria residing within epithelial cells, suggesting that it too reduces SPI-1 expression once the invasion phenotype is no longer required (Boddicker and Jones, 2004). Besides these two examples of negative regulators for which the likely site of expression is known, several other SPI-1 repressors have been identified, all of which reside outside SPI-1. HilE interacts with HilD to repress *hilA* (Baxter *et al.*, 2003). Hha, a histone-like protein, binds to and represses *hilA* (Fahlen *et al.*, 2001), while the small nucleoid-binding protein H-NS represses *hilA* as well (Schechter *et al.*, 2003). The global regulator polynucleotide phosphorylase (PNPase) is also a negative regulator of SPI-1 genes, as well as those of SPI-2. It has been proposed that control by PNPase is important in establishing persistent *Salmonella* infections (Clements *et al.*, 2002). As is true for invasion induction, SPI-1 genes can also be repressed by small signaling molecules. Mutation of two genes that encode dinucleoside polyphosphate hydrolases, *ygdP* and *apaH*, reduces invasion by repressing SPI-1 genes. Products of *ygdP* and *apaH* ordinarily degrade dinucleoside polyphosphates (Np_nN), whose functions in bacteria are not completely understood. In the mutant strains, levels of Np_nN rise, suggesting that these dinucleoside polyphosphates function as negative regulators of invasion (Ismail *et al.*, 2003).

Environmental repression of invasion

As described above, the environment of the distal small intestine, the region productive for infection, is likely to induce invasion. It is also clear that environmental cues can be used by *Salmonella* to repress the invasion phenotype at times during which invasion is not required. An example of repression by a constituent of the intestinal tract itself is that produced by bile. Bile is secreted into the proximal small intestine, an area not used for invasion. It represses SPI-1 genes high in the regulatory cascade,

working at or above the level of BarA/SirA (Prouty and Gunn, 2000). Similarly, short chain fatty acids can repress invasion. As discussed above, one short chain fatty acid, acetate, is found in high concentrations in the distal small intestine. However, the levels of two others, propionate and butyrate, rise in the cecum and colon, where *Salmonella* fails to invade. Different from acetate, these two fatty acids repress SPI-1 gene expression. A combination of all three fatty acids that mimics the conditions of the colon also efficiently represses invasion, indicating that the colonic environment prevents invasion of this portion of the intestine (Lawhon *et al.*, 2002). The environment within macrophages is also known to reduce SPI-1 gene expression. The PhoP/PhoQ two-component regulator that represses *hilA* is activated in response to the limiting Mg^{2+} found within macrophages (Garcia Vescovi *et al.*, 1996). Also repressive to SPI-1 are cationic peptides, known to exist in macrophages, suggesting a second mechanism by which invasion determinants are silenced once bacteria have reached the macrophage (Bader *et al.*, 2003).

Conclusion

Salmonella devotes a substantial portion of its regulatory capacity to the control of invasion, with more than two dozen genes now recognized as required for optimal invasion gene expression, epithelial cell penetrations, or virulence (Table 1). This investment in the regulation of SPI-1 genes suggests that the stringently controlled and appropriately timed expression of invasion determinants is essential to the infectious process. The number of negative regulators identified suggests further that the repression of invasion at inappropriate times (e.g. in portions of the intestinal tract not productive for invasion, or within macrophages, after invasion has occurred) is as important to bacterial survival as is its well-timed induction. It is also clear that SPI-1 genes are under the control of a number of global regulators, indicating that control of invasion has been integrated with that of numerous other bacterial functions. The likely implication of this integrated control is that conditions present in the intestinal tract of animal hosts induce the coordinated expression of invasion genes and other determinants that promote *Salmonella* survival in this environment. Although the environmental stimuli identified so far are consistent with those found in the intestinal tract of animals, much remains to be learned about the mechanisms by which environmental signals induce the many genetic regulators that control invasion.

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