INFECTION AND IMMUNITY, Mar. 2005, p. 1377–1385 Vol. 73, No. 3
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.3.1377–1385.2005

The *fimYZ* Genes Regulate *Salmonella enterica* Serovar Typhimurium Invasion in Addition to Type 1 Fimbrial Expression and Bacterial Motility

M. Aaron Baxter and Bradley D. Jones*

Department of Microbiology, Roy J. and Lucille A. Carver School of Medicine, University of Iowa, Iowa City, Iowa

Received 7 September 2004/Returned for modification 15 October 2004/Accepted 4 November 2004

An important step in *Salmonella enterica* serovar Typhimurium virulence is the ability to invade the intestinal epithelium. The invasion process requires a large number of genes encoded on *Salmonella* pathogenicity island 1 (SPI-1) at centisome 63 as well as genes located in other positions throughout the chromosome. Expression of the invasive phenotype is tightly regulated by environmental cues that are processed by a complex regulatory scheme. A central player in the invasion regulatory pathway is the HilA protein, which is transcriptional activator belonging to the OmpR/ToxR family. A number of positive regulators (*hilC*, *hilD*, *fis*, *sirA*/*barA*, *csrAB*, *phoBR*, *fadD*, *envZ*/*ompR*, and *fliZ*) and negative regulators (*hha*, *hilE*, *lon*, *ams*, *phoP*^{c} and *pag*) have been identified that are able to alter expression of *hilA* transcription. Recent work has found that *hilA* transcription requires the HilD protein for activation. Other work has emphasized the importance of HilE as a negative regulator of *hilA*. Overexpression of *hilE* superrepresses *hilA* transcription, as well as the invasive phenotype. Two-hybrid experiments suggest that HilE exerts its regulatory influence on *hilA* through protein-protein interactions with HilD as the protein does not bind to the *hilA* promoter nor does it affect *hilD* transcription. As it seems likely that *hilE* plays an important role in translating environmental signals into invasion gene regulation, we have attempted to identify how the *hilE* gene itself is regulated. Our results indicate that the *fimYZ* genes, response regulatory proteins involved in type 1 fimbrial gene expression and recently implicated in motility gene regulation, are important activators of *hilE* expression. These findings indicate that invasion gene expression is coregulated with motility and adherence and provide experimental evidence that the expression of these virulence phenotypes is a subset of the overall regulation of bacterial physiology.

Pathogenic *Salmonella* species are an important cause of disease throughout the world and cause infections ranging from self-limiting gastroenteritis to life threatening typhoid fever. Efforts to understand the mechanisms by which *Salmonella* spp. establish infection in a human host have identified several interactions that include adherence, invasion, and the ability of the bacteria to grow within host cells. Collectively, the various virulence mechanisms of the bacteria determine the ability to interact with and colonize the host in the process known as infectious disease.

Adherence to tissue culture cells is mediated in *Salmonella* spp. by mannose-sensitive type 1 fimbriae (12, 13). Type 1 fimbrial structures are assembled by transporting the structural proteins through the bacterial membrane in the chaperone-usher pathway, followed by assembly into the rigid appendages that facilitate adherence to host cells (25, 49). The structural components of *Salmonella* type 1 fimbriae are homologous to type 1 fimbriae of *Escherichia coli*, but the regulatory mechanisms governing expression of *Salmonella* type 1 fimbriae appear to be unique. The *Salmonella fim* gene cluster contains three genes, *fimW*, *fimY*, and *fimZ*, that affect expression of the structural subunit gene *fimA*. Genetic experiments have demonstrated that both FimY and FimZ are necessary for expression of the *fim* gene cluster, but DNA binding experiments have demonstrated that FimZ is able to bind to the *fimA* promoter in the absence of the FimY protein (47, 55). Interestingly, *fimZ* belongs to the response regulator family of proteins with highest homology to *bvgA* of *Bordetella pertussis*, but no sensor kinase has been identified as its partner (55).

Salmonella invasion into host cells occurs through a process by which effector proteins are transferred into cells via the action of a type III secretion system (15, 16). The expression of the invasive phenotype is tightly regulated by a variety of positive and negative regulatory genes, in response to environmental conditions. The *hilA* gene is a member of the OmpR/ToxR family of transcriptional activators that play a critical role in the transcription of genes encoding components of the type III secretion system as well as the *invF* gene, which specifically activates genes encoding effector proteins involved in the invasion process (4, 29). In addition to the *hilA*-dependent pathway of invasion gene activation that many laboratories have described and characterized, other groups have reported *hilA*-independent invasion gene activation pathways (1, 20, 42). That work has demonstrated that AraC-like proteins (RtsA/B, HilC, and HilD) can activate a subset of invasion genes in the absence of the *hilA* gene. However, the *hilD* and *hilC* genes (19, 42, 43), which encode AraC-like transcriptional activators, are also important positive regulatory elements of *hilA*. Mutation of *hilD* leads to a significant decrease in *hilA* expression (14-fold) and a >50-fold decrease in invasiveness. Recent work has demonstrated that both HilD and HilC bind to sequences upstream of the *hilA* promoter (39, 44) and that the presence of HilD is required for *hilA* activation even in the absence of several negative regulatory elements (9). Several other genes

* Corresponding author. Mailing address: Department of Microbiology, Roy J. and Lucille A. Carver School of Medicine, University of Iowa, Iowa City, IA 52242-1109. Phone: (319) 353-5457. Fax: (319) 335-9006. E-mail: bradley-jones@uiowa.edu.

TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Description[a]	Reference(s) or source
Escherichia coli DH12S	*mcrA* Δ*(mrr-hsdRMS-mcrBC)* F′ *lacI*^{q} ΔM15	Invitrogen
Salmonella enterica serovar Typhimurium		
BJ2710	SL1344 derivative containing LT2 *fimH* gene	This work
BJ2710 *fimZ-kan*	*fimZ*::kan in BJ2710, Kan^r	This work
BJ2462	*hilE*::cam in SL1344, Cam^r	6
LB5010	Strain LT2 containing a complete *fim* gene cluster	56
LBZ100	LB5010 *fimZ*::kan, Kan^r	56
SL1344	Wild-type virulent strain	54
SL1344 *fimZ-kan*	*fimZ*::kan in SL1344 transduced from LBZ100, Kan^r	This work
SL1344H3	SL1344 strain containing a *fimH*::kan insertion, Kan^r	8
Plasmids		
pACYC184	Cam^r Tet^r	10
pISF182	*fimYZ* cloned into pACYC184, Cam^r	55
pLS31	pRW50 vector encoding −497 to +420 of *hilA* fused to *lacZY*, Tet^r	43
pKD3	pANTSγ vector containing the chloramphenicol template gene cloned from pSC140, Amp^r	17
pKD46	Temperature-sensitive red helper plasmid expressing *araC-P*$_{araB}$ and γβ*exo* from λ phage, Amp^r	17
pMAB54	pGEM-T vector encoding *hilE* promoter from −886 to +121, Amp^r	This work
pMAB55	pGEM-T vector encoding *hilE* promoter from −886 to +121 and promoterless *lacZY* genes downstream of the promoter, Amp^r	This work
pMAB56	pZC320 vector encoding *hilE* promoter from −886 to +121, Amp^r	This work
pMAB69	*hilE* promoter −886 to +121 cloned in pRW50, Tet^r	This work
pMAB95	pGEM-T encoding the *hilE* promoter fragment −191 to +121, Amp^r	This work
pMAB97	pGEM-T vector encoding the *hilE* promoter fragment −886 to −271, Amp^r	This work
pMAB98	pGEM-T vector encoding the *hilE* promoter from −886 to +121 minus the −271 to −191 region, Amp^r	This work
pMAB99	pMAB98 ligated into pRW50, Amp^r Tet^r	This work
pMAB102	pRW50 *lacZY*-expressing vector with the *hilE* promoter missing −271 to −191 relative to the putative translation start site inserted upstream to *lacZY*, Tet^r	This work
pMRP9-1	GFP-expressing plasmid, Cam^r	E. P. Greenberg
pRTP::Tn5	Tn*5* transposon inserted onto plasmid pRTP1 which carries the *rpsL* gene (*Str*^{s}) Amp^r Kan^r	27, 46
pRW50	*lacZ* reporter vector, Tet^r	31
pZC320	Mini-F, Amp^r	45

[a] Tet^r, tetracycline resistant; Amp^r, ampicillin resistant; Kan^r, kanamycin resistant; Cam^r, chloramphenicol resistant.

have been identified as being important in activation of *hilA* expression, including *csrAB*, *sirA*/*barA*, *phoBR*, *fadD*, *envZ*, *fliZ*, and *fis* (2, 3, 26, 32, 53). The mechanism of action of these genes in invasion regulation is under investigation.

Several elements that negatively affect *hilA* expression have also been identified. The *phoP*(Con) mutant carries a point mutation in the *phoQ* gene that results in constitutive phosphorylation of *phoP*. This event has a dominant negative effect on *hilA* expression and results in a noninvasive phenotype (5, 7). The *hha* gene has been identified as a negative modulator of *hilA* expression as *hha* mutations increase *hilA* expression and overexpression of *hha* significantly decreases *hilA* expression and the invasiveness of *Salmonella* spp. (23). A mutation in the Lon protease has also recently been shown to decrease both *hilA* expression and *Salmonella* invasiveness (48).

A search for repressors of *hilA* identified *ams*, *pag*, and *hilE* as negative regulatory elements of *hilA* (22). Mutation of each of these genes results in overexpression of *hilA*. Recently, more extensive characterization of the *hilE* gene has been performed (6). That work demonstrated that overexpression of *hilE* completely represses *hilA* and results in a noninvasive phenotype. Efforts to identify the mechanism of regulation failed to show that the HilE protein binds to the *hilA* promoter or that *hilE* regulates *hilD* transcription. However, bacterial two-hybrid experiments demonstrated that HilD and HilE bind to one another, suggesting that a possible mechanism of action for HilE is to disrupt HilD activation of *hilA* by disrupting its function. Finally, the *hilE* gene was found to reside in a region of the *Salmonella* chromosome that resembles a pathogenicity island and is apparently unique to *Salmonella* strains.

Due to the evidence indicating that *hilE* plays a role in controlling *hilA* expression, we have performed experiments aimed at understanding how HilE is involved in the regulatory cascades that upregulate or downregulate expression of the SPI-1 *Salmonella* invasion genes. Those experiments have revealed that the *fimYZ* genes regulate *hilE* expression. These findings indicate that *fimYZ* are important components of a global regulatory network, as work by others has demonstrated that *fimZ* (and *fimY)* also regulates both motility and adherence (14).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Bacteria were routinely grown in Lennox broth (Gibco-BRL) containing the appropriate antibiotics added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; streptomycin, 100 μg/ml; chloramphenicol, 10 μg/ml; and tetracycline, 25 μg/ml. For the β-galactosidase assays, bacterial cultures were shaken at 250 rpm at 37°C to an optical density at 600 nm of ≈0.4 to 0.5, which corresponds to ≈4×10^8 to 5×10^8 CFU/ml. For the invasion assays, low-oxygen conditions were created by inoculating 5 ml of Lennox broth with 10 μl of a stationary-phase culture and incubating statically overnight at 37°C until an optical density at 600 nm of 0.4 to 0.5 was reached (27, 41). Plasmid purifications were performed with kits manufactured by Qiagen Inc., and molecular manipulations were performed with standard protocols (34).

Plasmid and strain construction. A *hilE* reporter plasmid, pMAB56, was created by amplifying the region from −886 to +121 in relation to the putative *hilE* translation start site from the chromosome with the primers hilE5′ (5′-GGATCCTTTGCGGATTACTGCCGTT-3′) and hilE3B (5′-AAGCTTCTTC

AATACCGTCCAGTT-3′) and ligating the DNA fragment upstream of the *lacZY* genes to form plasmid pMAB55. The *hilE-lacZY* fragment was then removed from pMAB55 with NsiI and SphI and cloned into pZC320 (45) to form the single-copy reporter pMAB56. Sequencing of the insert confirmed the presence and sequence of the *hilE* promoter. The *hilE* reporter pMAB69 was created by amplifying the *hilE* promoter (−886 to +121) from the chromosome with the primers hilE5′/Eco (5′-GAATTCTTTGCGGATTACTGCCGTT-3′) and hilE3B/Bam (5′-GGATCCCTTCAATACCGTCCAGTT-3′) and cloning the fragment upstream of the *lacZY* genes in the vector pRW50 (31).

The *hilE* reporter pMAB102, with the putative *fimZ* binding sites from −271 to −191 deleted relative to the translation start site, was constructed by amplifying the *hilE* promoter fragment from −191 to +121 with the primers hilEdel695 (5′-GAATTCGATATTTCTTTTTTGATATGGTTC-3′) and hilE3B/Bam. The resulting fragment was ligated into pGEM-T to create pMAB95. The *hilE* promoter fragment from −886 to −271 was amplified with the primers hilE5′/Eco and hilE15 (5′-AGATCTCCTTTTCACATCAATGGGTTTT-3′), and the resulting fragment was ligated into pGEM-T to form pMAB97.

By overlapping PCR, the fragments from pMAB95 and pMAB97 were used to create the deleted *hilE* promoter fragment (30). Briefly, pMAB95 was amplified with the primers hilE5′Eco and hilE21 (5′-TAAGAACCATATCAAAAAAGA TGGGTTTTTTAGACTTTTG-3′), while in a separate reaction pMAB97 was amplified with the primers hilE20 (5′-CAAAAGTCTAAAAAACCCATCTTTT TTGATATGGTTC-3′) and hilE3B/Bam. Primers hilE20 and hilE21 contain a tail that is homologous to the other PCR fragments on either side of the −271 to −191 deleted region. After amplification, the two PCR products were mixed and amplified with the primers hilE5′Eco and hilE3B/Bam. This PCR product was then ligated into pGEM-T, creating pMAB98. The reporter vectors pRW50 and pMAB98 were then digested with BamHI followed by ligation to create pMAB99. Finally, the pGEM-T portion of pMAB99 was removed by digesting the plasmid with EcoRI, followed by religation of the ends to create pMAB102, which contains the *hilE* promoter missing the −271 to −191 region relative to the putative translation start site, fused to a *lacZY* reporter.

Plasmid pISF187 is a derivative of plasmid pISF182 in which a universal translational terminator was introduced into a unique EcoRV site to inactivate the *fimY* gene (55). Subsequent work revealed that the plasmid carries a functional *fimZ* gene, as it could complement a *fimZ* mutant. Plasmid pISF189 is a derivative of plasmid pISF182 in which a universal translational terminator was introduced into a unique PvuI site to inactivate the *fimZ* gene (55). Subsequent work revealed that the plasmid carries a functional *fimY* gene, as it could complement a *fimY* mutant.

Tissue culture conditions and cell invasion assays. HEp-2 tissue culture cells (38) were maintained in RPMI 1640 medium containing 10% fetal bovine serum. The cells were passaged every 2 to 4 days as required. Invasion assays were conducted with the bacteria being grown under inducing conditions with previously described protocols (27, 41).

β-Galactosidase assays. β-Galactosidase assays were conducted on bacterial cultures with the standard method described by Miller (36).

P22-mediated transductions. Phage P22 HT *int* was used to move antibiotic resistance gene insertions between strains as described previously (18). Transductants were selected on Lennox agar containing the necessary antibiotic and 10 mM EGTA to prevent P22 reinfection. Transductants were purified twice on Lennox EGTA agar prior to use of the colonies.

Tn*5* transposon mutagenesis. In order to screen for mutations that increased *hilE* expression, a previously described protocol was used (22). Briefly, EE251 cells were transformed with pRTP1::Tn*5*. This plasmid contains a Tn*5* transposon (kanamycin resistance), the wild-type *rpsL* gene, which confers streptomycin sensitivity to the bacteria, and an ampicillin resistance marker. The bacteria were plated onto Lennox agar with ampicillin and kanamycin at a concentration of 500 CFU per plate. Colonies were replica plated onto Lennox streptomycin kanamycin agar and grown overnight at 37°C. Growth on Lennox agar with streptomycin and kanamycin indicated that Tn*5* had moved randomly from pRTP1 into the *S. enterica* serovar Typhimurium chromosome and that the delivery plasmid, pRTP1, had been lost. Next, pMAB69, a *hilE-lacZY* reporter plasmid, was transformed into the pools of Tn*5* mutants followed by plating on MacConkey plates with tetracycline and kanamycin.

Colonies that exhibited an increased red colony phenotype compared to wild-type *Salmonella* colonies were isolated. To identify the site of the Tn*5* insertion, chromosomal DNA was isolated from colonies with increased *hilE* expression. Chromosomal DNA was digested with BamHI to completion, and fragments 2 to 5 kb in size were gel purified. The vector pACYC184 (10) was digested with BamHI, and the isolated chromosomal fragments were ligated into the vector. The ligated DNA was transformed into DH12S and plated on Lennox-kanamycin agar to identify a clone carrying the kanamycin resistance gene from Tn*5* and flanking chromosomal DNA. To identify the junction between the Tn*5* sequence and the chromosome, the oxregA primer (5′-TTCAGGACGCTACTTGTG-3′) was used to sequence the cloned DNA. The sequence obtained was compared to the annotated *S. enterica* serovar Typhimurium LT2 genomic sequence (35) to identify the Tn*5* insertion site.

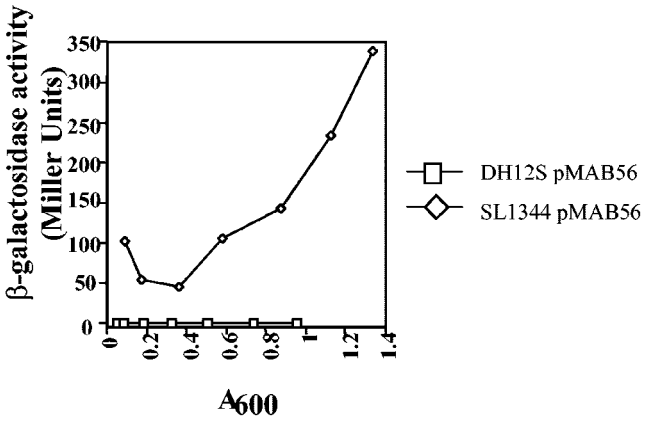

FIG. 1. Expression profile of a *hilE-lacZY* reporter throughout the growth curves of *E. coli* and *S. enterica* serovar Typhimurium. Strains were grown with shaking overnight in Lennox broth and then reinoculated into 20 ml of Lennox broth. Samples were taken every 40 min after the cultures began actively growing. Levels of β-galactosidase activity were determined for each sample taken. Plasmid pMAB56 is a single-copy mini-F plasmid that carries 1,000 bp of the putative *hilE* promoter fused upstream of a promoterless *lacZY* gene. Experiments were performed at least three times.

RNA isolation and primer extension. To determine where *hilE* transcription begins, we first isolated RNA by incubating DH12S/pMAB69/pISF182 and DH12S/pMAB102/pISF182 by growing the two *E. coli* strains overnight in 125 ml of Lennox broth. The bacteria were pelleted, and the total RNA was isolated (11). For primer extension analysis, the primer hilE23 (5′-GAACGTTCCATT TTCCAGCCA-3′) was end labeled with [γ-^{32}P]ATP, and the primer extension reaction was performed with the Primer Extension System avian myeloblastosis virus reverse transcriptase kit (Promega).

To determine the exact nucleotide start point for *hilE* transcription, the previously labeled primer hilE23 was used in sequencing reactions performed with the Fmol DNA cycle sequencing system (Promega). The products for the primer extension reactions and the products from the sequencing reactions were electrophoresed on a 6% acrylamide–7 M urea sequencing gel. The sequencing gel was dried onto filter paper and visualized after exposure to film.

RESULTS

HilE-lacZY expression in *S. enterica* serovar Typhimurium and in *Escherichia coli*. The *hilE-lacZY* reporter plasmid pMAB56 was created to measure *hilE* expression throughout the growth cycle in *Salmonella enteritidis* serovar Typhimurium and additionally whether *hilE* could be expressed within *Escherichia coli*. Previous searches have shown that *hilE* is a *Salmonella*-specific gene (6). When the reporter carried by *S. enterica* serovar Typhimurium strain SL1344 was assayed for activity, it was clear that *hilE* expression was regulated, since expression varied significantly over the course of the growth curve. At inoculation, β-galactosidase levels were relatively high and then decreased as the culture entered the early stages of logarithmic growth (Fig. 1). It is likely that the levels of β-galactosidase at early time points are residual from the stationary phase of the starting culture and that the subsequent decrease in activity is a result of cellular division. The *hilE-lacZY* levels were the lowest (45.5 Miller units) when the culture density was between optical densities at 600 nm of 0.17 and 0.36. Measurements of β-galactosidase levels throughout

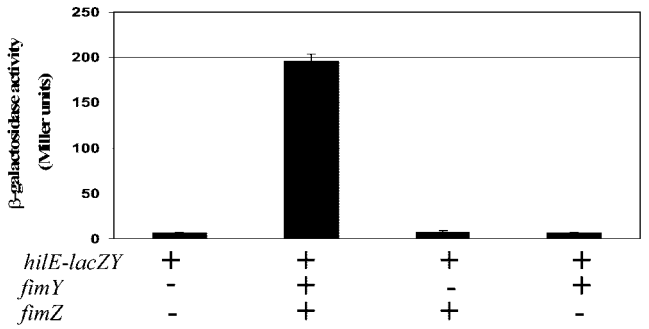

FIG. 2. Overexpression of *fimYZ* activates *hilE-lacZY* expression in *E. coli*. Strains were grown with shaking in Lennox broth to the late stationary phase. Each strain carried plasmid pMAB69, which is a low-copy-number *hilE* reporter carried on the pRW50 vector. Plasmid pISF182 has the *fimYZ* genes cloned into pACYC184 and expressed from the tetracycline promoter. Plasmids pISF187 (*fimZ*$^+$ *fimY*) and pISF189 (*fimZ fimY*$^+$) are identical to pISF182 except for the insertion of a transcriptional terminator (see Materials and Methods). *E. coli* carrying only pISF187 or only pISF189 was not induced for *hilE-lacZY* expression, indicating that both *fimY* and *fimZ* are necessary for the expression of *hilE*. Expression levels were determined by measuring β-galactosidase activity. Experiments were performed at least three times.

the rest of the growth cycle showed a continual increase in *hilE-lacZY* expression into the stationary phase.

Analysis of the expression of *hilE-lacZY* from plasmid pMAB56 indicated that the *hilE* gene is virtually not expressed in *E. coli* throughout the growth curve. This result suggests that *E. coli* lacks regulatory factors necessary for *hilE* expression. Unpublished work from our laboratory is consistent with this idea since BLAST searches of the genome databases have consistently failed to identify any gene with significant similarity to *hilE*. Current information indicates that *hilE* is a *Salmonella*-specific gene, and genes that control its expression may also have regulatory properties unique to *Salmonella* spp.

Identification of the response regulators *fimYZ* as activators of the *hilE* repressor gene. As our results suggest that *hilE* is controlled by a *Salmonella*-specific factor, we began a screen for *S. enterica* serovar Typhimurium genes that affect *hilE-lacZY* expression by performing Tn*5* mutagenesis (22). We identified several mutants that displayed increased *hilE-lacZY* expression. Two isolates that overexpressed *hilE-lacZY* from the plasmid reporter sevenfold more than the *S. enterica* serovar Typhimurium parent strain were selected for further characterization. Identification of the insertion sites of each of the Tn*5* insertions, by cloning and sequencing, revealed that each was located in a different position upstream of the *fimY* and *fimZ* genes, which are involved in type 1 fimbrial gene regulation (47, 50, 56) and regulation of *Salmonella* motility (14).

To test the possibility that the Tn*5* chromosomal insertions were increasing *fimY* and *fimZ* expression, we introduced a medium-copy plasmid (p15A replicon) that expresses *fimYZ* into *E. coli* and assayed what effect increasing the expression of *fimYZ* would have on *hilE-lacZY* expression. As shown in Fig. 2, the presence of the *fimYZ*-overexpressing plasmid, pISF182, in DH12S/pMAB69 increased expression of the *hilE-lacZY* reporter 31-fold. Next, we examined whether *fimY* only, *fimZ* only, or both *fimY* and *fimZ* were required for *hilE* activation. It has been shown previously that FimZ can bind to the promoter of the *fimA* gene cluster but that FimY is also required to activate gene expression of *fimA* (47, 55). Accordingly, we assayed *hilE-lacZY* expression in *E. coli* with the *fimY fimZ*$^+$ plasmid pISF187 and the *fimY*$^+$ *fimZ* plasmid pISF189. Both the *E. coli* strain DH12S/pMAB69/pISF187 (*fimY fimZ*$^+$) and the *E. coli* strain DH12S/pMAB69/pISF189 (*fimY*$^+$ *fimZ*) expressed *hilE-lacZY* at levels comparable to that of the *E. coli* strain containing the *hilE-lacZY* reporter alone (Fig. 2). These results indicate that both *fimY* and *fimZ* are required to induce *hilE-lacZY* expression in *E. coli*.

We next determined if overexpression of *fimYZ* increases *hilE-lacZY* expression in *S. enterica* serovar Typhimurium. Analysis of *S. enterica* serovar Typhimurium strain SL1344 containing the *hilE* reporter pMAB69 showed that *hilE-lacZY* expression increased 5.1-fold (55.3 ± 2.5 units versus 280.5 ± 6.5 units) by introduction of the *fimYZ*-expressing plasmid pISF182 (Fig. 3). Additionally, deletion of the *fimZ* gene affected *hilE-lacZY* expression. A strain lacking a functional *fimZ* gene, BJ2710 *fimZ*-kan, had 2.7-fold-reduced expression of β-galactosidase from pMAB69 compared to the parent strain (Fig. 3). These results indicate that the *fimYZ* genes function to activate expression of the *Salmonella* invasion gene repressor *hilE*.

Overexpression or deletion of *fimYZ* affects the expression of the invasion regulator *hilA* in *S. enterica* serovar Typhimurium. Previously, our research group has shown that variations in *hilE* expression have a direct effect on the level of *hilA* expression in *S. enterica* serovar Typhimurium (6). To extend those observations, we wanted to determine if deletion or overexpression of the *fimYZ* genes would have corresponding effects on *hilA* expression. We conducted a β-galactosidase assay with the *hilA-lacZY* reporter pLS31. When wild-type *S. enterica* serovar Typhimurium SL1344 was assayed, it expressed 437.4 ± 13.5 Miller units from the *hilA-lacZY* reporter (Fig. 4). Chromosomal deletion of *fimZ* in the SL1344 strain increased *hilA*::*lacZY* expression 1.6-fold (715 ± 11.5 Miller units). Overexpression of the *fimYZ* genes from plasmid pISF182 decreased *hilA-lacZY* expression 3.3-fold (131.8 ± 3.2 Miller units) compared to the wild-type SL1344/pLS31 (437.4 ± 13.5 Miller units), which is consistent with effects observed on the *hilE-lacZY* reporter.

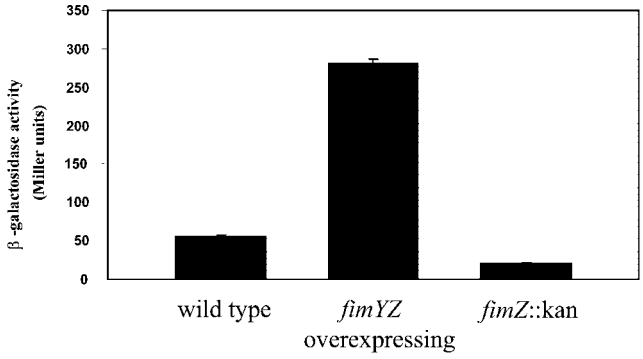

FIG. 3. Overexpression of *fimYZ* in *S. enterica* serovar Typhimurium leads to activation of a *hilE-lacZY* reporter. SL1344 (wild type), SL1344/pISF182 (*fimYZ* overexpressing), and SL1344 *fimZ*-kan, each containing the *hilE-lacZY* reporter pMAB69, were grown with shaking in Lennox broth to the late stationary phase. Levels of expression were measured by β-galactosidase activity. Each assay was performed at least three times.

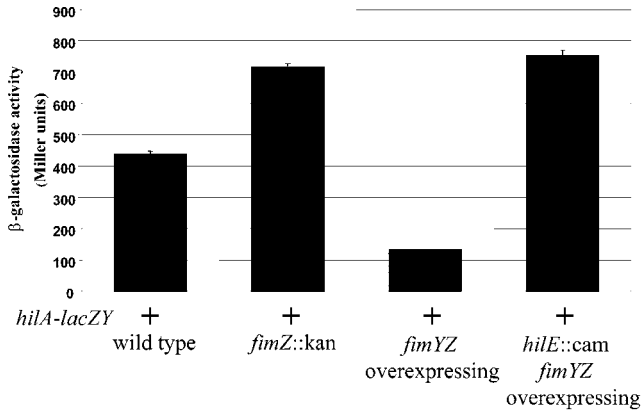

FIG. 4. Regulatory influence of *fimYZ* on *hilA-lacZY* expression. Deletion of *fimZ* increased *hilA-lacZY* expression, while overexpression of *fimYZ* within *S. enterica* serovar Typhimurium repressed an *hilA-lacZY* reporter. A deletion of the *hilE* gene eliminated the effect of *fimYZ* overexpression on *hilA* expression. Cultures were grown to the late stationary phase in Lennox broth. Levels of *lacZ* activity were measured by the β-galactosidase assay. Each assay was performed in triplicate and repeated three times.

To demonstrate in a more direct manner that the *hilE* gene is mediating the effects of *fimZ* on *hilA*, an *hilE* mutant *Salmonella* strain was constructed by the method developed by Datsenko and Wanner (17). The expression of the *hilA-lacZY* reporter was then measured in the *hilE* mutant strain when *fimYZ* was expressed from pISF182. Repression by *fimYZ* was lost in this strain, as β-galactosidase activity from *hilA-lacZY* was 1.7-fold higher than from the wild-type strain with *hilA-lacZY*, which was nearly identical to expression levels when a *fimZ* mutation was present. These results provide additional evidence that the *fimYZ* genes are acting as negative regulatory elements on *hilA* transcription via the activity of the HilE invasion gene repressor.

Overexpression or deletion of *fimYZ* affects the ability of *S. enterica* serovar Typhimurium to invade tissue culture cells in vitro. The experiments described above have demonstrated that increasing expression (by an overexpressing plasmid) or reducing expression (by deletion) of *fimYZ* has effects on both *hilE* and *hilA* expression levels. We next examined whether alterations in regulatory gene expression correlated with changes in the ability of *S. enterica* serovar Typhimurium to invade HEp-2 tissue culture cells. The *S. enterica* serovar Typhimurium strain BJ2710 was used as the invasive parental control, and invasiveness was arbitrarily set at 100% ± 4.2% when the bacteria were grown in invasion-inducing conditions (Fig. 5). Strain BJ2710/pISF182, which overexpresses *fimYZ*, had dramatically reduced levels of invasiveness (≈100-fold). This reduction in cellular entry indicates that the activities of *fimYZ* are required for determining whether *S. enterica* serovar Typhimurium will express the invasion machinery. Finally, the requirement for *hilE* in the repressing activity of *fimYZ* on *S. enterica* serovar Typhimurium invasion was assessed. The invasiveness of strain SL1344 *hilE*/pISF182 was measured and found to restore cellular entry to 96% ± 14.6%. Therefore, the repression of invasion observed in the *S. enterica* serovar Typhimurium strain carrying the *fimYZ* plasmid pISF182 requires a functional *hilE* gene.

Deletion of sequences in the *hilE* promoter eliminates the ability of *fimYZ* to induce *hilE* expression in *Escherichia coli*. As a result of our findings, we began an analysis of the *hilE* promoter in an effort to determine how the *fimYZ* genes exert their influence on the transcription of *hilE*. Published work has shown that the FimZ protein binds to the *fimA* promoter at a DNA region with a tandem repeat of the motif AATAAGA separated by 20 bp (56). Analysis of the *hilE* promoter identified the same AATAAGA sequence as that observed in the *fimA* promoter, although the repeats were separated by 47 bp. This motif was found to be 202 bp upstream of the putative translational start site of the *hilE* protein. Experiments were initiated to determine whether deletion of the putative FimZ binding site would eliminate the ability of *fimYZ* gene products to induce *hilE* expression.

We first deleted the putative FimYZ binding sequences in the *hilE* promoter with an overlapping PCR protocol described previously (30). The *hilE-lacZY* reporter plasmid pMAB102 is identical to the wild-type *hilE-lacZY* reporter plasmid pMAB69 except for the deletion of 80 bp encompassing the putative FimYZ binding site. Following construction of pMAB102, we performed experiments to measure the importance of the putative FimYZ binding sites in activation of *hilE* transcription.

E. coli carrying the *hilE-lacZY* reporter plasmid pMAB69 had typically low expression of the fusion reporter, which was induced 26.4-fold by the presence of the *fimYZ* plasmid pISF182 (Fig. 6A). *E. coli* carrying the *hilE-lacZY* fusion plasmid with the promoter deletion, pMAB102, expressed 135 ± 5.9 Miller units of β-galactosidase from *hilE*. Introduction of pISF182 (*fimYZ*) did not increase expression from the *hilE* promoter but decreased it slightly to 116.9 ± 5.4 Miller unit of β-galactosidase. Apparently, the deletion of the putative FimYZ binding sites had the unanticipated consequence of increasing the basal level expression of the *hilE* promoter in *E. coli*. Since basal expression of the *hilE* promoter, carrying the deletion of

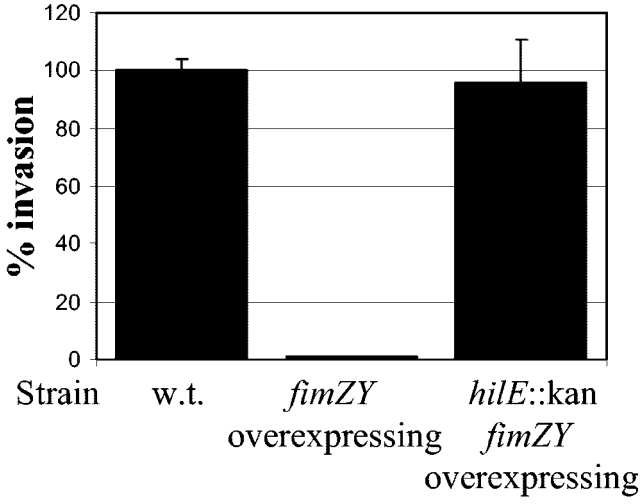

FIG. 5. Overexpression of *fimYZ* leads to repression of invasion of HEp-2 cells. Strains were grown under low-oxygen and high-osmolarity conditions. The deletion of *hilE* restored *S. enterica* serovar Typhimurium invasiveness in HEp-2 cells even when *fimYZ* were overexpressed. Values are presented as a percentage of invasion by the wild-type strain, with the invasiveness of that strain being set to 100%.

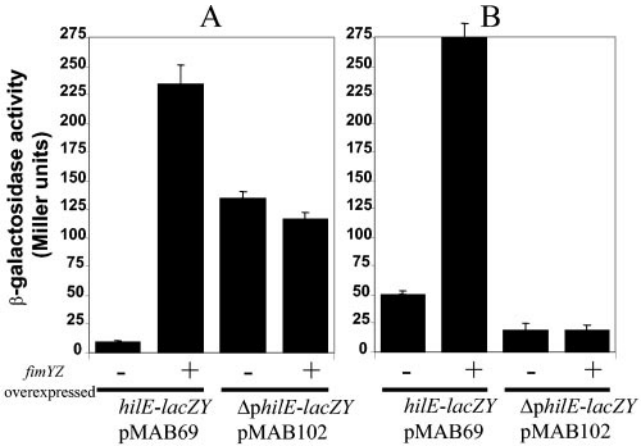

FIG. 6. FimYZ activation of the *hilE* promoter requires the presence of sequences in the *hilE* promoter in *E. coli* and *S. enterica* serovar Typhimurium. Expression values for the *hilE-lacZY* reporter plasmids in *E. coli* are shown in panel A. The *E. coli* strains DH12S/pMAB69, DH12S/pMAB69/pISF182, DH12S/pMAB102, and DH12S/pMAB102/pISF182 were grown with shaking overnight in Lennox broth. Expression values for *hilE-lacZY* reporter plasmids in *S. enterica* serovar Typhimurium are shown in panel B. The *S. enterica* serovar Typhimurium strains SL1344 Δ*hilE*/pMAB69, SL1344 Δ*hilE*/pMAB69/pISF182, SL1344 Δ*hilE*/pMAB102, and SL1344 Δ*hilE*/pMAB102/pISF182 were grown with shaking overnight in Lennox broth. Plasmid pMAB69 encodes the *hilE-lacZY* reporter. Plasmd pMAB102 encodes the *hilE-lacZY* reporter with putative FimYZ binding sites deleted from the promoter. Plasmid pISF182 encodes functional *fimY* and *fimZ* genes. β-Galactosidase assays were performed at least three times.

the putative FimYZ binding sites, increased in *E. coli*, we examined the behavior of the *hilE-lacZY* reporter plasmids in *S. enterica* serovar Typhimurium. As shown in Fig. 6B, the *fimYZ*-overexpressing plasmid pISF182 increased *hilE-lacZY* expression greater than fivefold in *S. enterica* serovar Typhimurium. However, the *hilE* promoter deletion construct was unresponsive to overexpression of *fimYZ*, and the levels of expression of *hilE* in the pMAB102 deletion plasmid remained low when present in *S. enterica* serovar Typhimurium. We therefore conclude that the AATAAGA tandem repeat in the *hilE* promoter is required for *fimYZ* induction of *hilE* transcription in *S. enterica* serovar Typhimurium.

Mapping of the *hilE* transcriptional start site. With the above information, we wanted to confirm that the *hilE* transcriptional start site was in close proximity to the putative FimYZ binding sites. With a primer extension assay, we mapped the transcriptional start site of the *hilE* mRNA. As shown in Fig. 7, the *hilE* transcript initiates at two independent sites, designated P1 and P2. The first transcript, designated P1, initiates at an adenosine which is 55 bp upstream of the putative ATG start codon. The P1 transcript starts 147 bp downstream of the putative FimYZ binding site motif. With the FimYZ binding site being quite far upstream of the P1 transcript, it seemed unlikely that the binding of FimYZ would play a significant role in activating transcription of the P1 *hilE* mRNA transcript. While speculative, it is possible that the increase in *hilE* expression seen in the pMAB102 *hilE* promoter deletion plasmid may be due to the increased availability of this site for *hilE* mRNA initiation.

The second mRNA transcript, designated P2, initiates at an adenosine that is 148 bp upstream of the putative ATG start codon. The P2 mRNA transcript starts 54 bp downstream of the putative *fimZ* binding site motif. The positioning of the putative FimYZ binding site in relation to the P2 transcriptional mRNA start site would seem to make it more likely to be the FimYZ binding site that is involved in activating *hilE* transcription.

To test this hypothesis, we performed primer extension analysis on mRNA isolated from an *E. coli* strain carrying

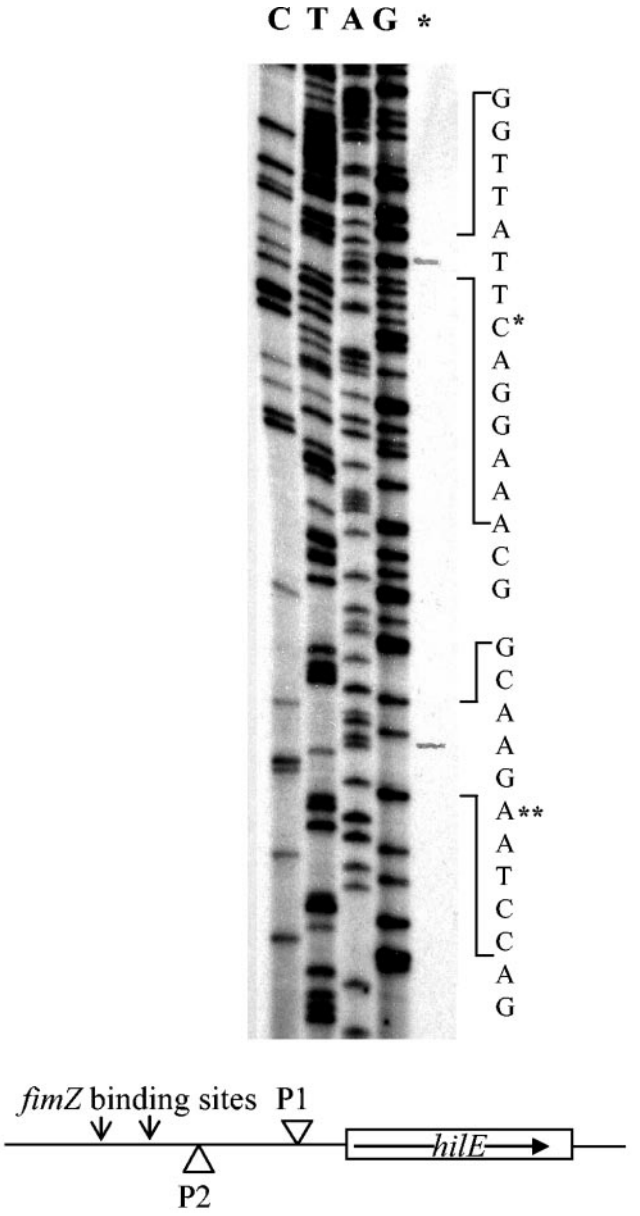

FIG. 7. Mapping the *hilE* transcriptional start site in relation to the putative translation start site for *hilE* and the putative FimYZ binding site. The primer extension products from pMAB69 and pMAB102 were primed with hilE23 and run alongside dideoxy sequencing reactions (lanes marked CTAG). The 5′ end of the *hilE* mRNA appears to initiate at two positions. The first mRNA initiates at an adenosine 55 bp upstream of the putative ATG start codon. The second transcription start site initiates at an adenosine 148 bp upstream of the putative ATG start codon. This second transcript starts 54 bp downstream of the putative FimYZ binding sites.

pMAB102, the *hilE* promoter deletion plasmid. The results of this assay seem to confirm our hypothesis. The primer extension product from the P1 *hilE* mRNA template indicated that mRNA was transcribed at a low level from this promoter, but no primer extension product was visible from the P2 promoter template on the sequencing gel (data not shown). With the results from the β-galactosidase assay and from mapping the *hilE* transcription start sites, our data indicate that FimYZ do bind to the *hilE* promoter and act to initiate the transcription of the *hilE* mRNA, leading to increased *hilE* expression within the bacterial cell.

DISCUSSION

The ability of *Salmonella enterica* serovar Typhimurium to invade occurs through the combined action of the secreted effector proteins being transported through a functional type III secretion system (15, 16). Since the process of invasion requires the expression of many different proteins, the bacteria require that the expression of these proteins be tightly regulated. The invasive phenotype is regulated by a variety of environmental conditions. A significant amount of work has been devoted to studying the genes that control the regulation of SPI-1. Characterization of *hilA* and *invF* has shown that the products of these genes are critical to the regulation of invasion as they are the transcriptional activators of the type III secretion system and secreted effector proteins (4, 29). Other genes within SPI-1, *hilD* and *hilC*, have also been identified as important regulators of *hilA* (19, 42, 43). Research has continued to identify other genes that play roles in the ability to activate or repress the transcription of *hilA*.

We have identified the *hilA* repressor, *hilE*, as being important in the control of *hilA* expression (6, 22). In this study, we performed experiments in an effort to identify genes that play a role in the activation of *hilE* expression and the subsequent repression of *hilA* expression and *Salmonella* invasiveness. This search has identified the fimbrial activator genes *fimYZ* as important transcriptional activators of *hilE* (55).

In this paper, we have found that the *hilE* gene is not expressed in *E. coli* but is significantly induced in *S. enterica* serovar Typhimurium, indicating that a specific factor is required for its activation. We observed that expression of *hilE-lacZY* is lowest as the bacterial culture begins logarithmic growth. The early logarithmic stage of growth has been shown by several groups, including our own, to be the point at which invasion gene expression is maximally induced by the *hilA* transcriptional activator (4, 5, 29, 41). Subsequently, *hilE* expression is induced as the bacteria approach the stationary phase of growth. While it is unclear why *S. enterica* serovar Typhimurium represses invasion gene expression at later stages of the growth cycle, this observation is also consistent with published work. Combined, these observations suggest that *hilE*-repressing activity is likely to be controlled by signals generated during the late mid-log and stationary phases of growth.

Subsequent work revealed that the *fimYZ* genes are factors that regulate *hilE* transcription. Overexpression of *fimYZ* induced *hilE* transcription 31-fold in *E. coli* and 5.1-fold in *S. enterica* serovar Typhimurium. Both *fimY* and *fimZ* are required for *hilE* activation. Assays were performed in which only *fimY* or *fimZ* was overexpressed, and in neither instance did *hilE-lacZY* levels increase. These findings are consistent with other research results in which the expression of the type 1 fimbrial gene operon did not occur without both *fimY* and *fimZ* being present (50, 56).

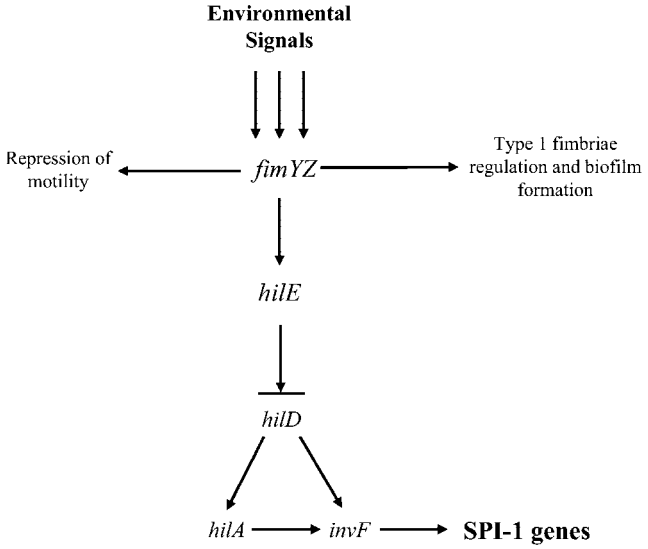

FIG. 8. Model for processing environmental signals that affect SPI-1 invasion gene expression. An environmental signal activates (or deactivates) FimYZ, which then activate (or deactivate) *hilE* transcription. Activation of *fimYZ* also leads to expression of type 1 fimbriae and an adhesive phenotype. The activation of *hilE* transcription produces HilE protein, which then leads to repression of *hilA* expression and subsequent expression of the other SPI-1 genes through its interaction with the HilD regulatory protein.

Work on the *fimY* gene has found that a *fimY S. enterica* serovar Typhimurium mutant is nonfimbriate (50). Furthermore, transcriptional studies have revealed that *fimA*, *fimY*, and *fimZ* all require functional *fimY* and *fimZ* genes for transcription, indicating that both are positive regulators (50, 56). Interestingly, *fimZ* has also been implicated in regulating bacterial motility. In those experiments, overexpression of *fimZ* alone significantly decreased *S. enterica* serovar Typhimurium motility (14). To ensure that *fimYZ*-mediated effects on *hilE* were transmitted through the invasion regulatory cascade, we examined what effects the overexpression or deletion of *fimYZ* would have on *hilA* expression. β-Galactosidase assays showed that increased activation of *fimYZ* led to a 3.3-fold decrease in *hilA* reporter expression. The deletion of *fimZ* from *S. enterica* serovar Typhimurium increases *hilA* reporter expression by 1.6-fold. Deletion of *hilE* alleviated the effects of *fimYZ* overexpression on the *hilA* reporter. Furthermore, when *fimYZ* was overexpressed, *Salmonella* invasiveness was reduced 100-fold, which was reversed by deletion of *hilE*.

Our results indicate that *fimYZ* play an active role in the control of *S. enterica* serovar Typhimurium pathogenesis, and we propose a working model for *Salmonella* invasion gene regulation that incorporates these newly described functions of *fimYZ* (Fig. 8). The *fimYZ* genes were originally identified as regulators that are required for expression of type 1 fimbriae (47, 55). More recently, it has been shown that the level of *fimZ* activation is inversely related to the motility of *S. enterica* serovar Typhimurium (14). Our work now shows that the *fimYZ*

genes also control the expression of *hilE*. Induction of *hilE* transcription, via *fimYZ*, leads to repression of *hilA* expression with corresponding repression of downstream invasion genes. These findings highlight that *fimYZ* are components of a global regulon in *S. enterica* serovar Typhimurium that potentially controls the expression of a wide range of phenotypes. Future efforts in the laboratory will be directed at characterizing the role of these regulators in pathogenic *S. enterica* serovar Typhimurium.

Analysis of the FimZ amino acid sequence reveals some features about the protein. First, FimZ is homologous to known response regulators. However, unlike most other two-component regulator systems, a sensor kinase protein partner has not been identified for FimZ (56). FimZ has sequences that resemble phosphorylation sites described for other response regulators, although the protein has not been proven to be phosphorylated. Past studies on *S. enterica* serovar Typhimurium pathogenesis have identified other two-component regulatory systems that have the ability to regulate *hilA* expression. A *phoQ* mutation (*pho-24*) that causes the *phoPQ* two-component system to be hyperactivated has been shown to superrepress *hilA* expression and invasion (5, 37, 40). The *phoPQ* two-component system responds to magnesium and calcium concentrations within the environment. When magnesium levels are at micromolar levels, PhoQ becomes activated via phosphorylation and subsequently transfers phosphate to PhoP in a phosphorelay reaction to activate the response regulator (24, 28, 51). The mechanism by which the *pho-24* mutation exerts its effect on *hilA* transcription is currently unknown.

The *phoBR* system has also been identified as a two-component regulatory system that alters *hilA* expression levels (32). This system responds to extracellular phosphate levels. In low-phosphate conditions, the *pho* regulon becomes upregulated, leading to increased expression of many different gene operons (52). It has been speculated that phosphate levels within the small intestine are elevated which leads to repression of the *phoBR* system and the upregulation of *hilA* (32). Analysis of the gene regulated by the *phoBR* two-component system has shown that PhoB binds to a site known as the *pho* box and that, in many cases, binding increases when the PhoB protein is phosphorylated (21, 33). A screen of the *fimZ* promoter has found, upstream of the *fimZ* transcriptional start site, a sequence that is 78% identical to the *pho* box (unpublished data). This suggests that *phoBR* may be involved in regulating *fimZ*. Activation of *fimZ* by *phoBR* could lead to increased *hilE* expression and subsequent repression of *hilA*. Future research will be directed at determining what effects, if any, the *phoBR* and *phoPQ* two-component systems have on *fimZ* and *hilE* expression.

ACKNOWLEDGMENTS

We thank Jennifer Boddicker and Nate Ledeboer for careful review of the manuscript.

REFERENCES

1. **Akbar, S., L. M. Schechter, C. P. Lostroh, and C. A. Lee.** 2003. AraC/XylS family members, HilD and HilC, directly activate virulence gene expression independently of HilA in *Salmonella typhimurium.* Mol. Microbiol. **47:**715–728.
2. **Altier, C., M. Suyemoto, and S. D. Lawhon.** 2000. Regulation of *Salmonella enterica* serovar Typhimurium invasion genes by *csrA.* Infect. Immun. **68:** 6790–6797.
3. **Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer.** 2000. Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. Mol. Microbiol. **35:**1872–1882.
4. **Bajaj, V., C. Hwang, and C. A. Lee.** 1995. *hilA* is a novel *ompR*/*toxR* family member that activates the expression of *Salmonella typhimurium* invasion genes. Mol. Microbiol. **18:**715–727.
5. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703–714.
6. **Baxter, M. A., T. F. Fahlen, R. L. Wilson, and B. D. Jones.** 2003. HilE interacts with HilD and negatively regulates *hilA* transcription and expression of the *Salmonella enterica* serovar Typhimurium invasive phenotype. Infect. Immun. **71:**1295–1305.
7. **Behlau, I., and S. I. Miller.** 1993. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. **175:**4475–4484.
8. **Boddicker, J. D., N. A. Ledeboer, J. Jagnow, B. D. Jones, and S. Clegg.** 2002. Differential binding to and biofilm formation on, HEp-2 cells by *Salmonella enterica* serovar Typhimurium is dependent upon allelic variation in the *fimH* gene of the *fim* gene cluster. Mol. Microbiol. **45:**1255–1265.
9. **Boddicker, J. D., B. M. Knosp, and B. D. Jones.** 2003. Transcription of the *Salmonella* invasion gene activator, *hilA*, requires HilD activation in the absence of negative regulators. J. Bacteriol. **185:**525–533.
10. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
11. **Chuang, S. E., D. L. Daniels, and F. R. Blattner.** 1993. Global regulation of gene expression in *Escherichia coli.* J. Bacteriol. **175:**2026–2036.
12. **Clegg, S., and G. F. Gerlach.** 1987. Enterobacterial fimbriae. J. Bacteriol. **169:**934–938.
13. **Clegg, S. and D. L. Swenson.** 1994. Fimbriae, p. 105–114. In P. Klemm (ed.), Adhesion, genetics, biogenesis, and vaccines. CRC Press, Boca Raton, Fla.
14. **Clegg, S., and K. T. Hughes.** 2002. FimZ is a molecular link between sticking and swimming in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **184:** 1209–1213.
15. **Collazo, C. M., and J. E. Galán.** 1997. The invasion-associated type III system of *Salmonella typhimurium* directs the translocation of Sip proteins into the host cell. Mol. Microbiol. **24:**747–756.
16. **Darwin, K. H., and V. L. Miller.** 1999. Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. Clin. Microbiol. Rev. **12:**405–428.
17. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
18. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced Bacterial Genetics: A manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Eichelberg, K., W. D. Hardt, and J. E. Galán.** 1999. Characterization of SprA, an AraC-like transcriptional regulator encoded within the *Salmonella typhimurium* pathogenicity island 1. Mol. Microbiol. **33:**139–152.
20. **Ellermeier, C. D., and J. M. Slauch.** 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **185:**5096–5108.
21. **Ellison, D. W., and W. R. McCleary.** 2000. The unphosphorylated receiver domain of PhoB silences the activity of its output domain. J. Bacteriol. **182:** 6592–6597.
22. **Fahlen, T. F., N. Mathur, and B. D. Jones.** 2000. Identification and characterization of mutants with increased expression of *hilA*, the invasion gene transcriptional activator of *Salmonella typhimurium.* FEMS Immunol. Med. Microbiol. **28:**25–35.
23. **Fahlen, T. F., R. W. Wilson, J. D. Boddicker, and B. D. Jones.** 2001. Hha is a negative modulator of *hilA* transcription, the *Salmonella typhimurium* invasion gene transcriptional activator. J. Bacteriol. **183:**6620–6629.
24. **Groisman, E. A.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. **183:**1835–1842.
25. **Hultgren, S. J., S. Normark, and S. N. Abraham.** 1991. Chaperone-assisted assembly and molecular architecture of adhesive pili. Annu. Rev. Microbiol. **45:**383–415.
26. **Johnston, C., D. A. Pegues, C. J. Hueck, A. Lee, and S. I. Miller.** 1996. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. Mol. Microbiol. **22:**715–727.
27. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect. Immun. **62:**3745–3752.
28. **Kato, A., H. Tanabe, and R. Utsumi.** 1999. Molecular characterization of the PhoP-PhoQ two-component system in *Escherichia coli* K-12: identification of extracellular Mg^{2+}-responsive promoters. J. Bacteriol. **181:**5516–5520.
29. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. USA **89:**1847–1851.
30. **Lefebvre, B., P. Formstecher, and P. Lefebvre.** 1995. Improvement of the gene splicing overlap (SOE) method. BioTechniques **19:**186–188.

31. **Lodge, J., J. Fear, S. Busby, P. Gunasekaran, and N. R. Kamini.** 1992. Broad host range plasmids carrying the *Escherichia coli* lactose and galactose operons. FEMS Microbiol. Lett. **74:**271–276.
32. **Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee.** 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar typhimurium. J. Bacteriol. **182:**1872–1882.
33. **Makino, S., C. Sasakawa, K. Kamata, T. Kurata, and M. Yoshikawa.** 1986. A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *S. flexneri* 2a. Cell **46:**551–555.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852–856.
36. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Miller, S. I., A. M. Kurkal, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
38. **Moore, A. E., L. Sabachewsky, and H. W. Toolan.** 1955. Culture characteristics of four permanent lines of human cancer cells. Cancer Res. **15:**598.
39. **Olekhnovich, I. N., and R. J. Kadner.** 2002. DNA-binding activities of the HilC and HilD virulence regulatory proteins of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **184:**4148–4160.
40. **Pegues, D. A., M. J. Hantman, I. Behlau, and S. I. Miller.** 1995. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. Mol. Microbiol. **17:**169–181.
41. **Penheiter, K. L., N. Mathur, D. Giles, T. Fahlen, and B. D. Jones.** 1997. Non-invasive *Salmonella typhimurium* mutants are avirulent because of an inability to enter and destroy M cells of ileal Peyer's patches. Mol. Microbiol. **24:**697–709.
42. **Rakeman, J. L., H. R. Bonifield, and S. I. Miller.** 1999. A HilA-independent pathway to *Salmonella typhimurium* invasion gene transcription. J. Bacteriol. **181:**3096–3104.
43. **Schechter, L. M., S. M. Damrauer, and C. A. Lee.** 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the *hilA* promoter. Mol. Microbiol. **32:**629–642.
44. **Schechter, L. M., and C. A. Lee.** 2001. AraC/XylS family members, HilC and HilD, directly bind and derepress the *Salmonella typhimurium hilA* promoter. Mol. Microbiol. **40:**1289–1299.
45. **Shi, J., and D. P. Biek.** 1995. A versatile low-copy-number cloning vector derived from plasmid F. Gene **164:**55–58.
46. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
47. **Swenson, D. L., and S. Clegg.** 1992. Identification of ancillary *fim* genes affecting *fimA* expression in *Salmonella typhimurium.* J. Bacteriol. **174:**7697–7704.
48. **Takaya, A., T. Tomoyasu, A. Tokumitsu, M. Morioka, and T. Yamamoto.** 2002. The ATP-dependent *lon* protease of *Salmonella enterica* serovar Typhimurium regulates invasion and expression of genes carried on *Salmonella* pathogenicity island 1. J. Bacteriol. **184:**224–232.
49. **Thanassi, D. G., and S. J. Hultgren.** 2000. Assembly of complex organelles: pilus biogenesis in gram-negative bacteria as a model system. Methods **20:** 111–126.
50. **Tinker, J. K., and S. Clegg.** 2000. Characterization of FimY as a coactivator of type 1 fimbrial expression in *Salmonella enterica* serovar Typhimurium. Infect. Immun. **68:**3305–3313.
51. **Vescovi, E. G., Y. M. Ayala, C. E. Di, and E. A. Groisman.** 1997. Characterization of the bacterial sensor protein PhoQ. Evidence for distinct binding sites for Mg^{2+} and Ca^{2+}. J. Biol. Chem. **272:**1440–1443.
52. **Wanner, B. L.** 1996. Phosphorus assimilation and control of the phosphate regulon, p. 1357–1381. In. F. C. Neidhardt, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
53. **Wilson, R. L., S. J. Libby, A. M. Freet, J. D. Boddicker, T. F. Fahlen, and B. D. Jones.** 2001. Fis, a DNA nucleoid-associated protein, is involved in *Salmonella typhimurium* SPI-1 invasion gene expression. Mol. Microbiol. **39:** 79–88.
54. **Wray, C., and W. J. Sojka.** 1978. Experimental *Salmonella typhimurium* in calves. Res. Vet. Sci. **25:**139–143.
55. **Yeh, K. S., L. S. Hancox, and S. Clegg.** 1995. Construction and characterization of a *fimZ* mutant of *Salmonella typhimurium.* J. Bacteriol. **177:**6861–6865.
56. **Yeh, K. S., J. K. Tinker, and S. Clegg.** 2002. FimZ binds the *Salmonella typhimurium fimA* promoter region and may regulate its own expression with FimY. Microbiol. Immunol. **46:**1–10.

Editor: J. B. Bliska