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HilE Interacts with HilD and Negatively Regulates *hilA* Transcription and Expression of the *Salmonella enterica* Serovar Typhimurium Invasive Phenotype

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The ability of *Salmonella enterica* serovar Typhimurium to traverse the intestinal mucosa of a host is an important step in its ability to initiate gastrointestinal disease. The majority of the genes required for this invasive characteristic are encoded on *Salmonella* pathogenicity island 1 (SPI1), and their expression is controlled by the transcriptional activator HilA, a member of the OmpR/ToxR family of proteins. A variety of genes (*hilC*, *hilD*, *fis*, *sirA/barA*, *csrAB*, *phoB*, *fadD*, *envZ/ompR*, *fliZ*, *hilE*, *ams*, *lon*, *pag*, and *hha*) have been identified that exert positive or negative effects on *hilA* expression, although the mechanisms by which these gene products function remain relatively unclear. Recent work indicates that the small DNA-binding protein, Hha, has a significant role in repressing *hilA* transcription and the invasive phenotype, particularly in response to osmolarity signals. We have characterized the *Salmonella*-specific gene, *hilE*, and found that it plays an important regulatory role in *hilA* transcription and invasion gene expression. Mutation of *hilE* causes derepression of *hilA* transcription, and overexpression of *hilE* superrepresses *hilA* expression and the invasive phenotype. Bacterial two-hybrid experiments indicate that the HilE protein interacts with HilD, suggesting a possible mechanism for HilE negative regulation of *hilA* gene expression and the *Salmonella* invasive phenotype. Finally, we have found that the *hilE* gene resides on a region of the serovar Typhimurium chromosome that has many characteristics of a pathogenicity island.

Salmonella enterica serovar Typhimurium is a gram-negative bacterium that primarily causes enteropathogenic infections that range from self-limiting gastroenteritis of the small intestine to systemic disease of the host lymphatic system. Pathogenic salmonellae first enter the host environment after the ingestion of contaminated food or water. Subsequently, the bacteria travel into the small intestine, where they invade the specialized M cells of the epithelium of Peyer's patches (10, 33, 50) and the absorptive enterocytes of the epithelium (58). After epithelial invasion, serovar Typhimurium organisms move quickly to the draining regional lymph nodes of the mouse before spreading to the liver and spleen, where rapid growth results in lethality (32, 39, 58). Within hosts to which serovar Typhimurium is not adapted, the bacteria remain localized to the intestinal epithelium where their presence is associated with inducing proinflammatory and cytotoxic signal transduction pathways, as well as neutrophil migration and recruitment (9, 38, 43, 46, 61).

A critical step in the establishment of *Salmonella* infection is the ability of the bacteria to invade the apical surface of epithelial cells within the small intestine. The entry process occurs after the bacteria bind to the surface of the host cell and induce actin rearrangements (ruffles) on the apical membrane that engulf the bacteria (21, 24). The majority of the genetic elements required for the invasive phenotype of *S. enterica* serovar Typhimurium (50) localize to a 40-kb region of the chromosome at centisome 63 termed *Salmonella* pathogenicity island 1 (SPI1) (reviewed in reference 12). Many of these SPI1 genes encode components of a type III secretion system, a system that functions by translocating specific *Salmonella* proteins into the host cell targeted for bacterial entry (29). Genes, both inside and outside of SPI1, encode secreted effector proteins that are responsible for inducing the host cell cytoskeletal changes that lead to uptake of the bacteria (22, 27, 28, 35, 63).

The expression of the serovar Typhimurium invasion genes is tightly regulated by a variety of environmental signals, including oxygen levels, osmolarity, pH, and phase of growth, that are believed to modulate the *Salmonella* invasive phenotype within the host intestinal environment (18, 25, 36, 56). The SPI1-encoded *hilA* gene encodes an OmpR/ToxR-like transcriptional activator that appears to play a central role in modulating the expression of the type III secretion apparatus proteins and the secreted effector proteins in response to environmental signals (5, 6). Importantly, the expression of *hilA* is modulated by the same environmental conditions that regulate the invasive phenotype. In addition, overexpression of *hilA* confers a hyperinvasive phenotype, and overexpression of *hilA* also counteracts the effects of repressing signals (37). Therefore, modulation of *hilA* expression by environmental signals appears to be a primary method of regulating the invasive phenotype of *Salmonella* (5, 6, 37). Results from our laboratory and from others (5, 50) reveal that null mutations in *hilA* cause a dramatic attenuation of invasion of tissure culture cells and M cells of the Peyer's patches, in addition to attenuating mouse virulence after oral inoculation. These results establish that *hilA* is required for the transcriptional activation

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Source or reference
Strains		
E. coli DH12S	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) F′ *lacI*^{q} ΔM15	Gibco-BRL
S. enterica serovar Typhimurium		
BJ66	*orgA*::Tn*5lacZY* in SL1344	31
BJ70	*hilA*::Tn*5lacZY* in SL1344; Tet^r	50
BJ1714	*invF*::Tn*5lacZY* in VV302; Tet^r	62
BJ1894	*orgA*::Tn*5lacZY* in VV302; Tet^r	This work
BJ2121	*hilE2*::Tn*5* in SL1344; Kan^r	This work
BJ2390	*hilE*::*cam* in BJ1714; Cm^r	This work
BJ2462	*hilE*::*cam* in SL1344; Cm^r	This work
BJ2492	*hilA*::Tn*5lacZY-080 hilE*::*cam* in SL1344; Tet^r Cm^r	This work
EE658	*hilA*::Tn*5lacZY-080* in SL1344; Tet^r	6
TF76	*hilE2*::*Tn5* in BJ70; Tet^r Kan^r	This work
VV302	Δ*hilA-523* in SL1344	5
Plasmids		
pACYC184	Cm^r Tet^r	8
pJB1	pZC320 vector encoding serovar Typhimurium *hilD* from its own promoter; Amp^r	62
pJB3	pZC320 vector encoding serovar Typhimurium *hilD* driven by the *lac* promoter; Amp^r	Jones lab
pLS31	pRW50 vector encoding −497 to +420 of *hilA* fused to *lacZY;* Tet^r	54
pMAB59	pGEM-T vector carrying *hilE;* Amp^r	This work
pMAB60	pACYC derivative carrying *hilE;* Cm^r	This work
pMAB62	pZC320 carrying *hilE;* Amp^r	This work
pMAB70	pACYC184 vector in which the *tet* gene has been deleted; Cm^r	This work
pMMB66EH	IncQ *lacI*^{q} *bla* (Amp^r)	23
pMRP9-1	GFP-expressing plasmid; Cm^r	E. P. Greenberg
pTF140	pMMB66EH vector carrying *hilE;* Amp^r	This work
pZC320	mini-F; Amp^r	57

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

of essential invasion genes and therefore plays a crucial role in *Salmonella* invasion and virulence.

Due to the central role of *hilA* in invasion gene activation, many groups have searched for genes that affect *hilA* expression. A large number of positive effectors have been identified that include *hilC*/*sirC*/*sprA* (17, 52, 54); *hilD* (54); *sirA*/*barA* (2, 30); *fis* (62); *csrAB* (2); and *phoB*, *fadD*, and *fliZ* (42). Two of these genes, *hilC* and *hilD*, encode AraC-like transcriptional activators that activate *hilA* transcription in response to specific conditions (54) and have recently been shown to bind to the upstream regulatory sequences of *hilA* (55). The regulatory activities of FadD, FliZ, PhoB, and EnvZ/OmpR were also recently shown to require the *hilA* upstream regulatory sequences (55). Additional work has revealed that EnvZ/OmpR affected the transcription of *hilC* but none of the identified regulators had any significant effect on *hilD* transcription. It has been hypothesized that these positive regulators modulate *hilD* posttranscriptionally or that they modulate the activity of *hilA*-negative regulators (41, 49). A more complete understanding of these and other positive *hilA* regulators awaits further experimentation.

Negative regulators of *hilA* have also been identified. Transposon mutagenesis has been used to identify *ams*, *hilE*, and *pag* as negative modulators of *hilA* expression (19). Another search for negative modulators of *hilA* transcription was conducted by introducing a *S. enterica* serovar Typhimurium chromosomal gene bank into a serovar Typhimurium *hilA*::Tn*5lacZY* reporter strain and screening for a decrease in the expression of the *hilA* reporter. That work identified the *hha* gene as a negative regulator of *hilA* since it was found to repress both *hilA* expression and the *Salmonella* invasive phenotype (20). Further work demonstrated that purified Hha protein could bind to a *hilA* DNA promoter fragment.

In the present study, we have focused on characterizing the negative regulator, *hilE*. We have found that overexpression of the *hilE* gene significantly represses *hilA* expression and a *hilE* mutant significantly overexpresses *hilA*. Correlative effects are observed on the invasive phenotype of serovar Typhimurium. A series of experiments have been performed to determine the mechanism by which HilE represses *hilA* expression. Efforts to demonstrate binding of purified HilE protein to the *hilA* promoter were unsuccessful. However, bacterial two-hybrid studies revealed a binding interaction between the HilE and HilD proteins, suggesting that the HilE mechanism of *hilA* regulation is through interactions with the HilD activator. Finally, we have discovered that the *hilE* gene resides on a region of the serovar Typhimurium chromosome that possesses the characteristics of a pathogenicity island.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in the present study are shown in Table 1. Bacteria were routinely grown in Luria broth (LB; Gibco-BRL) containing the appropriate antibiotics added at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 25 μg/ml, streptomycin at 100 μg/ml, and tetracycline at 25 μg/ml. *S. enterica* serovar Typhimurium strains were grown in LB (1% NaCl) or TYE broth (0% NaCl) for high-osmolarity or low-osmolarity conditions, respectively. High-oxygen-repressing conditions were created by inoculating 5 ml of LB or TYE broth with 10 μl of a stationary-phase bacterial culture. The culture was shaken at 225 rpm at

TABLE 2. PCR primers used in this study

Primer	Sequence
hilE3B	5′-AAGCTTCTTCAATACCGTCCAGTT-3′
hilE5′	5′-GGATCCTTTGCGGATTACTGCCGTT-3′
hilE8	5′-GGATCCATACAGAGACACCAACGAAATG-3′
hilE9	5′-CGGCCGGTCCTCATCGCCACAGCG-3′
hilE5W′	5′-GTTATAGCAGATTGTCGGTATTTAATCTGGTATACAGAGACACCAACGAACATATGAATATCCTCCTTA-3′
hilE3W′	5′-ATTTCGCTATACAGCATCGCCCACTGCGAGTCCGCAAGCTTGTTTTGTCCGTGTAGGCTGGAGCTGCTTC-3′
hns5W′	5′-TCTATTATTAGCTCAACAAACCACCCCAATATAAGTTTGAGATTACTACACATATGAATATCCTCCTTA-3′
hns3W′	5′-GGCAAAAAAAATCCCGCCAGCGGCGGGATTTTAAGCATCCAGGAAGTAAAGTCTAGGCTGGAGCTGCTTC-3′

37°C until an optical density at 600 nm (OD_{600}) of 0.1 to 0.14 (~10^8 CFU/ml) was reached. Low-oxygen conditions were created by inoculating 5 ml of LB or TYE broth with 10 μl of a stationary-phase culture, followed by incubation statically overnight at 37°C until an OD_{600} of 0.4 to 0.5 was reached, which corresponds to about 4×10^8 to 5×10^8 CFU/ml (31, 50). Strains with the *hilA-lacZY* reporter plasmid pLS31, the *hilE*-expressing plasmid pMAB60, the *hilD*-expressing plasmids pJB1 or pJB3, as well as other control plasmids, were grown in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Plasmid constructions. A plasmid carrying a functional *hilE* gene was obtained by amplifying the *hilE* gene from the *Salmonella* chromosome with PCR by using the primers hilE8 and hilE9 (see Table 2 for primer sequences) and cloning the 553-bp PCR product into pGEM-T (Promega) to create plasmid pMAB59. The *hilE* gene was subcloned into the cloning vectors pMMB66EH (23) and pACYC184 (8) to create the low-copy-number *hilE* plasmids pTF140 and pMAB60, respectively, and into the single-copy vector pZC320 (57) to produce pMAB62. The control vector pMAB70 was created by cutting pACYC184 with *Bsp*MI and *Eco*RV, thereby deleting the *tet* gene from the original vector. The *hilD*-expressing plasmid pJB1 contains 1,039 bp upstream of the putative *hilD* translation initiation codon and the entire 930 bp encompassing the *hilD* coding region (62). The *hilD*-expressing plasmid pJB3 was created by PCR amplifying *hilD*, including the upstream ribosome-binding site, from *S. enterica* serovar Typhimurium. This PCR fragment was then cloned into the single-copy vector pZC320 so that *hilD* can be expressed from the *lac* promoter (7).

Plasmid cloning vectors used in the bacterial two-hybrid experiments were pDP804 and pMS604 as described by Dmitrova et al. (15). Derivatives of these plasmids, containing HilE or HilD protein fusion constructs, were made as follows. A set of PCR primers were synthesized with which to amplify the *hilE* gene so that when it was cloned into pDP804 it formed a fusion protein with $LexA_{1-87}$408, and another set of primers was used to amplify the *hilD* gene so that when cloned into pMS604 it formed a fusion protein with $LexA_{1-87}$. These plasmid constructs were sequenced prior to use, confirmed to have the desired fusion protein sequence, and then introduced into the *Escherichia coli* reporter strain SU202 for use in the experiment.

Restriction digestions, DNA ligations, bacterial electroporations, and PCR amplifications reactions were done by using standard conditions and according to standard protocols.

Computer mapping studies and DNA sequencing. The sequence information for *S. enterica* serovar Typhimurium was obtained from the Washington University School of Medicine genome sequence database (http://genome.wustl.edu/gsc/bacterial/salmonella.shtml), and the genome sequence information for *S. enterica* serovar Typhi came from the database maintained at the Sanger Centre (http://www.sanger.ac.uk/Projects/S_typhi/blast_server.shtml). BLAST searches (4) were conducted on sequences found at the National Center for Biotechnology Information database (http://www.ncbi.nlm). Fluorescence automated sequencing (Perkin-Elmer and the University of Iowa DNA Facility) was used to sequence *hilE* to verify that mutations had not been introduced into the gene sequences during the PCR amplification and cloning process.

β-Galactosidase assays. β-Galactosidase assays were conducted on bacterial cultures by the method of Miller et al. (44).

P22-mediated transductions. Antibiotic resistant gene insertions were moved between strains by transduction with P22 HT int^- as previously described (14). Transductants were selected on LB agar containing the appropriate antibiotic and 10 mM EGTA to prevent reinfection by P22. Transductants were purified twice on LB EGTA agar prior to use of the colonies.

Tissue culture conditions and cell invasion assays. HEp-2 tissue culture cells (47) were maintained in RPMI 1640 medium containing 10% fetal bovine serum. The cells were passaged every 2 to 4 days as needed. Invasion assays were conducted, with bacteria grown in various growth conditions, by using previously described protocols (31, 50).

Modified invasion assay and confocal imaging. Strains expressing green fluorescent protein (GFP) were used to infect HEp-2 cells on coverslips at a multiplicity of infection of 100. After 60 min of infection, the tissue culture medium was replaced with medium containing 100 μg of gentamicin/ml and then incubated for 90 min. Next, the HEp-2 cells were extensively washed with 1× phosphate-buffered saline (PBS) and fixed to the coverslips by 4% formaldehyde treatment, washed with 1× PBS, permeabilized with 0.2% Triton X-100, and stained with rhodamine phalloidin (Molecular Probes) at a 1:500 dilution. The coverslips were washed and placed cell-side down into 3 μl of VectaShield anti-quench medium (Vector Laboratories, Inc.) on a microscope slide. A Bio-Rad MRC-600 confocal scanning laser microscope was employed to visualize the rhodamine stained HEp-2 cells and GFP-expressing bacteria and confocal images are presented as a composite of 10 to 15 sections taken in the *x-y* plane at 1-nm sections throughout the HEp-2 cells. In three independent experiments, the bacteria within about 100 to 150 HEp-2 cells were counted for each strain in each growth condition.

Creation of defined chromosomal mutations in the *hilE* genes. Three *hilE*::Tn*5* serovar Typhimurium mutants were previously obtained and identified by our laboratory (19). However, to eliminate the possibility of Tn*5* transposon effects, a defined Typhimurium *hilE* mutant was constructed by using the procedure described by Datsenko and Wanner (13). Briefly, PCR primers were synthesized with 50 bp of homology to the 5′ and 3′ ends of the *hilE* gene. In addition, the hilE5W′ primer was synthesized so that it carried priming site 2 of pKD3 (13), and the hilE3W′ primer was synthesized so that it carried priming site 1 of pKD3 (see Table 2 for primer sequences). PCR amplification was performed with these primers by using plasmid pKD3 as the template, and the expected 1.1-kb fragment was obtained. The linear PCR fragment was purified and electroporated into SL1344 carrying pKD46 and mutants were selected on L cam plates at 37°C. Several chloramphenicol-resistant (Cm^r), ampicillin-sensitive (Amp^s)colonies were purified and found by PCR to have the transformed fragment recombined into the *hilE* gene on the chromosome. Subsequently, the Cm^r gene within the *hilE* chromosomal sequences was excised by introduction of plasmid pCP20 (temperature-sensitive replicon, Amp^r), which expresses the *flp* recombinase gene after thermal induction. Colonies that were Cm^s and Amp^s were shown to have excised the Cm^r gene by PCR, and therefore these colonies carried a complete deletion of the *hilE* gene in the chromosome.

RESULTS

Overexpression or mutation of *hilE* affects the expression of a *hilA*::Tn*5lacZY* reporter and the ability of *Salmonella* to invade HEp-2 cells in inducing conditions. Previous work from our laboratory identified mutations in a new gene, designated *hilE*, that resulted in upregulation of a *hilA* reporter (19). In an effort to more thoroughly characterize the role of *hilE* in regulating *Salmonella* invasion gene expression, we performed experiments to measure the effect of the *hilE* gene on *hilA* expression and on the invasiveness of *S. enterica* serovar Typhimurium. The expression of a *hilA*::Tn*5lacZY* reporter was compared in three different strains: BJ70 (*hilA*::Tn*5lacZY*), TF76 (*hilA*::Tn*5lacZY*, *hilE*::Tn*5*) and TF76 pMAB62. Plasmid pMAB62 is a single-copy plasmid that expresses the *hilE* gene from the *lac* promoter. As shown in Fig. 1, BJ70 expressed 543 ± 17 U of β-galactosidase activity after growth in low-oxygen, high-osmolarity conditions, whereas strain TF76 expressed

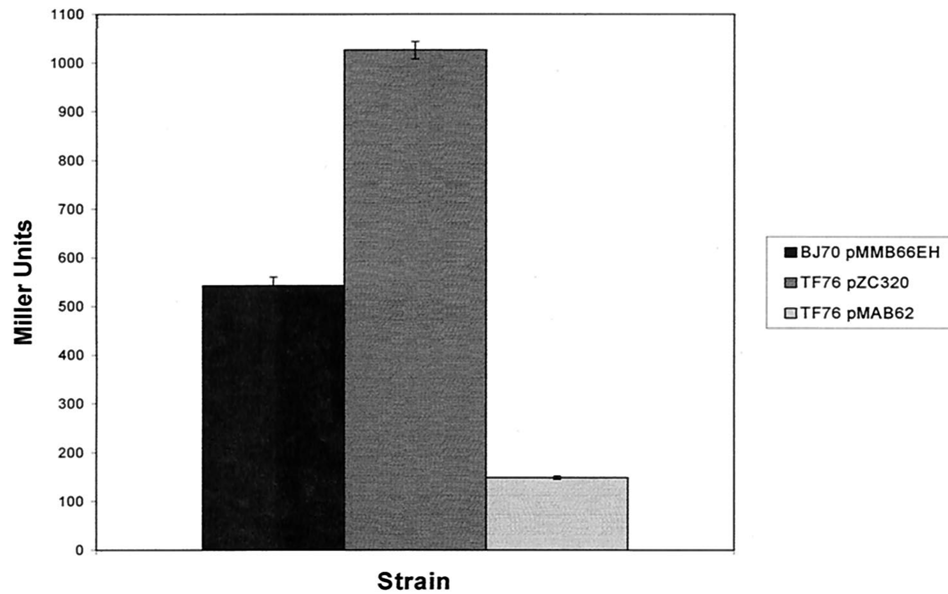


FIG. 1. Overexpression of *hilE* represses a *hilA*::Tn*5lacZY* reporter construct in *S. enterica* serovar Typhimurium. Strains were grown statically in low-oxygen, high-osmolarity conditions to an OD_{600} of ~0.5 before we quantitated the β-galactosidase activity from each strain. Strain BJ70 pMMB66EH is the parent strain that carries the *hilA*::Tn*5lacZY* reporter. Strain TF76 is an isogenic strain of BJ70 that carries a mutation in the *hilE* gene. Plasmid pMAB62 is the single-copy mini-F plasmid that expresses the *hilE* gene from the *lac* promoter.

1,027 ± 18 U after growth in the same conditions. Expression of *hilE* from the single-copy plasmid, in the absence of a functional chromosomal copy of *hilE*, resulted in 148 ± 3 U of β-galactosidase expression. Therefore, after growth under conditions that induce *hilA* the absence of *hilE* led to a 1.9-fold increase in *hilA* expression, whereas single-copy *hilE* expression from the *lac* promoter repressed *hilA* expression ~3.7-fold compared to the parent strain and ~6.9-fold compared to the *hilE* mutant strain.

Next, experiments were performed to determine whether the effects of the *hilE* mutation on the ability of serovar Typhimurium to invade HEp-2 tissue culture cells would be similar to those observed on *hilA*::Tn*5lacZY* reporter expression. The invasiveness of SL1344 carrying either empty pACYC184 vector or empty pZC320, SL1344 pMAB60, SL1344 pMAB62, and SL1344 *hilE2*::Tn*5*, were assessed in a HEp-2 tissue culture cell invasion assay after growth of the bacterial strains under inducing conditions. SL1344 pZC320 and SL1344 pACYC184 are the invasive parental controls, and the invasiveness of these strains was arbitrarily set at 100% (Fig. 2). The invasiveness of SL1344 pMAB60, which carries the *hilE* gene on a low-copy vector, was reduced to 4.5% (~22-fold reduction in invasion) and the invasiveness of strain SL1344 pMAB62, which carries the *hilE* gene on a single-copy mini-F vector, was reduced to 5.3% (~19-fold reduction in invasion). As a control, the invasiveness of the *hilE* deletion strain, BJ2121, was measured and found to increase 1.7-fold to 170%, which was consistent with previous findings from our laboratory. These results establish that relatively small changes in *hilE* expression have significant effects on both the expression of the *hilA* gene and the invasive phenotype of serovar Typhimurium for tissue culture cells. These results suggest that *hilE* exerts a negative effect on the expression of SPI1 gene expression by reducing the levels of *hilA* expression which, in turn, downregulates the expression of *hilA*-dependent invasion genes.

Mutation of *hilE* leads to significant derepression of *hilA*::Tn*5lacZY* expression and HEp-2 cell invasion in nonin-

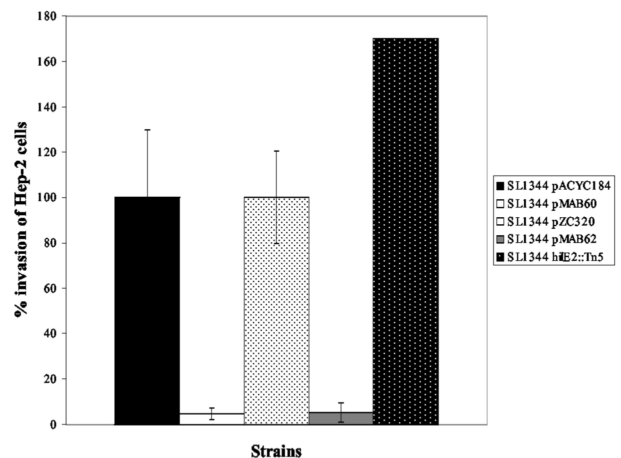


FIG. 2. Overexpression of *hilE* represses *Salmonella* tissue culture invasion after growth in inducing conditions. SL1344 is the invasive parent strain, and its invasiveness with either vector (pACYC184) or (pZC320) was compared to SL1344/pMAB60, which overexpresses *hilE* from the medium-copy plasmid, pACYC184, or SL1344/pMAB62, which overexpresses *hilE* from the single-copy plasmid, pZC320. Strains were grown in low-oxygen, high-osmolarity conditions before we assayed their ability to invade HEp-2 tissue culture cells. Invasion numbers are presented as a percentage of wild-type invasion, with wild-type invasion standardized to 100%. The SL1344 *hilE2*::Tn*5* mutant was included as a control to show the effect of mutating the chromosomal *hilE* gene on *Salmonella* invasion.

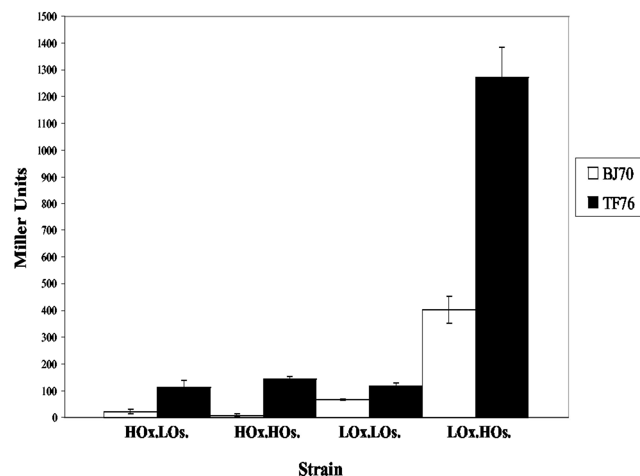


FIG. 3. Mutation of *hilE* derepresses *hilA*::Tn*5lacZY* expression in repressing environmental conditions. Cultures of BJ70 (*hilA*::Tn*5lacZY*) (□) or TF76 (*hilA*::Tn*5lacZY*, *hilE2*::Tn*5*) (■) were incubated in either high-oxygen, low-osmolarity (HOx.LOs.); high-oxygen, high-osmolarity (HOx.HOs.); low-oxygen, low-osmolarity (LOx.LOs.); or low-oxygen, high-osmolarity (LOx.HOs.) conditions before we quantitated levels of β-galactosidase from each culture. These experiments were performed three times.

ducing environmental conditions. Although the experiments described above demonstrated that a *hilE* mutation derepressed both *hilA* expression and invasion after growth in inducing conditions, we were also interested in determining what effect a mutation in *hilE* would have on *hilA* expression and invasion after growth in different repressing growth conditions. The expression of β-galactosidase from the parent strain BJ70 (*hilA*::Tn*5lacZY*) and the *hilE* derivative TF76 (*hilA*::Tn*5lacZY*) were compared after growth in the following repressing conditions: high oxygen and low osmolarity, high oxygen and high osmolarity, and low oxygen and low osmolarity. In addition, β-galactosidase was quantitated from each strain after growth in low-oxygen, high-osmolarity conditions as a control. Under conditions in which both oxygen and osmolarity were noninducing, the *hilA*::Tn*5lacZY* reporter in BJ70 gave 22 ± 7 U, and the mutation in *hilE* (TF76) increased *hilA*::Tn*5lacZY* expression 113 ±27 U (a 5.1-fold increase) (Fig. 3). When osmolarity was inducing but oxygen was noninducing, the parent strain gave 9.0 ± 6 U of activity, and *hilA*::*lacZY* expression in the *hilE* mutant increased 12.8-fold to 145 ± 8 U. Growth of BJ70 in the noninducing condition of low oxygen and low osmolarity gave 68 ± 3 U of activity, and the β-galactosidase units from the *hilE* mutant (TF76) were 1.7-fold higher at 118 ± 14 U. Expression of the *lacZ* reporter under inducing conditons (low oxygen and high osmolarity) gave 403 ± 50 U for strain BJ70 and 1,272 ± 109 U for strain TF76, an induction of 3.2-fold. These data indicate that the *hilE* mutation derepresses *hilA* expression in all three repressing conditions examined, but the derepression is most significant in response to high-oxygen conditions, when *hilA* expression was derepressed 5.1- and 12.8-fold, depending on whether the osmolarity was low (repressing) or high (inducing).

Since the *hilE* mutation significantly increased *hilA* expression in environmental conditions that were normally repressing, we examined whether the *hilE* mutation would increase tissue culture invasiveness after growth in repressing conditions. The invasiveness of SL1344 (parent strain) and BJ2462 (*hilE*) for HEp-2 tissue culture cells was compared after growth in conditions of low oxygen and low osmolarity, low oxygen and high osmolarity, high oxygen and low osmolarity, and high oxygen and high osmolarity. The invasiveness of each strain was quantitated, and the ratio of BJ2462 to SL1344 invasiveness was determined for each condition as shown in Fig. 4. Although the *hilE* mutant strain was more invasive for HEp-2 cells under all conditions tested, the increase in invasiveness of strain BJ2462 was not as much as we expected based upon the increase in *hilA* expression that we observed. Since the tissue culture invasion assay is a measure of both cellular entry and survival, we performed invasion assays with *Salmonella* that contained the GFP-expressing plasmid pMRP9-1, so that we could directly enumerate the bacteria that were within the tissue culture cells. Examination of the cells indicated that there were apparent differences in the number of cells containing the *hilE* mutant strain, as well as the numbers of bacteria within individual cells (Fig. 5). Virtually no bacteria could be detected in cells infected with SL1344 grown in conditions of high oxygen and high osmolarity or of high oxygen and low osmolarity (Fig. 5A and C), whereas it was possible to find clusters of bacteria within cells infected with BJ2462 grown in the same conditions (Fig. 5B and D). By counting 100 to 150 HEp-2 cells per environmental condition, we were able to determine that there was a 6.2-fold increase in the number of internalized BJ2462 compared to SL1344 when the bacteria were grown under high-oxygen, high-osmolarity conditions. The difference between BJ2462 and SL1344 was 15-fold when we compared bacteria that were grown under high-oxygen, low-osmolarity conditions. In low-oxygen, low-

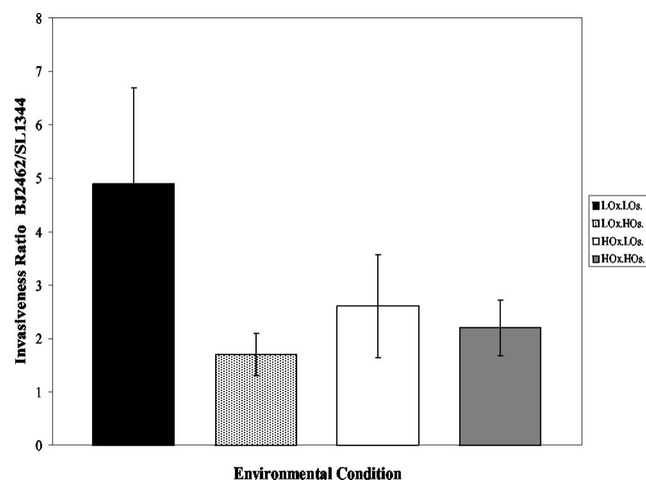
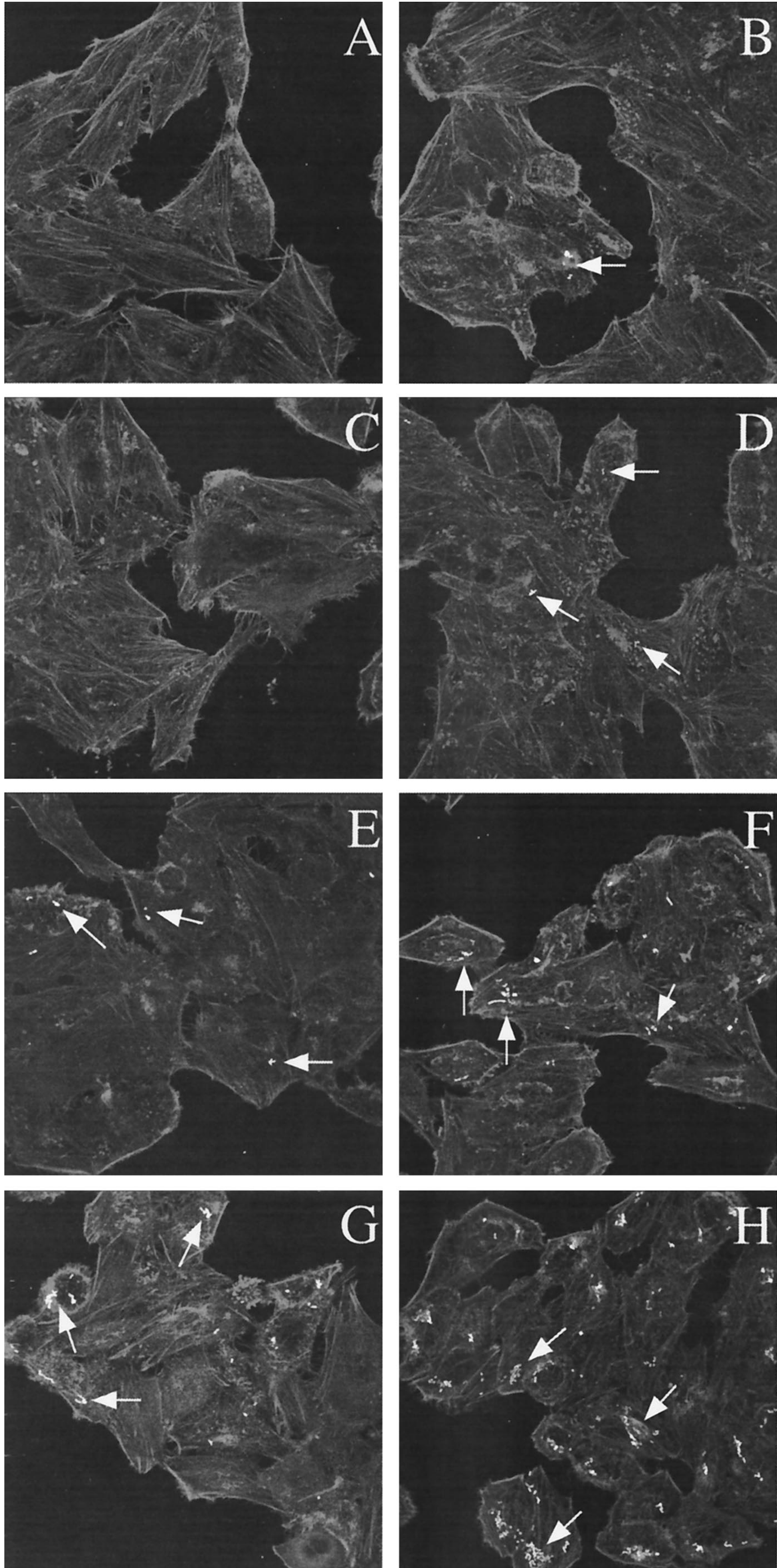


FIG. 4. *S. enterica* serovar Typhimurium with a *hilE* mutation is significantly more invasive in repressing environmental conditions than the wild-type invasive strain. The tissue culture invasiveness of the parent strain SL1344 and the *hilE* mutant BJ2462 were compared after growth in low oxygen and low osmolarity (LOx.LOs.); low oxygen and high osmolarity (LOx.HOs.), high oxygen and low osmolarity (HOx.LOs.), and high oxygen and high osmolarity (HOx.HOs.). In each experiment, the *hilE* mutant was more invasive than the wild-type strain, and the data are represented as a ratio of the percent invasion of BJ2462 to the percent invasion of SL1344 for each of the growth conditions shown.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osmolarity conditions, small groups of bacteria were observed in cells infected with SL1344 (Fig. 5E), a result similar to that seen with BJ2462 grown in either high-oxygen, high-osmolarity conditions or high-oxygen, low-osmolarity conditions. In contrast, many clusters of bacteria were observed (~6.3-fold more) for BJ2462 after growth in low-oxygen, low-osmolarity conditions (Fig. 5F). After growth in low-oxygen, high-osmolarity conditions, the difference in internalized bacteria between SL1344 and BJ2462 was ~6.4-fold (Fig. 5G and H). The discrepancies between the invasiveness determined for strains by the gentamicin assay and that by the microscopic assay are puzzling and interesting and are currently under investigation in the laboratory.

Identification of the mechanism by which *hilE* regulates *hilA* expression. In an effort to identify the mechanism by which *hilE* exerts its regulatory effect, we first analyzed the *hilE* nucleotide sequence and the HilE protein sequence by using a variety of computer programs to identify homologues and to characterize any motifs that would suggest how *hilE* functions. These searches failed to reveal any similarity to other gene or protein sequences present in the database except in *Salmonella* serovars. Next, since we had recently demonstrated that another negative regulator of *hilA*, Hha, bound to *hilA* promoter sequences (20), we tested whether purified HilE might also bind to *hilA* promoter sequences. Unfortunately, those efforts failed to demonstrate binding of HilE to *hilA* promoter sequences (data not shown).

Another possible mechanism of action for the HilE repressor is by regulating transcription of the HilD activator protein. We examined this possibility by transforming plasmids encoding *hilE*, *hilD*, and/or *hilA-lacZY* into *E. coli* DH12S and measuring the levels of β-galactosidase expression from the *hilA-lacZY* reporter. As expected, the *hilA-lacZY* reporter was not expressed at significant levels in *E. coli* DH12S (27.0 ± 6.7 Miller units; Fig. 6, lane 1) (54). When plasmids encoding the *hilD* gene under the control of the *lac* promoter or its own promoter were introduced into this strain, high-level induction of the *hilA-lacZY* reporter was observed (2,051.1 ± 397.2 U [Fig. 6, lane 3] and 625.4 ± 191.1 U [Fig. 6, lane 5], respectively). However, upon introduction of pMAB60, a plasmid encoding *hilE*, expression of *hilA-lacZY* was completely repressed (Fig. 6, lanes 4 and 6) whether *hilD* was expressed from the *lac* promoter or its own promoter. This result indicates that HilE represses *hilA* transcription by a mechanism other than modulation of *hilD* transcription since HilE was able to fully repress *hilA* even when the HilD activator was expressed from the *lac* promoter.

Two-hybrid analysis indicates that HilE and HilD interact with each other. A system has been developed that is based upon LexA protein binding to sites upstream of the *sulA* gene (15). The system relies on a wild-type LexA DNA-binding domain and a mutant LexA-binding domain that, when brought together by heterologous protein-protein interactions, dimerize and bind to an altered DNA-binding site upstream of a *sulA-lacZY* reporter. Protein-protein binding results in repression of the *sulA* promoter and a significant reduction in β-galactosidase production from the *sulA-lacZY* reporter. Plasmids were constructed, as described in Materials and Methods, that encode a $LexA_{1-87}$408-HilE fusion protein or a $LexA_{1-87}$ WT-HilD fusion protein. The *E. coli* SU202 *sulA-lacZY* reporter strain was transformed with both the plasmid encoding the $LexA_{1-87}$408-HilE fusion protein and the $LexA_{1-87}$WT-HilD fusion protein. In addition, the appropriate empty vectors were transformed into the reporter strain as controls. As seen in Table 3, the reporter strain constitutively expressed high levels of β-galactosidase (2,502 ± 47 U). When either the LexA′-HilE plasmid or the LexA-HilD plasmid was present in *E. coli* SU202 with only a control vector plasmid, there was no reduction in β-galactosidase activity, and in fact a slight increase in expression was observed in some of the experiments. However, when both the LexA′-HilE plasmid and the LexA-HilD plasmid were present in the *E. coli* reporter strain there was a 4.8-fold reduction in *sulA-lacZY* reporter activity. This reduction in β-galactosidase activity is indicative of a protein-protein interaction between HilE and HilD that leads to dimerization of the LexA DNA-binding proteins and repression of the *sulA* promoter. This system has also been used to

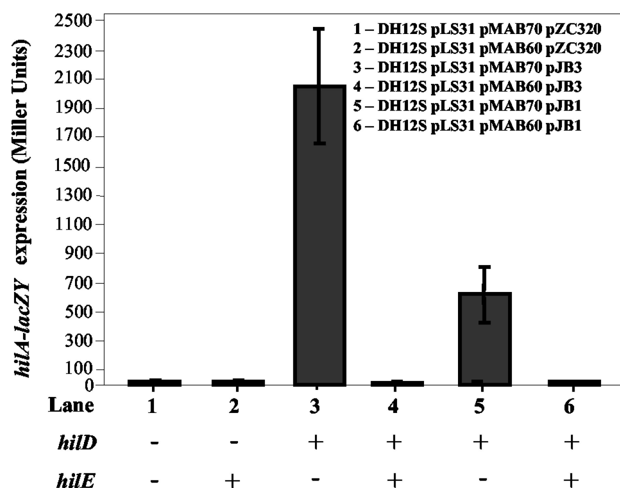

FIG. 6. The *hilE* regulator is able to repress the expression of a *hilA*::*lacZY* reporter within *E. coli* DH12S independently of *hilD* transcription. Lane 1, DH12S/pLS31/pMAB70/pZC320 (*hilA*::*lacZY hilE hilD*); lane 2: DH12S/pLS31/pMAB60/pZC320 (*hilA*::*lacZY hilE*$^+$ *hilD*); lane 3, DH12S/pLS31/pMAB70/pJB3 (*hilA*::*lacZY hilE hilD*$^+$); lane 4, DH12S/pLS31/pMAB60/pJB3 (*hilA*::*lacZY hilE*$^+$ *hilD*$^+$); lane 5, DH12S/pLS31/pMAB70/pJB1 (*hilA*::*lacZY hilE hilD*$^+$); lane 6, DH12S/pLS31/pMAB60/pJB1 (*hilA*::*lacZY hilE*$^+$ *hilD*$^+$). pJB3 expresses *hilD* under the control of the *lac* promoter, whereas pJB1 expresses *hilD* under the control of its own promoter.

FIG. 5. Comparison of the invasiveness of strains SL1344 and BJ2462 for HEp-2 cells by using fluorescence confocal microscopy. The tissue culture invasiveness of the parent strain SL1344 and the *hilE* mutant BJ2462 were compared in a modified invasion assay as described in Materials and Methods after growth in high oxygen and high osmolarity (A and B), high oxygen and low osmolarity (C and D), low oxygen and low osmolarity (E and F), and low oxygen and high osmolarity (G and H). Confocal microscopy was employed to visualize the rhodamine-stained HEp-2 cells and GFP expression. In three independent experiments, the bacteria within ca. 100 to 150 HEp-2 cells were counted for each strain in each growth condition. Cells infected with SL1344 are shown in panels A, C, E, and G, and cells infected with BJ2462 are shown in panels B, D, F, and H.

TABLE 3. Repression of the *sulA-lacZY* reporter caused by dimerization of the $LexA_{1-87}$408-HilE fusion protein and the $LexA_{1-87}$WT fusion protein

Strain	Mean β-galactosidase activity from the *sulA-lacZY* reporter ± SD	Fold repression of β-galactosidase from *sulA-lacZY* (wild type vs experimental)
SU202 (*sulA-lacZY*)	2,503 ± 47	1
SU202/pLexA′-HilE+ pDP804	2,694 ± 102	0.93
SU202/pLexA-HilD+pMS604	2,789 ± 98	0.90
SU202/pLexA′-HilD pLexA-HilD	523 ± 56	4.8

demonstrate an interaction between the regulatory proteins FimZ and FimW that control the expression of *Salmonella* type 1 fimbriae (59). The interaction between FimZ and FimW was found to repress *sulA-lacZY* expression by ~12-fold, indicating a stronger interaction between FimZ and FimW than between HilE and HilD.

The *hilE* gene can repress *invF* expression in a *hilA*-independent manner. Two transcriptional regulators, *hilA* and *invF*, both encoded within SPI1, are required for activation of genes required for *Salmonella* invasion (5, 34). Previous work has shown that *invF* expression, and a subset of invasion proteins, can be activated in the absence of a functional *hilA* gene (16, 52). This activation appears to be HilD dependent (40). Since we have evidence that HilE may regulate invasion gene expression by binding to the HilD protein, we were interested in determining whether *hilE* could repress *invF* transcriptional activation in the absence of *hilA*. Such a finding would provide additional evidence that *hilE* regulates HilD activity. We previously constructed a Typhimurium strain that has the genotype Δ*hilA invF*::Tn*5lacZY* that was designated BJ1714. A derivative of BJ1714 was made that lacks a functional *hilE* gene, designated BJ2390. Both strains were grown in inducing low-oxygen, high-osmolarity conditions, and the *invF*::Tn*5lacZY* reporter was quantitated. The parent strain BJ1714 expressed the *invF* reporter at low levels (34.5 ± 0.2 U) and expression increased to 187.2 ± 0.6 U when a *hilE* mutation was present, an increase of 5.4-fold (Fig. 7). Introduction of a *hilE*-expressing plasmid, pMAB62, resulted in undetectable levels of β-galactosidase from the *invF*::Tn*5lacZY* reporter, a repression of >187-fold. As controls for the experiment, BJ1894/pZC320 and BJ1894/pMAB62 strains were constructed that lack *hilA* and carry an *orgA*::Tn*5lacZY* fusion. The *orgA* gene has been shown to require a functional *hilA* gene for expression, and these strains should be unregulated by *hilE* in the absence of *hilA*. As expected, *hilE* had no effect at the *orgA* promoter in the absence of *hilA* as the wild-type and *hilE* overexpressing strains had 32.3 ± 1.3 and 27.9 ± 0.2 U of activity, respectively. These results indicate that *hilE* is capable of repressing the *hilA*-independent activation of *invF* transcription, presumably by modulating HilD activity.

The *hilE* gene resides near centisome 98 of the *Salmonella* chromosome on sequences that have the characteristics of a pathogenicity island. In an effort to gain understanding about the origins of the *hilE* regulator, the gene was mapped on the chromosomes of serovar Typhimurium and serovar Typhi by using the genomic databases available at the Washington University School of Medicine (http://genome.wustl.edu/gsc/bacterial/salmonella.shtml) and the Sanger Centre (http://www.sanger.ac.uk), respectively. It was hoped that information gleaned from neighboring genes might be used to gain additional information into the function of *hilE*. Mapping efforts placed the *hilE* gene at centisome 98 between the *miaE* and *hsdS* genes, which are separated by >40 kb (Fig. 8) (53). Analysis of this region revealed that a large portion of the sequence is found only in serovars of *Salmonella*. Due to this observation, we analyzed this region of the chromosome by using criteria that have been used by others to identify pathogenicity islands. The ~40-kb region of DNA between *miaE* and *hsdS* contains two large segments of DNA that are specific for *Salmonella* serovars (Fig. 8). Comparison of region 1 in serovar Typhimurium (~26 kb in size) to the same region in serovar Typhi (~35 kb in size) revealed that these sequences bear no significant homology to one another, which was not completely surprising since their sizes differ substantially. In contrast, region 2 of both serovars (~10 kb in size for each strain) is virtually identical (99% homologous) to each other. The *hilE* gene is situated near the end of region 2, nearer to the *hsdS* gene. One criterion that has been used for the identification of pathogenicity islands in *Salmonella* strains is the absence of a homologous sequence in the closely related strain, *E. coli* (45). We were able to establish that both regions 1 and 2 are absent in this region of the *E. coli* genome, since sequences that flank each side of *Salmonella* region 1 and *Salmonella* region 2 are contiguous in the *E. coli* chromosome. Another criterion for pathogenicity islands is whether the sequence of interest has a percent G+C content substantially different from the average G+C content of the entire chromosome, which is 52 to 54% for *Salmonella* strains (48). Analysis of the Typhimurium region 1 found the sequence to have a 48.4% G+C content and analysis of the Typhi region 1 revealed a 46.5% G+C content. Region 2 has percent G+C contents of 48.1% for Typhimurium and 48.4% for Typhi. A third criterion for pathogenicity islands is the presence of insertion sequence (IS) element remnants or tRNA genes that were used for the recombination of these islands into the

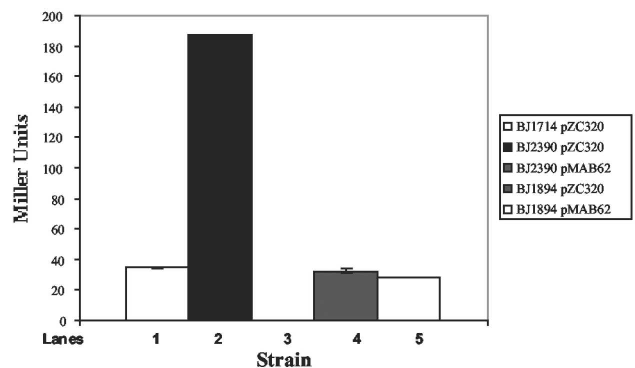

FIG. 7. The *hilE* regulator exerts a negative influence on *invF* transcription in a *hilA*-independent manner. Strains BJ1714/pZC320 (Δ*hilA invF*::Tn*5lacZY*), BJ2390/pZC320 (Δ*hilA invF*::Tn*5lacZY hilE*::*cam*), BJ2390/pMAB62 (Δ*hilA invF*::Tn*5lacZY hilE*::*cam hilE*$^{+}$), BJ1894/pZC320 (Δ*hilA orgA*::Tn*5lacZY*), and BJ1894/pMAB62 (Δ*hilA orgA*::Tn*5lacZY*, *hilE*$^{+}$) were grown in low-oxygen, high-osmolarity growth conditions to an OD_{600} of ~0.45, and then the β-galactosidase activity was quantitated for each strain.

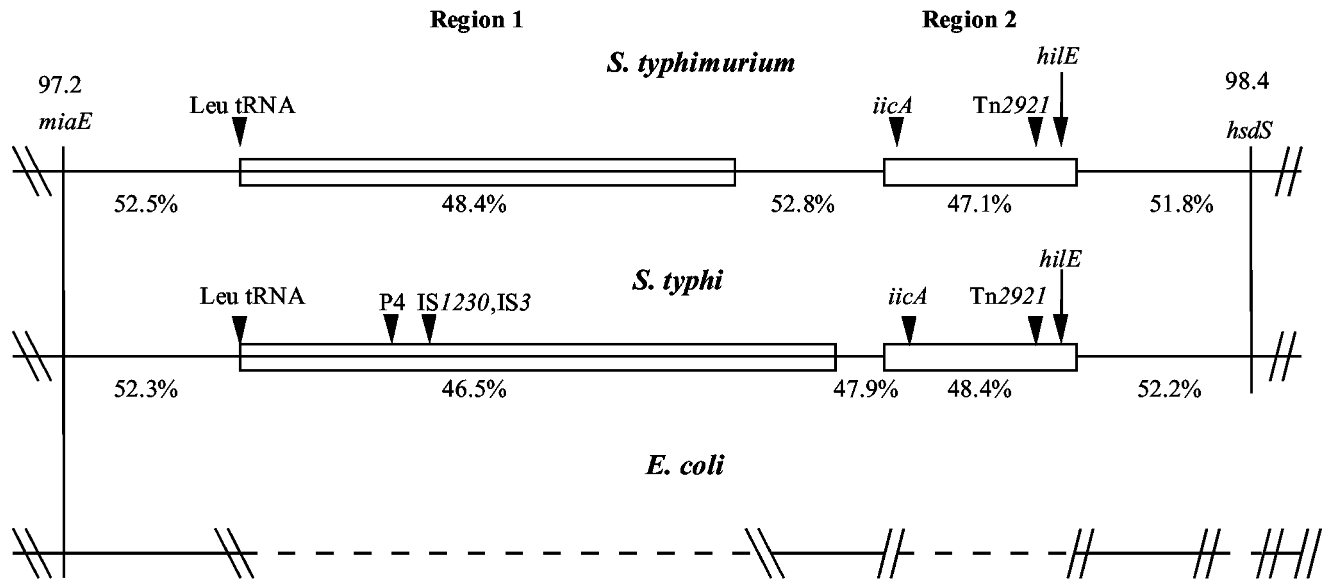

FIG. 8. Comparison of serovar Typhimurium, serovar Typhi, and *E. coli* chromosomal regions surrounding the *hilE* gene at centisome 98 on the chromosome. Regions 1 and 2 depicted in the figure are aligned for serovar Typhimurium, serovar Typhi, and *E. coli*. The percent G+C for each region, the positions of known genes, and the positions of the tRNA and IS elements within each region are indicated. The open boxes indicate similar sequences for region 2, and the region 1 sequences for each strain have a different pattern to note the lack of sequence similarity.

genome (3, 26). In region 1, both serovar Typhimurium and serovar Typhi contain a Leu tRNA gene at the left end of the insert region. Serovar Typhi also contains additional IS*1230* and P4 phage sequences in region 1 that may have been used for the insertion of this DNA. In region 2, both genomes contain the remnants of a Tn*2921* element within the insert. Analysis of DNA sequences of region 1 and region 2, as well as the intervening sequences, revealed the presence of a large number of putative open reading frames with no known functions, in addition to some genes with known functions. The Typhi region 1 contains the fimbria-encoding *sef* genes previously identified within *S. enterica* serovar Enteriditis (11, 60). In addition to *hilE*, region 2 in both Typhimurium and Typhi encodes the *iicA* gene (for induced intracellularly A gene), which has been shown to be induced upon *Salmonella* internalization into host cells (51). *hilE* and *iicA* are separated by ~6 kb of DNA on the chromosome.

DISCUSSION

We have added additional detail here to the developing picture of *Salmonella* invasion gene regulation. Previous work from our laboratory has identified several negative regulatory elements that act, directly or indirectly, on *hilA* transcription, including *hha* (20) and *ams*, *pag*, and *hilE* (19). Other laboratories have identified a variety of positive activators, including *hilC*/*sirC*/*sprA* (17, 52, 54); *hilD* (54); *sirA*/*barA* (2, 30); *fis* (62); *csrAB* (1); and *phoB*, *fadD*, and *fliZ* (42). Experimental evidence is beginning to focus our understanding of how this large number of regulators function together to effectively control *hilA* expression in response to environmental stimuli. Recently, it was found that controlled overexpression of *hilD* overcomes the effects of all repressing conditions, as well as mutations in many positive effectors of *hilA* (42). In addition, mutations in many of the positive activators (i.e., *fadD*, *fliA*, *envZ*, and *hilC*) no longer affect *hilA* expression in the absence of *hilD*, although mutations in these genes do not signficantly affect *hilD* transcription. These results suggest that many of the positive activators function by affecting *hilD* activity posttranscriptionally. Consistent with this observation, Lucas et al. (42) noted that *csrA* and *ams* have been identified as regulators of *hilA* expression. Altier et al. have shown that overexpression or mutation of *csrA* results in reduced levels of *hilD* transcript, presumably by destabilization of mRNA (1). RNase E, which is encoded by *ams*, exerts a negative influence on *hilA* expression, and it is possible that it exerts its regulatory effect by selectively degrading mRNA of specific genes such as *hilD* and/or *hilA*. Fis, another identified activator of *hilA*, may be an exception to this proposed mechanism of regulation as preliminary data from our laboratory indicates that Fis footprints specific sequences on the *hilA* promoter (unpublished data).

Work on negative regulation of *hilA* is also beginning to yield new information. The work described here has provided clear evidence that the *hilE* gene is an important negative regulator of *hilA* expression. Our experiments reveal that overexpression of *hilE* from a single-copy plasmid significantly decreases *hilA* expression and HEp-2 invasion both in inducing and in repressing growth conditions. Conversely, deletion of the *hilE* gene in the chromosome leads to a significant increase in *hilA* expression in inducing and repressing growth conditions, particularly in high oxygen. The increase in *hilA* expression translates into increased invasiveness in repressing growth conditions, although the increases in invasion are not as large as we had expected. One possible explanation for this inconsistency may be that the *hilE* mutation makes serovar Typhimurium more susceptible to killing by the intracellular environment of cells. Future experiments will address this possibility. The *hha* gene has been recently identified as a negative modulator of *hilA* (20). Hha is a small nucleoid associated DNA-

binding protein that has been shown to regulate hemolysin expression in *E. coli* by binding to the DNA. In addition, work from our laboratory demonstrated that purified Hha protein is able to bind to DNA upstream of the *hilA* gene (20). Genetic experiments have shown that a strain with a *hha* mutation is significantly, but not completely, derepressed for *hilA* expression under repressing environmental conditions, especially low osmolarity. Importantly, we have demonstrated that *hilA* derepression, in the *hilE* mutant, is most profound in high-oxygen repressing conditions. These results suggest that HilE and Hha respond to different environmental conditions to provide overlapping control of *hilA* transcription and expression of the invasive phenotype.

An important finding has come from our efforts to identify the mechanism of HilE repression of *hilA*. We were unable to demonstrate that purified HilE could bind to the *hilA* promoter in gel shift mobility assays. We performed genetic experiments to determine whether HilE acted by repressing *hilD* transcription. However, HilE was able to repress HilD-mediated activation of *hilA* whether *hilD* was transcribed from its own promoter or from the *lac* promoter, suggesting that HilE was not repressing *hilD* transcription as part of its mechanism to regulate *hilA*. Finally, we have found that HilE and HilD bind to each other in a two-hybrid assay. A consistent observation with this finding, also described in this report, is that HilE represses *hilA*-independent activation of *invF*. A *hilA*-independent activation pathway of *invF* and genes that *invF* regulates has been described (16, 52). Other workers have published work that suggests that HilD mediates this activation pathway (40). Our observation that HilE is also involved in regulating this pathway is consistent with the idea that HilE mediates its regulatory effects through protein-protein interactions with HilD.

Another aspect of *hilE* worth further investigation is that it appears to be a *Salmonella* specific regulator, similar to *hilC* and *hilD*, since we were unable to find any homologs for *hilE* in gene libraries. In contrast to the positive regulators of *hilA*, *hilE* is not encoded within SPI1 and instead appears to be encoded on a new island of DNA at centisome 98 with many genes of unknown function. In addition, the *iicA* gene (51) resides ca. 6 kb from the *hilE* gene. Although no specific function has been ascribed to *iicA*, the gene has been shown to be induced by intracellular conditions. One possibility is that, in addition to regulating invasion gene expression, *hilE* may play a role in *iicA* expression, which would provide a plausible explanation for the phenotype of the *hilE* mutant in the tissue culture invasion assay.

A model for invasion gene regulation through the *hilA* transcriptional activator has been proposed (41). Many of the positive activators of *hilA* expression are now believed to function by posttranscriptional modification of *hilD*, although the activity of the Fis protein may be more direct (62). HilC and HilD have recently been shown to bind to the *hilA* promoter, and it is believed that the binding of one or both of these proteins displaces repressors of *hilA* expression (55), although both proteins are members of the AraC family of transcriptional activators. Recent work from our lab has identified the Hha protein as a repressor of *hilA* that possesses the ability to bind to the *hilA* promoter DNA, which is consistent with the proposed model (20). In this report we have further characterized another gene, *hilE*, that possesses the ability to negatively regulate *hilA* expression. Our results indicate that HilE functions not by binding to the *hilA* promoter but by binding to HilD. This interaction suggests that *hilA* repression, at least by HilE, occurs by inhibition of HilD activity. Future work will be aimed at characterizing in detail the precise role of HilE and its interactions with HilD in the regulation of *hilA* and the *S. enterica* serovar Typhimurium invasive phenotype.

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