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Hha Is a Negative Modulator of Transcription of *hilA*, the *Salmonella enterica* Serovar Typhimurium Invasion Gene Transcriptional Activator

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An early step in the establishment of *Salmonella enterica* serovar Typhimurium murine infection is the penetration of the intestinal mucosa of the small intestine. The majority of the genes responsible for the *Salmonella* invasive phenotype are encoded on *Salmonella* pathogenicity island 1, and their transcription is controlled by the *hilA* transcriptional activator. The expression of *hilA* is regulated by environmental signals including oxygen, osmolarity, pH, and growth phase such that the presence of any one suboptimal condition results in repression of *hilA* expression and the invasive phenotype. We have conducted a search for negative regulators of *hilA* by introduction of a *Salmonella enterica* serovar Typhimurium chromosomal DNA gene bank into a *Salmonella enterica* serovar Typhimurium *hilA*::Tn*5lacZY* reporter strain. This screen has identified the *hha* gene as a regulator that exerts a negative influence on *hilA* expression. Plasmid-encoded *hha* significantly reduces *hilA*::Tn*5lacZY* chromosomal expression, as well as expression of the invasion genes *invF*, *prgH*, and *sipC*. An *hha* null mutation results in substantial derepression of both chromosomally encoded and plasmid-encoded *hilA*::Tn*5lacZY* expression. Introduction of plasmid-encoded *hha* into strain SL1344 results in attenuation of invasion using in vitro and in vivo assays. Importantly, purified Hha protein was found to bind to a *hilA* DNA promoter fragment, suggesting that the regulatory activity of the Hha protein occurs at the *hilA* promoter. These data add detail to the developing model of the regulation of *Salmonella* invasion genes.

Pathogenic *Salmonella* species cause infections in humans ranging from self-limiting gastroenteritis to lethal systemic disease. After ingestion of *Salmonella* in contaminated food or water, the bacteria access the small intestine and invade the specialized M cells of the follicle-associated epithelium of Peyer's patches (6, 30, 47) and the absorptive enterocytes (53). Subsequently, host-adapted species move to the regional lymph nodes before spreading to the liver and spleen, where unchecked growth can result in death due to enteric fever (29, 36). Organisms that are unable to grow within the lymphatic system of the host remain localized to the intestinal epithelium, where they induce substantial inflammation that contributes to the pathology of localized gastroenteritis.

A critical step in initiation of salmonellosis is the ability to invade the intestinal cells of the host. The entry process occurs by rearrangement of the cellular membrane in the form of actin ruffles that engulf the bacteria (15). Many genetic elements responsible for the invasive phenotype of *Salmonella enterica* serovar Typhimurium localize to a 40-kb region of the chromosome at centisome 63, termed *Salmonella* pathogenicity island 1 (SPI-1) (reviewed in reference 8). Many of the SPI-1 genes encode structural components of a secretion system and are homologous to type III secretion systems found in both plant and animal pathogens including *Pseudomonas*, *Rhizobium*, *Erwinia*, *Yersinia*, *Shigella*, and enteropathogenic *Escherichia coli* (19). These systems function by translocating virulence proteins into eukaryotic cells (23). Loci within and outside SPI-1 encode proteins that are secreted through the type III secretion apparatus of SPI-1. These secreted proteins induce cellular changes in tissue culture cells and are responsible for the ability of *Salmonella* to invade tissue culture cells (14, 21, 22, 32, 56).

The induction of *Salmonella* invasion genes is tightly regulated by environmental signals that are believed to be important in the expression of the invasive phenotype in the host environment. The conditions that have been shown to repress *Salmonella* invasion include high oxygen, low osmolarity, low pH, and stationary-phase growth (11, 16, 34, 51). The *hilA* gene, located in SPI-1, encodes a transcriptional activator that modulates expression of the type III secretion apparatus proteins and the secreted effector proteins (2). Importantly, expression of *hilA* is modulated by the same conditions that regulate the invasive phenotype. In addition, overexpression of *hilA* confers a hyperinvasive phenotype and overexpression also counteracts the effects of repressing signals. Therefore, modulation of *hilA* expression by environmental signals appears to be a primary method of regulating the invasive phenotype of *Salmonella* (2, 3, 35). Results from our laboratory and from the works of others (2, 47) reveal that null mutations in *hilA* cause a dramatic attenuation of invasion of tissue culture cells and M cells of the Peyer's patches, in addition to attenuating virulence in mice following oral inoculation. These results establish that *hilA* plays a crucial role in expression of the *Salmonella* invasive phenotype.

Many genetic elements that exert regulatory effects on *hilA* have been identified including positive elements *hilC*/*sirC*/*sprA* (10, 48, 50), *hilD* (50), *sirA* (26), *fis* (55), *barA*, *csrAB* (1), and *phoB*, *fadD*, and *fliZ* (39) as well as negative elements such as *phoPQ* (5, 46). Recent work by Schechter et al. (50) provided

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evidence for the existence of factors that repress *hilA* transcription in response to environmental cues, although the putative factors were not identified. The results from a transposon mutagenesis screening conducted in our laboratory identified the *hupB* and *ams* genes, as well as two previously uncharacterized open reading frames, as negative modulators of *hilA* expression (12). As an alternative to the transposon mutagenesis approach, we have conducted a search for additional factors that negatively influence *hilA* transcription using a gene bank of *Salmonella enterica* serovar Typhimurium genomic DNA. We now report the identification and characterization of the role of the histone-like protein, Hha, in *hilA* transcription and expression of the *Salmonella* invasive phenotype.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown in Luria-Bertani (LB) high-osmolarity medium (1% tryptone, 1% NaCl, 0.5% yeast extract) or LB low-osmolarity medium (1% tryptone, 0.5% yeast extract). Antibiotics were added to the following final concentrations (in micrograms per milliliter) where appropriate: ampicillin, 100; chloramphenicol, 25; kanamycin, 50; and tetracycline, 20. Bacterial cultures for invasion assays were grown under oxygen-limiting conditions by inoculating 3 ml of LB broth with 10 μl of a stationary-phase culture and incubating statically overnight at 37°C to a density of $\approx 4 \times 10^8$ to 5×10^8 CFU ml^{-1}. High-oxygen conditions were obtained by inoculating 3 ml of LB broth with 10 μl of stationary-phase culture and shaking vigorously at 37°C to a density of $\approx 1.5 \times 10^8$ CFU ml^{-1} (31, 47).

Strain and plasmid construction. The plasmid pTF137 carries the *hha* gene from *Salmonella* strain SL1344 cloned into the PCR cloning vector pGEM-T (Promega). The *hha* gene was amplified from the SL1344 chromosome in a standard PCR using primers hha5′ (5′-AAGCTTTGTTGTTCAGCAGCTATG-3′) and hha3′ (5′-AAGCTTCCTGCTATTGCTATGTGA-3′). The product was ligated into pGEM-T as per the instructions of the manufacturer.

The *hha* null mutant strain TF79 was constructed by allelic exchange of *hha* with a kanamycin cassette using a suicide plasmid system developed in our laboratory. This technique employs a mini-F suicide vector, pBDJ129, which relies on the RepE protein for replication, and a second high-copy-number plasmid, pBDJ200, that carries the RepE protein DNA binding sites. Since pBDJ200 is a high-copy-number plasmid and pBDJ129 is a low-copy-number plasmid, cotransformation of these plasmids into the same bacterium results in inhibition of replication of the pBDJ129 derivative due to the sequestration of RepE protein by the pBDJ200-encoded RepE DNA binding sites. Selection for an antibiotic marker on the pBDJ129 derivative allows recovery of strains in which the plasmid has integrated into the chromosome. Creation of pBDJ129 derivatives with sequences homologous to the chromosome allows integration into specific sites in the chromosome by homologous recombination. Using this system, we constructed a pBDJ129 derivative with DNA ≈900 bp upstream and ≈900 bp downstream of the *hha* open reading frame. This was accomplished by PCR amplification of chromosomal DNA, using primer hha3 (5′-AGATCTTT CCTAGCTATTTTCTCC-3′) with hha5 (5′-TTTTAGTTAATGGTGGATCCG ACATAAATTCTAC-3′) and primer hha6 (5′-CCCCTCTTCAGGATCCAAA TTCATTCGTTA-3′) with hha4 (5′-AGATCTATTTCCGCCTACCACGA-3′), to amplify the upstream and downstream sequences, respectively. The hha5 and hha6 primers incorporate a *Bam*HI site at the 3′ end of the upstream fragment and the 5′ end of the downstream fragment, respectively. Also, hha3 and hha4 incorporate unique *Bgl*II sites into the 5′ end of the upstream and the 3′ end of the downstream PCR products, respectively. Both PCR products were digested with *Bam*HI and ligated to pGEM-T in a triple ligation. This created a plasmid carrying ≈900 bp of DNA upstream and ≈900 bp of DNA downstream of, but not including, the *hha* gene joined by a unique *Bam*HI site. A kanamycin cassette obtained from pUC4K (Pharmacia) was then ligated into the *Bam*HI site between the cloned PCR fragments. The kanamycin cassette and flanking DNA was cut from the pGEM-T vector with *Bgl*II and ligated into the unique *Bgl*II site in the pBDJ129 suicide vector. This created a suicide plasmid, designated pTF142, with DNA homologous to sequences upstream and downstream of *hha*, separated by a kanamycin cassette. This plasmid was introduced into BJ70, carrying pBDJ200, and kanamycin-resistant, chloramphenicol-resistant colonies were selected. Since pTF142 is unable to replicate in the presence of pBDJ200, transformants harboring the kanamycin and chloramphenicol resistance markers have integrated pTF142 into the chromosome, which was confirmed by PCR. A screen for spontaneous kanamycin-resistant, chloramphenicol-sensitive colonies yielded the *hha* null mutant strain in which a second crossover had occurred, leaving a deletion of the *hha* gene replaced with the kanamycin cassette. Construction of the *hha* null mutant was verified by PCR amplification of the region and sequencing of the fragment.

Various *Salmonella* strains were constructed by transduction of antibiotic markers using P22 HT int$^-$-mediated transduction (9). The *E. coli hha*::Tn*5* mutation was moved to a *lacZ* background by P1-mediated transduction of Tn*5* from *E. coli* YK4122H to *E. coli* GS162 to create *E. coli* strain BJ1575 (41).

Plasmid pTF141 was constructed in the same manner as plasmid pLS79, described in reference 50. Briefly, primers LS11 (5′-CGGATCCATGTTGAGT

TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Description	Source or reference(s)
E. coli		
DH12S	*mcrA* Δ(*mrr-hsd RMS-mcrBC*) F′ *lacI*^{q} *lacZ*ΔM15	Gibco BRL
YK4122H	5K *hha3*::Tn*5* Kn^r	18, 44
GS162	K-12 *lac* laboratory strain	G. Stauffer
BJ1575	GS162 *hha3*::Tn*5* Kn^r	This work
BW21355	K-12 F$^-$ Δ*lacX74*	B. Wanner
BJ1925	BW21355 *hha3*::Tn*5* Kn^r	This work
S. enterica serovar Typhimurium		
SL1344	Wild-type virulent strain	57
BJ68	SL1344 *sipC*::Tn*5lacZY* Tc^r	47
BJ70	SL1344 *hilA*::Tn*5lacZY* Tc^r	47
BJ72	SL1344 *invF*::Tn*5lacZY* Tc^r	47
BJ644	SL1344 *phoP*::Tn*10* Tc^r	49
BJ661	SL1344 with *pho-24* by P22 transduction from BJ2272 Cm^r	This work
BJ690	SL1344 Δ*phoP hilA*::Tn*5lacZY* Tc^r	This work
BJ2227	TF79 with *pho-24* by P22 transduction from BJ2272 Cm^r Km^r	This work
BJ2272	TA2367 (*pho-24*) with *pepT7*::Mud by P22 transduction from JE2761 Cm^r	This work
BJ2305	BJ690 with *hha*::*kan* by P22 transduction from TF79 Km^r Tc^r	This work
EE656	SL1344 *prgH*::Tn*5lacZY* Tc^r	3
JE2761	LT2 *pepT7*::Mud Cm^r	54
TA2367	LT2 *pho-24*	K. Sanderson
TF59	EE251 *pepT*::Mud 1(x)	12
TF79	BJ70 *hha*::*kan* Kn^r	This work
TF80	SL1344 *hha*::*kan* Kn^r	This work
TF81	LT2 *pho-24 hha*::*kan* Kn^r	This work
TF82	LT2 *hha*::*kan* Kn^r	This work
Plasmids		
pRW50	Low-copy-number *lacZYA* reporter vector, Tc^r	37
pGEM-T	High-copy-number PCR cloning vector, Ap^r	Promega
pTF120	Cosmid carrying serovar Typhimurium DNA carrying the *hha* gene, Ap^r	This work
pTF137	pGEM-T with *hha* from *S. typhimurium*, Ap^r	This work
pTF141	pRW50 derivative carrying nt −39 to +420 of *hilA* fused to *lacZYA* Tc^r	This work
pTF142	pBDJ129 derivative carrying *hha*::*kan* Cm^r Kn^r	This work
pLS31	pRW50 derivative plasmid carrying −497 to +420 of *hilA* fused to *lacZYA* Tc^r	50
pBDJ129	Suicide mini-F plasmid vector, Cm^r	33
pBDJ200	*repE* pBR322 derivative, Ap^r	33
pWAM582	Plasmid carrying the *hlyCABC* hemolysin genes and upstream promoter sequences	R. Welch

ATGAAATCATC-3′) and LS26 (5′-GAATTCGCAGCATTTACACCCCA-3′), which incorporate *Bam*HI or *Eco*RI sites into the PCR product, respectively, were used to amplify the *hilA* promoter from −39 bp upstream of the *hilA* transcriptional start site to +412 bp into the *hilA* transcript. The PCR product was cloned into pGEM-T (Promega) and then subcloned into pRW50 as a *Bam*HI-*Eco*RI fragment to create plasmid pTF141. The *hilA* promoter fragment in pTF141 was sequenced prior to use to confirm that it carried the proper nucleotide sequence.

Plasmid gene bank construction. A plasmid gene bank of *S. enterica* serovar Typhimurium SL1344 DNA was created by isolation of chromosomal DNA using a DNeasy Tissue kit (Qiagen). The DNA was partially digested with *Sau*3A1 and separated on a 0.7% agarose gel. Fragments in the range of 3 to 5 kb were cut from the gel, eluted using a QIA Quick Gel Extraction kit (Qiagen), and ligated into the *Bam*HI site in pBluescript (Stratagene).

β-Galactosidase assays. β-Galactosidase production from Tn*5lacZY* reporter constructs was quantitated using the method of Miller (41).

Hemolysin assays. The hemolytic activity of culture supernatants was determined in a hemoglobin release assay as described by Godessart et al. (18) with minor alterations. Briefly, cultures were inoculated (100 μl into 5 ml) from an overnight culture and grown with shaking at 37°C to an optical density at 600 nm (OD_{600}) of ≈0.6. Bacteria were removed by centrifugation, before mixing 300 μl of culture supernatant with 400 μl of phosphate-buffered saline and 300 μl of 1% washed bovine red blood cells. Samples were incubated at 40°C for 10 min, and the red blood cells were removed by centrifugation. The OD_{420} of each sample was measured to calculate the amount of hemoglobin released from the red blood cells. One hemolytic unit is defined as OD_{420}/OD_{600} of the bacterial culture × time (in hours) × volume of culture supernatant (in milliliters).

Tissue culture conditions and invasion assays. HEp-2 tissue culture cells (42) were maintained in RPMI 1640 (Gibco/BRL) containing 10% (vol/vol) fetal bovine serum and passaged every 2 to 3 days. Invasion assays were done as previously described (31).

Ligated loop experiments and preparation of samples for electron microscopy. In vivo invasion of murine intestinal tissue by *S. enterica* serovar Typhimurium strains was assessed by the use of ligated ileal loop experiments performed as previously described (47). Briefly, ligated intestinal loops that contained ileal Peyer's patches were prepared in anesthetized BALB/c mice and injected with approximately 4×10^8 bacteria. Following incubation of the ligated loops with bacterial inocula, the mice were sacrificed and sections of the infected intestinal tissue were removed. The tissue sections were prepared for scanning electron microscopy as previously described (25) and viewed with a Hitachi S-4000 field emission scanning electron microscope.

Purification of a Hha-maltose-binding fusion protein. The *hha* gene was amplified from the serovar Typhimurium chromosome with primers MBP-hha3 (5′-GTAGAAGAATTCTCTGATAAACCATTAACTAAA-3′) and MBP-hha2 (5′-TCCCAGATAACACAAGCTTGTTCTCTA-3′), which introduce an *Eco*RI and *Hin*dIII restriction enzyme site at the 5′ end and 3′ end of the *hha* gene, respectively. The *hha* PCR fragment was digested with *Hin*dIII and *Eco*RI, ligated to the pMAL-C2 vector (New England Biolabs) previously digested with *Hin*dIII and *Eco*RI and transformed into *E. coli* DH12S. Sequencing of plasmid DNA from selected colonies confirmed construction of the desired plasmid, designated TF143, encoding an Hha-MBP fusion. Hha-MBP was purified from *E. coli* induced for expression of the fusion protein by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) according to the directions of the manufacturer. Briefly, culture extracts were passed over an amylose column and washed extensively with column buffer to remove unbound proteins. The Hha-MBP was eluted with 5 ml of column buffer containing 10% maltose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified protein indicated that it was at least 95% pure (data not shown). The Hha portion of the fusion protein was removed from the MBP by digestion with factor Xa protease. The purified protein was quantitated using the Bradford protein quantitation kit (Bio-Rad) in preparation for gel mobility shift assays.

Gel mobility shift assay. The gel mobility shift assay was based on the methods of Fried and Crothers (13) and Garner and Revzin (17). A 457-bp *hilA* promoter DNA fragment, from nucleotides −497 to −39, was amplified by PCR with primers TFHilA5′ (5′-CGGAATTCGTCCAGATGACA-3′) and LS15R (5′-TGGGTGTAAATGCTGCTT-3′). A 479-bp *invF* fragment, from nucleotides −156 to +323 of the *invF* control region, was amplified by PCR with primers InvF5′ (5′-CTGCAGAACAATAAGCCAG-3′) and InvF3′ (5′-CGGATTCAGCATATGTCG-3′). These DNA fragments were labeled by first removing the 5′ phosphate group with calf intestinal phosphatase and then end labeling the DNA with phosphonucleotide kinase and [γ-^{32}P]-ATP. Unincorporated nucleotides and enzymes were removed with a PCR purification kit, and the specific activity of the labeled fragments was determined in a scintillation counter. The gel mobility shift assay was performed by preincubating the labeled DNA in 1× DNA binding buffer (18-μl total volume) at 37°C for 5 min and then adding 2-μl volumes of twofold serial dilutions of Hha protein in 1× DNA binding buffer. After the tubes were incubated at 37°C for 15 min, 1 μl of loading dye was added and the samples were loaded onto a nondenaturing 5% polyacrylamide–3% glycine gel.

Nucleotide sequence accession number. The *S. enterica* serovar Typhimurium *hha* nucleotide sequence determined in this study has been deposited in GenBank under accession number AF242359.

RESULTS

Identification of a plasmid gene bank clone that represses *hilA* expression. Colonies of serovar Typhimurium strain BJ70 *hilA*::Tn*5lacZY* exhibit a "fisheye" phenotype (red center with a white periphery) when grown on MacConkey lactose agar due to the oxygen regulation of the *lacZY* fusion (28). Expression of the *hilA* reporter in this strain is increased 2.5- to 3.5-fold by growth under inducing conditions (low oxygen and high osmolarity) compared to repressing conditions (high oxygen and low osmolarity). While these changes in *hilA* expression are relatively small, identical growth conditions are used to quantitate both β-galactosidase activity and tissue culture invasiveness. When *Salmonella* strains are cultured in these growth conditions to measure invasiveness, we consistently observe large changes in the ability of the strains to enter cells (≈500-fold). These reproducible findings give us a high level of confidence that small changes in *hilA* expression have a significant impact on downstream invasion gene transcription, invasion into mammalian cells, and host virulence. To identify factors that decrease expression of the *hilA* gene, we transformed strain BJ70 with a plasmid gene bank of the serovar Typhimurium SL1344 chromosome and plated ampicillin-resistant transformants on MacConkey lactose agar. White transformants that were able to significantly repress *hilA*::Tn*5lacZY* expression were isolated and retransformed into strain BJ70 to confirm the ability to repress *hilA* expression. One isolate, carrying a plasmid that was able to repress chromosomal *hilA*::Tn*5lacZY* expression approximately 75% compared to induced levels of expression, was obtained and selected for further study. The cloned DNA was sequenced, and the sequence obtained was used to search databases at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) to identify the cloned DNA fragments. The partial sequence of the plasmid, pTF120, identified a gene that was highly similar to the *E. coli hha* gene, a gene involved in the regulation of hemolysin production (18, 43) but which has not been previously described for *Salmonella* species. Interestingly, a functional homolog of *hha* in *Yersinia enterocolitica*, named *ymoA*, has been shown to be involved in virulence gene regulation (4, 7). To determine if the *hha* gene was responsible for the repression of *hilA*::Tn*5lacZY* by pTF120, we amplified *hha* from the serovar Typhimurium chromosome by PCR, cloned the product into pGEM-T to create plasmid pTF137, and introduced the plasmid into strain BJ70. Overexpression of *hha* from plasmid TF137 reduced chromosomal *hilA*::Tn*5lacZY* expression more than sevenfold. Overexpression of *hha* also superrepresses (25-fold) *hilA*::Tn*5lacZY* expression from a plasmid reporter, which is likely due to high-level expression of *hha* from the high-copy-number plasmid (Fig. 1). As a control, the effect of overexpression of *hha* on the growth of serovar Typhimurium was assessed in growth curve experiments. No sig-

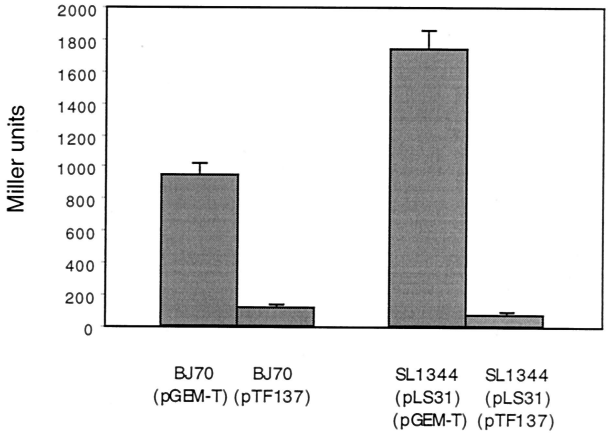

FIG. 1. Effect of multicopy *hha* on serovar Typhimurium *hilA*::Tn*5lacZY* expression. Strains were grown statically in LB broth to an OD_{600} of ≈0.4 before assaying β-galactosidase activity from the *hilA*::Tn*5lacZY* reporter. Strain BJ70 carries a chromosomal *hilA*::Tn*5lacZY* reporter, while pLS31 is a low-copy-number *hilA*::Tn*5lacZY* reporter plasmid (50). *Salmonella* SL1344 is the wild-type virulent strain.

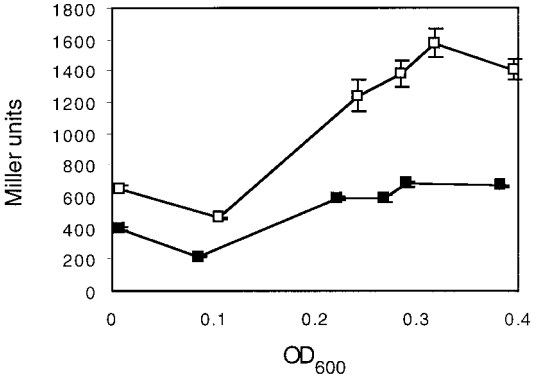

FIG. 2. Effect of a *hha* mutation on expression of a serovar Typhimurium *hilA*::Tn*5lacZY* reporter. Cultures of strain BJ70 (*hilA*::Tn*5lacZY*) (solid squares) or TF79 (*hilA*::Tn*5lacZY hha*::*kan*) (open squares) were incubated statically in LB broth. Samples were taken at various time points throughout growth and assayed for β-galactosidase activity. Data are representative of three independent experiments.

nificant differences in the growth curves of *Salmonella* containing pGEM-T or pTF137 were observed (data not shown). Thus, overexpression of Hha causes significant repression of *hilA* transcription without having a measurable effect on the growth rate of *Salmonella*.

Overexpression of *hha* significantly reduces expression of *invF*, *prgH*, and *sipC* invasion gene reporters. Since *hha* modulates *hilA* expression, we were interested in examining the effect of *hha* on invasion genes known to be regulated by *hilA*. To this end, we transformed strains containing chromosomal *invF*::Tn*5lacZY*, *prgH*::Tn*5lacZY*, or *sipC*::Tn*5lacZY* fusions with the vector pGEM-T or plasmid pTF137 and examined the effect of the plasmid on the expression of *invF*, *prgH*, or *sipC* by β-galactosidase assay. We found that, in addition to repressing *hilA*::Tn*5lacZY* expression, pTF137 caused significant decreases in chromosomal expression of *invF*::Tn*5lacZY* (159-fold), *prgH*::Tn*5lacZY* (11-fold), and *sipC*::Tn*5lacZY* (4-fold) fusions (data not shown). Thus, it seems that the repressing effect of Hha on *hilA* is transmitted to transcription of the *invF*, *prgH*, and *sipC* invasion genes. It is also possible that Hha has a repressing effect on transcription of downstream invasion genes independent of *hilA* transcriptional repression.

A serovar Typhimurium *hha* mutant is derepressed for *hilA* expression. To further explore the role of Hha in the regulation of *hilA*, we replaced the majority of the *hha* gene in BJ70 with a kanamycin cassette to create the *Salmonella hha* null mutant TF79. We compared the expressions of the *hilA*::Tn*5lacZY* chromosomal reporter in strains BJ70 (wild type) and TF79 (*hha*) at various points in the growth curve of *Salmonella* grown in LB broth. We observed derepression of the *hilA*::Tn*5lacZY* reporter throughout the growth curve with maximal derepression (≈2.5-fold) at an OD_{600} of ≈0.3 (Fig. 2). Interestingly, we observed that the *hilA*::Tn*5lacZY* reporter in TF79 still seemed to be regulated by some environmental signals. Therefore, to examine the role of *hha* in regulating *hilA* expression under high-oxygen or low-osmolarity conditions, growth experiments in which oxygen was a repressing signal, but not osmolarity, or osmolarity was a repressing signal, but not oxygen, were performed. The expression of the *hilA*::Tn*5lacZY* reporter was then quantitated in the parent strain or *hha* mutant background. As shown in Fig. 3, *hilA*::Tn*5lacZY* is significantly repressed in both the wild-type and *hha* mutant strains after growth under high-oxygen (high-osmolarity) conditions, although *hilA*::Tn*5lacZY* expression is about fourfold higher in the *hha* mutant. Under low-osmolarity (low-oxygen) conditions, *hilA*::Tn*5lacZY* expression was increased approximately sixfold in the *hha* mutant compared to the wild-type strain. In fact, the levels of expression of *hilA*::

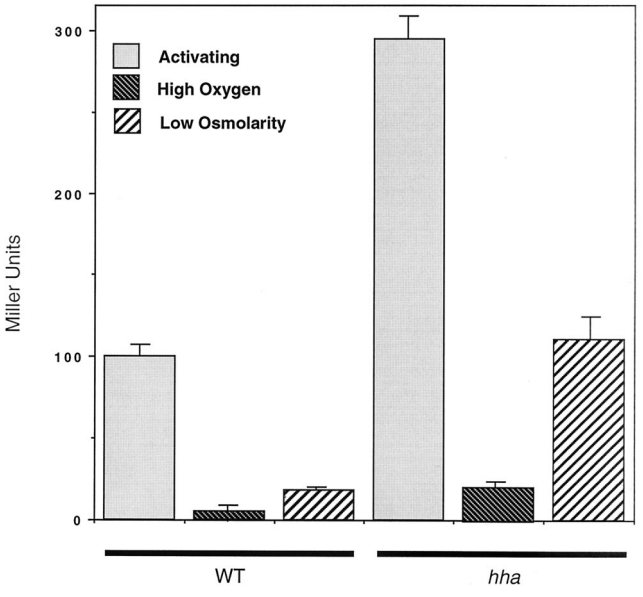

FIG. 3. Effects of an *hha* mutation on expression of *hilA*::Tn*5lacZY* after growth under repressing conditions. Cultures of wild-type (WT) (*hilA*::Tn*5lacZY*) or the *hha* mutant (*hilA*::Tn*5lacZY hha*::*kan*) were grown in LB medium with 1% NaCl under oxygen-limiting conditions (activating), LB medium with 1% NaCl with vigorous shaking (high oxygen), or LB medium with no NaCl under oxygen-limiting conditions (low osmolarity). β-Galactosidase of the *hilA*::Tn*5lacZY* reporter values were standardized, with the wild-type invasion after growth under inducing conditions being set to 100%.

Tn*5lacZY* in the *hha* mutant after growth under these conditions were slightly higher than those observed for the *hilA*::Tn*5lacZY* reporter in the wild-type strain when grown under fully inducing conditions. We also examined the effect of the absence of *hha* on expression of *orgA*::Tn*5lacZY*, *invF*::Tn*5lacZY*, and *sipC*::Tn*5lacZY* reporters under similar growth conditions and observed similar derepression of these reporters under normally repressing conditions (data not shown). Thus, deletion of *hha* significantly increases expression of *hilA* and genes (i.e., *orgA*, *invF*, and *sipC*) regulated by *hilA*. Taken together, these data indicate that *hha* encodes a negative regulator of the serovar Typhimurium *hilA* invasion transcriptional activator.

***Salmonella* Hha has homology to YmoA and RmoA and is a functional homolog of *E. coli* Hha.** The Hha protein is a member of a family of small nucleoid-associated histone-like proteins that include H-NS, HU1, HU2, and YmoA. To perform comparative studies, the nucleotide sequence of the serovar Typhimurium *hha* gene was determined. A BLAST search of the NCBI database with the predicted *Salmonella* Hha protein identified proteins with significant homology to Hha, including Hha from hemolytic *E. coli* (43), RmoA from wild-type *E. coli* (45), and YmoA from *Y. enterocolitica* (7). A boxshade analysis (24, 52) revealed high conservation of amino acid residues ranging from 50% identical and 64% similar (RmoA) to 99% identical and 99% similar (*E. coli* Hha). The identity levels at the DNA level ranged from 54% identical (*rmoA*) to 86% identical (*E. coli hha*) (data not shown). Interestingly, a group of 10 amino acids (SAADHRLAEL) is completely conserved in the C-terminal half of each of these proteins, although no putative function for this amino acid motif has been identified.

The *ymoA* and *hha* genes have been shown to be functionally interchangeable (4, 40). We were interested in determining whether the *Salmonella hha* gene could function in *E. coli* to cause repression of hemolysin activity. The *S. enterica* serovar Typhimurium *hha*-carrying plasmid clone completely eliminated the zones of clearing around colonies on blood agar plates, indicating that the hemolytic activity of the *E. coli* colonies was repressed (data not shown), and quantitative analysis revealed that the hemolytic activity was repressed 34-fold; 106 ± 4 (mean ± standard deviation) hemolytic units were observed for *E. coli* pWAM582 pGEM-T versus 3 ± 2 units for *E. coli* pWAM582 pTF137. These experiments demonstrate that the *hha* gene in *Salmonella* which we have identified is a functional homolog of *hha* in *E. coli* as it is able repress *E. coli* hemolytic activity.

Repression of serovar Typhimurium *hilA* expression by Hha requires *hilA* URS. The expression of *hilA* is known to be regulated by a variety of environmental and genetic signals with the condition that a single negative signal causes repression of *hilA* transcription (3). Recently, Schechter et al. (50) identified a URS between −497 bp and −39 bp of the *hilA* promoter that is necessary for regulation of *hilA* by oxygen, osmolarity, *pho-24* (*phoQ*^{c}), *sirA*, and *barA*. To examine the role of this URS in *hha* repression of *hilA*, we measured transcription of *hilA-lacZYA* from a wild-type promoter (pLS31) or one lacking the −497 to −39 region of the promoter (pTF141) in strains either lacking *hha* or overexpressing *hha*. A *hha* mutation increased expression of *hilA-lacZYA* from the wild-type promoter from 2,017 ± 62 units to 4,017 ±116 units (2-fold increase) and overexpression of *hha* from pTF137 reduced *hilA* expression to 136 ± 10 units (15-fold decrease). In contrast, a *hha* mutation had no significant effect on *hilA-lacZYA* expression from the deleted URS construct carried by pTF141 (wild-type expression, 6,971 ± 314 units, versus *hha* mutant expression, 7,112 ± 49 units). In addition, overexpression of *hha* from pTF137 only slightly decreased *hilA-lacZYA* expression from pTF141 (5,632 ± 33 units). These data indicate that *hha* repression of *hilA* is dependent on the −497 to −39 promoter region of *hilA*.

Since a *hha* gene nearly identical to that of *Salmonella* is also present in *E. coli*, we performed experiments to determine if Hha could act as a repressor of *hilA* in *E. coli*. The *hilA-lacZYA* reporter plasmid pLS31 or pTF141 was introduced into the *E. coli lac* strain GS162 or the isogenic *hha* mutant BJ1575. *E. coli* strain GS162 had 103 ± 4 units of β-galactosidase from pLS31 compared to 250 ± 18 units from BJ1575. However, a *hha* mutation did not significantly increase *hilA-lacZYA* expression from the plasmid (pTF141) with a deletion of −497 to −39 of the *hilA* promoter (wild-type expression, 215 ± 13 units; *hha* mutant expression, 252 ± 25 units). We noted that the levels of expression of *hilA-lacZYA* from pTF141 were similar to those of pLS31 in an *E. coli hha* mutant. However, the levels of *hilA-lacZYA* from pTF141 were significantly below those which we observed for *Salmonella* and which were reported for a very similar plasmid, pLS79, by Schechter et al. in *E. coli* strain BW21355 (50). We examined this discrepancy further by performing an identical set of expression studies in *E. coli* strain BW21355. The *hilA-lacZYA* reporter plasmid pLS31 or pTF141 was introduced into the *E. coli lac* strain BW21355 or the isogenic *hha* mutant BJ1925. *E. coli* strain BW21355 expressed 69 ± 4 units of β-galactosidase from pLS31 compared to 136 ± 1 units from BJ1925. However, *hilA-lacZYA* expression from pTF141 was ≈100-fold higher in strain BW21355 (8,299 ± 234 units), a level similar to that observed by Schechter et al. A *hha* mutation did not significantly increase *hilA-lacZYA* expression (10,373 ± 150 units) in *E. coli* strain BJ1925. Our experiments with serovar Typhimurium provide additional evidence for the role of *hha* in *hilA* regulation but suggest that *hilA* expression can be misregulated in certain strains of *E. coli*, although the reason for this difference is unclear.

Hha regulates the invasive phenotype of *S. enterica* serovar Typhimurium. We performed experiments designed to assess the regulatory role of the Hha protein on the *S. enterica* serovar Typhimurium invasive phenotype. First, the effect of overexpression of *hha* on *Salmonella* invasion was examined by quantitating the invasiveness of SL1344 pGEM-T, the *hha* mutant TF80 pGEM-T and TF80 carrying pTF137. While the invasive phenotype of TF80 was slightly higher than that of SL1344 after growth under inducing conditions, overexpression of Hha from plasmid pTF137 in strain TF80 caused a significant reduction (40-fold) in invasion compared to SL1344 (data not shown). Invasion of SL1344 carrying pTF137 was repressed in a manner similar to that observed for TF80 pTF137 (data not shown). Next, we compared the invasiveness of SL1344 and TF80 after growth under oxygen-repressing, osmolarity-repressing, both oxygen-repressing and osmolarity-repressing, or inducing conditions. As shown in Fig. 4, a mutation in the *hha* gene significantly increased the invasion of *Salmonella* when grown under normally repressing growth con-

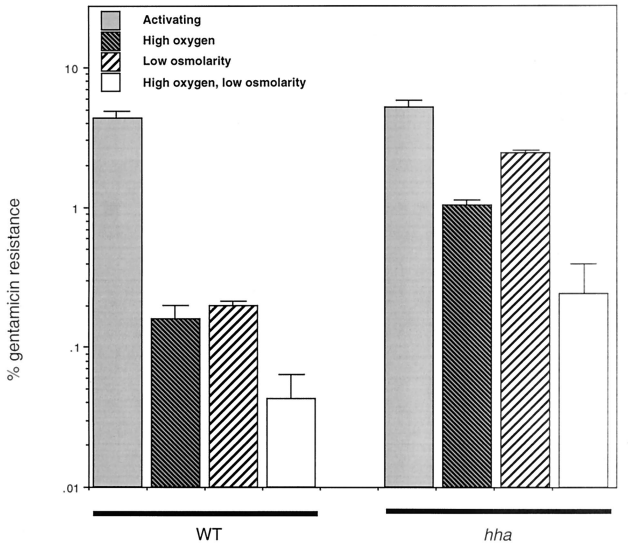

FIG. 4. Effects of an *hha* mutation on *Salmonella* SL1344 invasion after growth under repressing conditions. Cultures of SL1344 or TF80 were grown under activating low-oxygen, high-osmolarity growth conditions as the positive control or high-oxygen and high-osmolarity, low-oxygen and low-osmolarity, or high-oxygen and low-osmolarity repressing growth conditions before assaying for invasion of the bacteria into HEp-2 cells.

ditions. Invasion was 6-fold higher (0.16 versus 1.0%) ($P = 0.0005$) after growth under high-oxygen conditions, 12-fold higher (0.2 versus 2.45%) ($P < 0.0005$) after growth under low-osmolarity conditions, and 6-fold higher (0.043 versus 0.24%) ($P = 0.005$) after growth in high-oxygen, low-osmolarity conditions. These results correlate well with the increased levels of β-galactosidase expression observed with the *hilA-lacZY* reporter in the *hha* mutant (Fig. 3 and 4).

Wild-type *S. enterica* serovar Typhimurium invades and destroys M cells of the follicle-associated epithelium of Peyer's patches in a ligated loop model of infection (27, 30). Because *hilA* and many of the genes that it regulates are essential for M cell invasion, the murine ligated loop model was used to assess the effect of overexpression of Hha on invasion of M cells. Cultures of wild-type SL1344 transformed with the control vector pGEM-T or pTF137 were grown under conditions that induce invasiveness before the inocula were introduced into ligated intestinal loops of mice. Following processing of the tissue for microscopy, examination of the intestinal tissue by scanning electron microscopy revealed that more than 90% of M cells in murine ileal follicle-associated epithelium infected with wild-type bacteria had large apical membrane ruffles or were destroyed. By comparison, very few (<10%) of M cells infected with the strain overexpressing Hha displayed any membrane alterations (Fig. 5). These data establish that the presence of multicopy *hha* in wild-type *Salmonella* greatly reduces the ability of the strain to invade and destroy M cells in vivo.

Relationship of the regulatory activity of *hha* and *phoPQ* on *hilA* expression. A point mutation (*pho-24*) in the *phoQ* gene results in constitutive activation of *phoP* (20) and is known to cause significant repression of *hilA* transcription (3). Our work indicates that *hha* also plays a negative regulatory role in *hilA* expression. We were interested in determining whether *hha* negative effects were mediated by the *phoPQ* two-component system, whether constitutive *phoP* activity was mediated by the *hha* gene, or whether the two regulators exert separate effects on *hilA* expression. To this end, *Salmonella* strains that carry

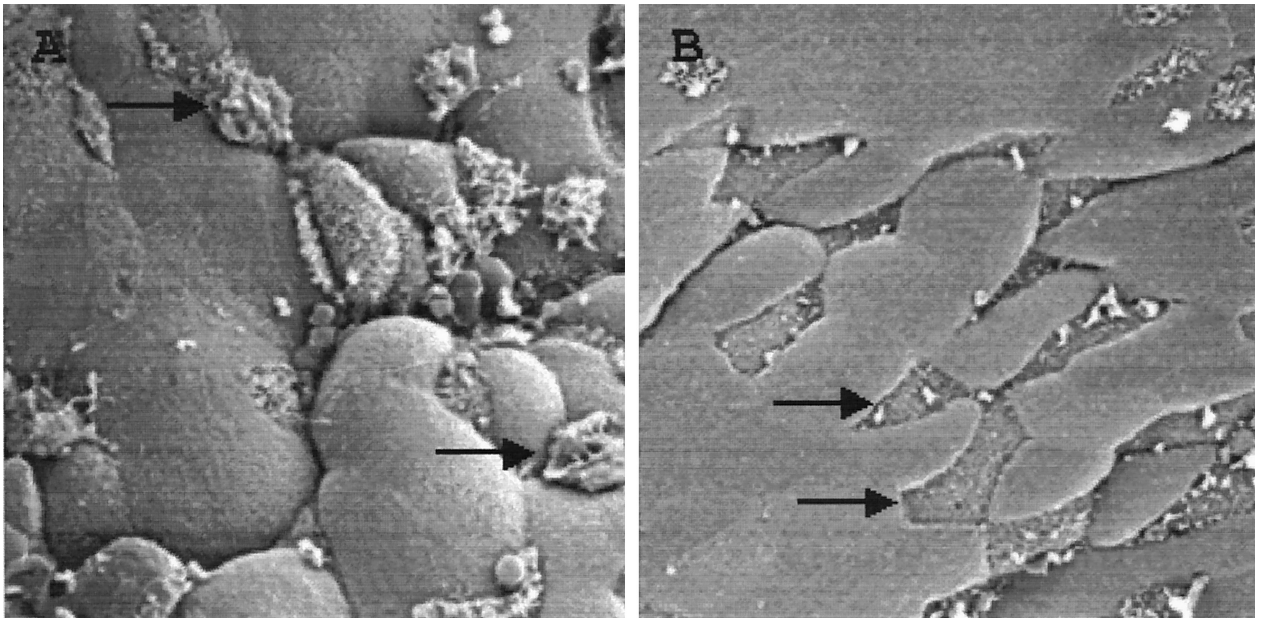

FIG. 5. Effect of multicopy *hha* on in vivo invasion. Intestinal ligated loops of BALB/c mice were prepared and injected with $\approx 4 \times 10^8$ CFU of SL1344 (wild type) containing the vector (pGEM-T) or *hha* (pTF137) plasmid. Following a 1-h incubation, infected intestinal tissue was harvested and prepared for viewing by scanning electron microscopy. (A) Murine Peyer's patch epithelium infected with SL1344 (pGEM-T). (B) Murine Peyer's patch epithelium infected with SL1344 (pTF137). Arrows, M cells.

TABLE 2. Effects of altered *phoPQ* and *hha* levels on *hilA*::Tn*5lacZY* expression

Strain (plasmid)	Strain genotype	Plasmid genotype	Units of β-galactosidase activity[a]
BJ70	*hilA*::Tn*5lacZY*	None	563 ± 3
TF79	*hilA*::Tn*5lacZY hha*::*kan*	None	1,528 ± 20
BJ690	*hilA*::Tn*5lacZY* Δ*phoP*	None	702 ± 12
BJ2305	*hilA*::Tn*5lacZY hha*::*kan* Δ*phoP*	None	1,531 ± 33
BJ70 (pGEM-T)	*hilA*::Tn*5lacZY*	Vector	575 ± 4
BJ70 (pTF137)	*hilA*::Tn*5lacZY*	*hha*$^+$	116 ± 4
BJ661 (pGEM-T)	*hilA*::Tn*5lacZY pho-24*	Vector	5 ± 1
BJ661 (pTF137)	*hilA*::Tn*5lacZY pho-24*	*hha*$^+$	2 ± 1
BJ690 (pTF137)	*hilA*::Tn*5lacZY* Δ*phoP*	*hha*$^+$	155 ± 2
BJ2227 (pGEM-T)	*hilA*::Tn*5lacZY hha*::*kan pho-24*	Vector	18 ± 8

[a] β-Galactosidase activity was determined after incubation in growth-inducing conditions.

mutations in either *phoP*, *hha* or both the *phoP* and *hha* genes were constructed in a BJ70 (*hilA*::Tn*5lacZY*) background. Other strains that carry the constitutive *phoP* mutation (*pho-24*), overexpress *hha,* or both carry *pho-24* and overexpress *hha* were constructed. In addition, we constructed and examined a strain that carries the *pho-24* mutation but has a mutation in *hha* as well as a strain that overexpresses *hha* but has a mutation in the *phoP* gene. β-Galactosidase experiments were performed to measure the effects of the different mutations on expression of the *hilA*::Tn*5lacZY* reporter (Table 2). The *Salmonella* strain carrying a mutation in *hha* (TF79) had 2.7-fold higher levels of expression of β-Galactosidase than did the parent strain BJ70. The strain carrying the *phoP* mutation, BJ690, had slightly increased levels of *hilA*::Tn*5lacZY* expression compared to BJ70. The *hha phoP* double-mutant strain had *hilA* levels of expression comparable to those of the *hha* single mutant (1,531 ± 33 units and 1,528 ± 20 units, respectively). Overexpression of *hha* from a plasmid resulted in 5.0-fold repression of the *hilA*::Tn*5lacZY* reporter, while the *pho-24* constitutive mutation decreased the *lacZY* reporter levels >100-fold. The strain overexpressing *hha* and carrying the *pho-24* mutation had β-galactosidase levels comparable to those of the strain carrying only the *pho-24* mutation. Interestingly, overexpression of *hha* still significantly repressed *hilA*::Tn*5lacZY* expression (3.7-fold) in a strain lacking a functional *phoP* gene, although not quite as well as when the *phoP* gene was intact. In addition, the *pho-24* constitutive mutation still induced efficient repression of *hilA*::Tn*5lacZY* expression (32-fold) in the absence of the *hha* gene, although not as efficiently as when a functional *hha* gene was present. These results confirm that both *phoPQ* and *hha* exert negative regulatory influences on *hilA* expression and indicate that each can significantly repress *hilA* expression in the absence of the other regulator.

The Hha protein binds to *hilA* upstream regulatory sequences. Based on the evidence presented here that *hha* is a negative modulator of *hilA* transcription and invasion, we investigated whether the Hha protein could bind directly to the *hilA* promoter. First, plasmid pTF143, which encodes Hha-MBP, was tested and found to possess the ability to repress *hilA*::Tn*5lacZY* expression 3.6-fold compared to the parent strain (data not shown). The result from this control indicated that Hha purified using the MBP system still possessed the ability to repress *hilA* expression. The ability of twofold dilutions of purified Hha protein to bind to and alter the gel mobility of a ^{32}P-labeled *hilA* promoter fragment (−497 to −39) was tested. As seen in Fig. 6A, 1.0 μmol of Hha retarded the mobility of virtually all of the radiolabeled *hilA* promoter fragment, 0.5 μmol of Hha shifted the majority of the DNA, and 0.25 μmol of Hha still shifted a substantial portion of the radiolabeled *hilA* promoter fragment. We next examined the ability of unlabeled *hilA* promoter DNA to compete for Hha binding to a ^{32}P-labeled *hilA* promoter fragment. Twofold dilutions of the unlabeled *hilA* promoter fragment (12.5 to 100 ng) were added to 100 ng of radiolabeled *hilA* promoter incubated with 1.0 μmol of Hha. As observed in Fig. 6B, the unlabeled *hilA* promoter DNA effectively competed for binding of the Hha protein, as the concentration of the unlabeled fragment approached that of the radiolabeled *hilA* fragment. Importantly, unlabeled nonspecific *Salmonella* chromosomal DNA did not compete for Hha binding to the *hilA* promoter (data not shown). As another specificity control, the *invF* promoter and portions of the open reading frame (−156 to +323) were amplified for use in a gel mobility shift assay. The *invF* promoter fragment was labeled and incubated with twofold dilutions of Hha protein (0.25 to 1.0 μmol of protein) or Fis protein (0.25 to 1.0 μmol of protein) under conditions similar to those described for the experiments illustrated in Fig. 6A. No shifting of the radiolabeled *invF* DNA was observed with the highest amounts of Hha protein used (1.0 μmol of protein), although the Fis protein was able to shift the *invF* promoter at 0.5 μmol of protein, providing additional evidence that Hha binding to the *hilA* promoter is specific under the conditions tested (data not shown).

DISCUSSION

We have used a plasmid gene bank approach to search for factors that down-regulate expression of *S. enterica* serovar Typhimurium *hilA* expression. A plasmid clone that has the ability to repress a *hilA*::Tn*5lacZY* reporter was identified, and subsequent work revealed that the plasmid carried the *hha* gene which was responsible for the *hilA*::Tn*5lacZY* repressing activity. An *S. enterica* serovar Typhimurium *hha* knockout mutant was constructed by allelic exchange, and the mutation was found to significantly derepress *hilA* expression. Hha-mediated derepression of *hilA* also resulted in significantly increased levels of tissue culture invasion after growth of the bacteria under high-osmolarity repressing conditions, as well as high-oxygen repressing conditions. Overexpression of *hha* repressed transcription of invasion genes known to be regulated by HilA (i.e., *invF*, *prgH*, and *sipC*). Finally, overexpression of *hha* repressed the invasiveness of *S. enterica* serovar Typhimurium for HEp-2 tissue culture cells and for murine M cells in an intestinal ligated loop model. These data lead us to conclude that Hha is a negative regulator of the *S. enterica* serovar Typhimurium invasion gene transcriptional activator *hilA*.

Interestingly, we found that the *invF*::*lacZY* reporter was derepressed significantly more (159-fold) than the *sipC*::*lacZY* reporter (4-fold). Since *invF* encodes a transcriptional activator of the *sipBCDA* operon, this result is somewhat surprising. One possible explanation for this difference is that basal levels

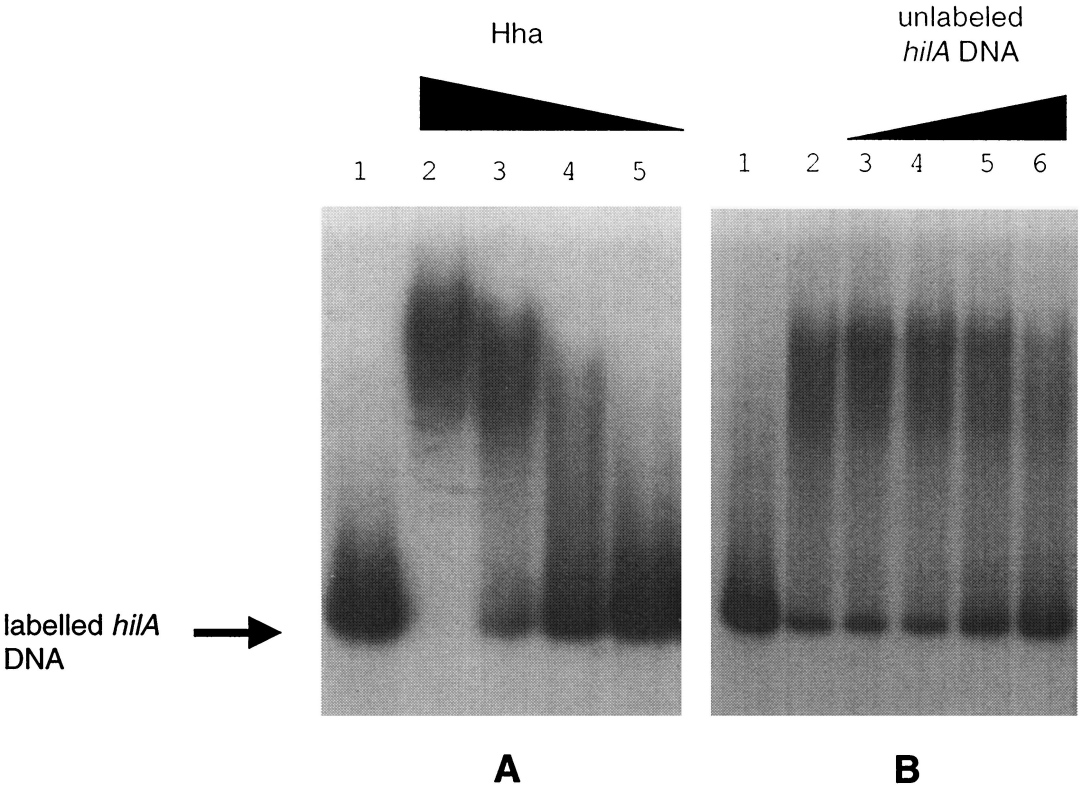

FIG. 6. Gel retardation assay of *hilA* promoter DNA with purified Hha protein. (A) ^{32}P-labeled *hilA* promoter DNA (100 ng) was incubated with various concentrations of Hha and then run on a nondenaturing 5% polyacrylamide-3% glycine gel. The concentrations of Hha used were as follows: lane 1, no protein; lane 2, 1 μmol; lane 3, 0.5 μmol; lane 4, 0.25 μmol; lane 5, 0.13 μmol. Bands were visualized by autoradiography. The arrow indicates the unshifted radiolabeled *hilA* band. (B) The binding of Hha to labeled *hilA* promoter DNA was competed with unlabeled *hilA* promoter DNA. Labeled *hilA* promoter DNA (100 ng) was mixed with 1 μmol of Hha and various amounts of unlabeled *hilA* promoter competitor DNA as indicated: lane 1, labeled *hilA* DNA only (control); lane 2, labeled *hilA* DNA with 1 μmol of Hha; lane 3, labeled *hilA* DNA with 1 μmol of Hha and 12.5 ng of unlabeled *hilA* promoter DNA; lane 4, labeled *hilA* DNA with 1 μmol of Hha and 25.0 ng of unlabeled *hilA* promoter DNA; lane 5, labeled *hilA* DNA with 1 μmol of Hha and 50.0 ng of unlabeled *hilA* promoter DNA; lane 6, labeled *hilA* DNA with 1 μmol of Hha and 100.0 ng of unlabeled *hilA* promoter DNA. Bands were visualized by autoradiography.

of *invF* and *sipC* differed substantially, with *sipC* expression levels under repressing conditions being significantly higher than those of *invF*. Thus, the apparent effect of the *hha* mutation on *sipC* expression was minimized due to the high basal levels of expression. Another possible explanation is that other regulatory factors are important for *invF* transcription, but not *sipC* transcription, and while the absence of Hha results in a significant derepression of *sipC* the additional factors still exert a significant effect on *invF* transcription. Future experiments will be performed to address these questions.

Work characterizing positive and negative regulation of *hilA* is in progress in many laboratories. It is now clear that there are multiple genes that contribute to the activation of *hilA*, including *hilC*/*sirC*/*sprA* (10, 48, 50), *hilD* (50), *sirA*, *barA* (1), *csrAB* (1), *fis* (55), and *phoB*, *fadD*, and *fliZ* (39). Schechter et al. (50) have demonstrated that *hilA* expression is repressed in the absence of *hilD*. However, deletion of the URS promoter sequence of *hilA* led to unregulated expression of *hilA*, even in the absence of *hilD*. More recently, it was found that PhoP/PhoQ, FadD, FliZ, PhoB, SirA, and EnvZ require the URS for modulation of *hilA* expression, but these factors are not responsible for regulating *hilA* in response to oxygen and osmolarity (38). It was hypothesized that these factors may function by altering the expression of *hilD* or the unidentified repressor(s). Our present findings indicate that the absence of *hha* leads to derepression of *hilA* under low-osmolarity conditions and partial derepression under high-oxygen conditions. Importantly, the derepression of *hilA* transcription under these conditions, in the *hha* mutant, leads to a corresponding increase in tissue culture invasion. Our present findings do not allow us to determine whether positive regulatory factors (i.e., *fadD*, *fliZ*, *sirA*, *barA*, *csrAB*, *phoB*, and *fis*) alter the expression or activity of *hha* or whether they modulate HilD activity posttranscriptionally. However, the activities of these regulators have been shown not to entail modulation of *hilD* transcription, although mutations in *pstS* and *sirA* were found to have a mild effect on *hilD* transcription (38). Interestingly, since a *hha* mutation did not totally derepress *hilA* expression under all repressing growth conditions, it is likely that another repressor (*hilE*, *ams*, *pag*, and/or another unidentified repressor) also plays an important role in modulating *hilA* expression in response to repressing growth conditions.

Despite the fact that a variety of environmental conditions are known to downregulate invasion gene expression, until recently the only gene known to repress *hilA* expression was the *phoQ* constitutive allele (5, 46). We have performed genetic experiments to determine if mutations in *phoPQ* and *hha* have similar effects on *hilA* transcription. The results suggest

that the mechanisms of regulation of *hha* and *phoPQ* on *hilA* transcription differ. A mutation in *hha* increases *hilA* transcription approximately threefold, and overexpression of *hha* decreases *hilA* transcription approximately fivefold. These data seem to be consistent with the idea that Hha binds at the *hilA* promoter to exert a regulatory effect on transcription of the gene. In contrast, deletion of *phoP* increases *hilA* transcription only slightly (≈20%) but the *pho-24 phoQ* constitutive mutation decreases *hilA* expression 115-fold. These results suggest that the high levels of phosphorylation activity present in strains with the *pho-24* mutation are required for *phoPQ* regulation of *hilA*. From the data it seems possible that phosphorylated PhoP is not responsible for the *hilA* repression caused by the *pho-24* mutation since a *phoP* mutation itself does not substantially derepress *hilA* transcription, although the data do not eliminate this possibility. Alternatively, hyperphosphorylation resulting from the *pho-24* mutation may downregulate the transcription or protein activity of critical positive regulatory factors such as *hilD*, *fis*, or any of the other identified factors to significantly reduce *hilA* transcription. It is also possible that hyperphosphorylation resulting from the *pho-24* mutation upregulates or increases the binding activity of negative regulatory factors. Answers to these detailed questions await further work.

Our group recently identified transposon insertions within *ams*, the gene encoding RNase E; *hupB*, a subunit of the nucleoid-associated protein HU; and an unidentified *pag* gene and a newly described *hilE* gene that cause upregulation of a *hilA*::Tn*5lacZY* reporter (12). While the mechanism by which these genes alter *hilA* expression is unknown, it is possible that some of these genes encode *hilA* repressors that may be the targets of the derepressors HilC and HilD. In the present work we have identified another gene, *hha*, that also appears to have the ability to repress *hilA* expression. Thus, the number of *hilA* negative regulators is approaching that of the identified positive regulators of *hilA*, suggesting that a mutation in any single repressor protein will not result in constitutive expression of *hilA*. Interestingly, Schechter et al. (50) recently postulated that small nucleoid-associated proteins are good candidates for *hilA* repressors because this family of proteins modulates expression of genes in response to conditions such as temperature, osmolarity, pH, and oxygen tension. Our recent findings appear to confirm this speculation.

We have provided evidence indicating that at least one activity of Hha is at the *hilA* promoter. In a model of *hilA* regulation proposed by Schechter et al. (50), a repressor protein present in both *E. coli* and *S. enterica* serovar Typhimurium acts at the −497 to −39 *hilA* promoter region to modulate *hilA* expression. Essentially identical Hha proteins (1-amino-acid difference) in *E. coli* and *S. enterica* serovar Typhimurium are functionally homologous, as *S. enterica* serovar Typhimurium Hha can repress expression of *E. coli* hemolysin. We have demonstrated that the *S. enterica* serovar Typhimurium Hha protein is able to repress expression of *hilA*::Tn*5lacZY* from a reporter plasmid. Further, the repressing activity of Hha requires the −497 to −39 region of the *hilA* promoter, since a reporter plasmid lacking this sequence was not repressed by Hha in *S. enterica* serovar Typhimurium. Finally, we have shown using a gel shift assay that purified Hha protein binds to *hilA* promoter DNA. This binding could be inhibited in competitive-binding studies with unlabeled *hilA* sequences but not by unlabeled nonspecific *Salmonella* chromosomal DNA sequences. Hha was also unable to bind to and shift a DNA fragment carrying the *invF* promoter. Collectively, these data provide evidence that Hha binds specifically to the *hilA* promoter, which we believe is the likely mechanism by which it represses *hilA* transcription. The specific sites of binding of the Hha protein await future experiments.

In summary, we report that the *S. enterica* serovar Typhimurium Hha protein is a repressor of *hilA* transcription. However, it seems clear that the Hha protein is only one of many repressors of *hilA*. Previous work by our group has identified several other genes that may encode proteins with *hilA*-repressing activity (12). In addition, *phoPQ* repression appears to be independent of the *hha* pathway. The identification of Hha as a regulator of *hilA* suggests that *hilA* may be regulated by alterations in DNA topology, which may be the common link between the effects of environmental signals on the invasive phenotype and the activity of the Hha repressor protein. Future work will be directed at more carefully defining the sites of interaction of regulator proteins at the *hilA* promoter as well as determining how repressors and activators interact with each other.

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