JOURNAL OF BACTERIOLOGY, Apr. 2000, p. 1872–1882 Vol. 182, No. 7
0021-9193/00/$04.00+0

Multiple Factors Independently Regulate *hilA* and Invasion Gene Expression in *Salmonella enterica* Serovar Typhimurium

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Received 11 November 1999/Accepted 19 January 2000

HilA activates the expression of *Salmonella enterica* serovar Typhimurium invasion genes. To learn more about regulation of *hilA*, we isolated Tn*5* mutants exhibiting reduced *hilA* and/or invasion gene expression. In addition to expected mutations, we identified Tn*5* insertions in *pstS*, *fadD*, *flhD*, *flhC*, and *fliA*. Analysis of the *pstS* mutant indicates that *hilA* and invasion genes are repressed by the response regulator PhoB in the absence of the Pst high-affinity inorganic phosphate uptake system. This system is required for negative control of the PhoR-PhoB two-component regulatory system, suggesting that *hilA* expression may be repressed by PhoR-PhoB under low extracellular inorganic phosphate conditions. FadD is required for uptake and degradation of long-chain fatty acids, and our analysis of the *fadD* mutant indicates that *hilA* is regulated by a FadD-dependent, FadR-independent mechanism. Thus, fatty acid derivatives may act as intracellular signals to regulate *hilA* expression. *flhDC* and *fliA* encode transcription factors required for flagellum production, motility, and chemotaxis. Complementation studies with *flhC* and *fliA* mutants indicate that FliZ, which is encoded in an operon with *fliA*, activates expression of *hilA*, linking regulation of *hilA* with motility. Finally, epistasis tests showed that PhoB, FadD, FliZ, SirA, and EnvZ act independently to regulate *hilA* expression and invasion. In summary, our screen has identified several distinct pathways that can modulate *S. enterica* serovar Typhimurium's ability to express *hilA* and invade host cells. Integration of signals from these different pathways may help restrict invasion gene expression during infection.

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that causes gastroenteritis in humans and a typhoid-like disease in mice. Some *S. enterica* serovar Typhimurium virulence factors are encoded on the 40-kb *Salmonella* pathogenicity island 1 (SPI1) located at centisome 63 (60). Many SPI1 genes were first identified for their roles in invasion, a process whereby bacteria induce their own uptake into normally nonphagocytic cells (34). These invasion genes have also been implicated in other processes that may contribute to *S. enterica* serovar Typhimurium virulence, including intestinal colonization (R. A. Murray, unpublished observations), destruction of M cells in Peyer's patches (49, 66), activation of cytokine expression in epithelial cells (45), induction of neutrophil migration across the intestinal epithelium (36, 58), and stimulation of apoptosis in macrophages (18, 44, 61).

SPI1 invasion genes encode a type III secretory apparatus and several secreted factors that are translocated by the secretion system directly into the cytosol of cultured epithelial cells (21, 33, 39) (Fig. 1). During invasion, the secreted effectors are thought to interact with eukaryotic proteins to activate signal transduction pathways, rearrange the actin cytoskeleton, and cause membrane ruffling and macropinocytosis in the host cell, ultimately inducing uptake of bacteria (17, 32, 33, 40, 45). Oxygen, osmolarity, bacterial growth state, and certain mutations affect *Salmonella*'s ability to invade cultured epithelial cells (10, 31, 53, 74, 79). Many of these conditions and mutations also affect expression of invasion genes (9, 65), suggesting that invasiveness may be modulated by regulating invasion gene expression. This regulation is thought to be mediated by several transcriptional regulators on SPI1, including InvF and HilA (9, 23, 29).

InvF is an AraC-like transcriptional regulator required for expression of secreted effectors encoded on SPI1 (Fig. 1), SPI5, and SopEΦ (23, 29). HilA is an OmpR-ToxR family member that appears to directly activate transcription of SPI1 genes encoding components of the type III secretory apparatus (8, 9). HilA also appears to directly activate *invF* expression, thereby indirectly activating expression of several secreted effectors. Interestingly, two SPI1 effectors may be directly activated by both HilA and InvF (23, 29). One effector, *sipC*, appears to have two promoters: a HilA-dependent promoter thought to be upstream of *spaS* and an InvF-dependent promoter located downstream of *spaS* (23). Thus, HilA directly or indirectly regulates expression of the type III secretion system and its secreted effectors, and this regulation is thought to be mediated by modulating *hilA* expression.

Changes in oxygen tension, osmolarity, and pH alter expression of *hilA* and SPI1 invasion genes (9), but the sensors and transcription factors involved in this regulation have not yet been identified. A point mutation (*pho-24*) in the extracellular cation sensor *phoQ* drastically reduces *hilA* and invasion gene expression (9, 65). Normally, the PhoQ sensor kinase is activated and phosphorylates its cognate transcriptional regulator, PhoP, when extracellular cation levels are low (82). In the *pho-24* mutant, PhoQ is active even when extracellular cation levels are relatively high, resulting in net phosphorylation of PhoP (37). The strong repressing effect of the *pho-24* mutation on *hilA* and invasion gene expression suggests that PhoP~P might repress *hilA* directly or indirectly in response to low

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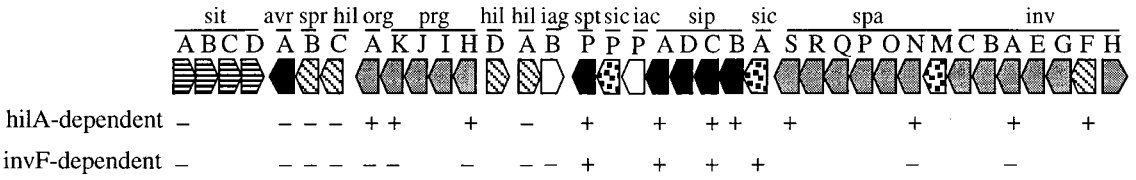

FIG. 1. Regulation of SPI1 genes by HilA and InvF. The proposed functions of SPI1 gene products are as follows: black boxes, secreted effectors; gray boxes, type III secretory apparatus; dotted boxes, chaperones involved in type III secretion; diagonally striped boxes, transcriptional regulators; horizontally striped boxes, iron uptake system; white boxes, function unknown (73). −, expression of the SPI1 gene is not affected by a mutation in *hilA* or *invF*; +, expression of the SPI1 gene is severely reduced by a mutation in *hilA* or *invF* (8, 9, 23, 29, 30; this work; S. M. Damrauer, unpublished observations).

extracellular cation levels. Expression of *hilA* and invasion genes is also reduced by disruptions in *sirA* or *barA* (1, 4, 48). The products of these genes resemble GacA and LemA, respectively. GacA and LemA are two-component regulatory factors implicated in *Pseudomonas* pathogenesis. It is thought that BarA regulates the activity of SirA in response to an unknown environmental cue, and SirA goes on to directly or indirectly activate expression of *hilA* and invasion genes.

Other mutations causing decreased *hilA* and invasion gene expression have been identified in *csrB* and in a second pathogenicity island called SPI2. In *Escherichia coli*, CsrB is an RNA thought to sequester CsrA, a protein that binds to and accelerates degradation of particular mRNAs (55, 84). Chromosomal disruptions in *S. enterica* serovar Typhimurium *csrB* or expression of *E. coli csrA* from a plasmid reduces *hilA* expression (4), suggesting that CsrA may degrade an mRNA whose product is involved in regulation of *hilA. hilA* expression is also reduced by insertions in SPI2 genes that encode structural components of a type III secretion system required by *S. enterica* serovar Typhimurium to survive inside macrophages (25). Thus, regulation of the SPI1 and SPI2 secretion systems may be interrelated. The mechanisms whereby CsrA-CsrB and SPI2 genes affect *hilA* expression are unknown.

Recent studies of the *hilA* promoter suggest that inhibition of *hilA* expression by high oxygen, low osmolarity, *pho-24*, or disruptions in *sirA* or *barA* requires region −39 to −314 upstream of the *hilA* transcriptional start site (72). An unknown repressor is thought to act at this site to inhibit *hilA* expression. *hilC*, also referred to as *sirC* (69) or *sprA* (30), and *hilD* are SPI1 genes predicted to encode AraC-like transcriptional regulators that may inhibit the repressor under appropriate conditions, allowing expression of *hilA* (72). *hilC* mutants exhibit a mild decrease in *hilA* expression and invasiveness, while a disruption in *hilD* drastically reduces *hilA* expression and significantly inhibits *S. enterica* serovar Typhimurium's ability to invade HEp-2 cells (69, 72). Thus, *hilD* seems to play a more important role than *hilC* in regulation of *hilA* expression and *S. enterica* serovar Typhimurium invasiveness in vitro. HilC may be capable of activating *invF* and *invA* expression directly, since high-level expression of *hilC* from a plasmid allows expression of these genes in the absence of *hilA* (30, 69).

Although many genes have been implicated in *hilA* regulation, only one screen designed to isolate mutations reducing invasion gene expression has been reported (48), and this screen was not saturating. Identification of such mutations might help uncover novel regulatory pathways or elucidate mechanisms involved in regulation of *hilA* and invasion genes by environmental conditions. Here we report the identification of Tn*5* insertions causing decreased *hilA* and/or invasion gene expression. Our results suggest that several pathways not previously implicated in regulation of *hilA* work independently of each other to modulate *hilA* expression and invasion of HEp-2 cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani (LB) medium composed of 0.5% Bacto-yeast extract, 1% Bacto-tryptone, and 1% NaCl. When appropriate, the medium was supplemented with antibiotics as follows: 100 to 200 μg of ampicillin per ml, 50 to 100 μg of kanamycin per ml, 10 μg of chloramphenicol per ml, and/or 10 μg of tetracycline per ml. Expression of *fliA*, *fliZY*, *fliZ*, and *fliY* was induced by growing cultures in medium containing 0.01 to 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). β-Galactosidase assays were performed with bacterial cultures grown under low-oxygen conditions as previously described (9, 54), and activities were quantified by the Miller method (59).

Tissue culture growth conditions and invasion assays. HEp-2 cells (ATCC CCL23) were maintained in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum. Invasion assays were performed on HEp-2 monolayers with oxygen-limited bacterial cultures as previously described (9, 54). To verify results from invasion assays, coverslip assays were performed on HEp-2 monolayers that were obtained by seeding approximately 5×10^4 cells per coverslip in a 24-well plate and allowing growth overnight at 37°C with 5% CO_2. After inoculation of coverslips with 100 μl of oxygen-limited bacterial cultures, plates were centrifuged at $100 \times g$ for 10 min and incubated at 37°C in a 5% CO_2 incubator for 1 h. Cells were then rinsed two times with phosphate-buffered saline (PBS), fixed with methanol for 5 min, and treated with Giemsa stain for 45 min. Following four rinses with distilled water, coverslips were dried, mounted, and examined by bright-field microscopy.

DNA methods. Restriction enzymes were purchased from New England Biolabs. Chromosomal DNA was isolated with Invitrogen's Easy-DNA kit. PCR was performed with r*Taq* polymerase from TaKaRa Shuzo Co. PCR products were cloned with a TA-cloning kit from Invitrogen. Plasmid DNA was purified on Qiagen columns. Enzymes and kits were used according to the manufacturers' instructions.

Bacterial strain construction. Marked mutations were moved into different strain backgrounds and tested for linkage to chromosomal markers by P22 transduction. Plasmids were passaged through r^-m^+ LT2 strain LB5000 (15, 70) before being electroporated into SL1344 derivatives by standard methods.

CL87 was constructed by generating a chromosomal *iagB87*::*lacZY* fusion in SL1344. A promoterless *lacZY* fragment was isolated by digesting pRS415 (76) with *Sma*I and *Stu*I. This 5.2-kb fragment was then ligated into an *Ssp*I site 90 bp downstream of the *iagB* translational start site. *iagB87*::*lacZY* was crossed into the SL1344 chromosome by allelic exchange with pMAK705 (38). The location and orientation of *lacZY* were confirmed by PCR with primers in *lacZ* and *hilA*.

Motility assays and flagellar staining. Motility was tested by assessing swarming phenotypes on motility agar (2.5% nutrient broth and 0.5% Bacto-agar) supplemented with antibiotics and IPTG when appropriate. To visualize flagella, cells were grown in tubes on a roller at 37°C to mid-log phase, and 1 drop of each culture was mixed with 5 to 10 μl of RYU Flagella stain (Remel). Cells were then examined by bright-field microscopy.

Tn*5* mutagenesis and screening of mutants. Pools of EE251::Tn*5* mutants were generated as previously described (54). To identify mutations that affect *hilA* and invasion gene expression, Tn*5* mutations were moved from EE251 pools into CL87 and EE633 by P22 phage transduction. Transductants were initially selected on LB agar supplemented with 100 μg of kanamycin per ml and 10 mM EGTA. Transductants were scraped off the plates, suspended in LB medium containing 10 mM EGTA, and replated on MacConkey lactose (MacLac) agar supplemented with 100 μg of kanamycin per ml and 10 mM EGTA. Colonies exhibiting a loss-of-red phenotype compared to their wild-type parents were restreaked on MacLac agar containing 10 mM EGTA to purify the bacteria from phage and to confirm the loss-of-red phenotype.

Identification of Tn*5* mutations. RL20, RL119, and RL224 chromosomal DNAs were digested with *Sal*I or *Eco*RI, ligated into pUC19, and transformed into DH5α. Plasmids from transformants which were resistant to both ampicillin and kanamycin were isolated and sequenced with primer IS50RL (GGTCACA TGGAAGTCAGATC), corresponding to a sequence internal to Tn*5*, or primer M13F (GTAAAACGACGGCCAG). Chromosomal DNAs from RL60, RL61, and RL134 were subjected to PCR with primers IS50RL and tar1 (CTGGCGG

TABLE 1. *S. enterica* serovar Typhimurium strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype	Source or reference
Strains		
SL1344 derivatives		
EE633	*sipA44*::Tn*5lacZY* (Tet^r)	9
EE636	*orgA*::Tn*5lacZY* (Tet^r)	9
EE637	*invF11-5*::Tn*5lacZY* (Tet^r)	9
EE638	*sipC11-6*::Tn*5lacZY* (Tet^r)	9
EE656	*prgH020*::Tn*5lacZY* (Tet^r)	9
EE658	*hilA4080*::Tn*5lacZY* (Tet^r)	9
EE659	*prgK100*::Tn*5lacZY* (Tet^r)	9
ST54	*fadF103*::Mu*d*J (*lac* Kan^r)	78
CL87	*iagB87*::*lacZY*	This work
CL87 derivatives		
RL353	*pst-4*::Tn*10* (Tet^r)	This work and reference 47
RL414	*fadD1*::Tn*5* (Kan^r)	This work
RL291	*pstS55*::Tn*5* (Kan^r)	This work
RL415	*fliA51*::Tn*5* (Kan^r)	This work
RL446	*fliA36*::Tn*5*B50 (Tet^r)	This work
RL424	*flhC4*::Tn*5* (Kan^r)	This work
RL60	*flhC36*::Tn*5* (Kan^r)	This work
RL61	*flhD37*::Tn*5* (Kan^r)	This work
EE710	*envZ182*::*cam* (Cam^r) from BA708	This work; S. Lindgren and B. A. Ahmer, unpublished data
EE719	*sirA2*::*kan* (Kan^r) from BA732	This work; B. A. Ahmer, unpublished data
RL516	*fadD1*::Tn*5 envZ182*::*cam* (Kan^r Cam^r)	This work
RL421	*fadD1*::Tn*5 pst-4*::Tn*10* (Kan^r Tet^r)	This work
RL451	*fadD1*::Tn*5 fliA36*::Tn*5*B50 (Kan^r Tet^r)	This work
RL423	*pst-4*::Tn*10 fliA51*::Tn*5* (Tet^r Kan^r)	This work
RL419	*pst-4*::Tn*10 envZ182*::*cam* (Tet^r Cam^r)	This work
RL462	*pst-4*::Tn*10 sirA2*::*kan* (Tet^r Kan^r)	This work
RL448	*fliA36*::Tn*5*B50 *envZ182*::*cam* (Tet^r Cam^r)	This work
RL524	*fliA36*::Tn*5*B50 *sirA2*::*kan* (Tet^r Kan^r)	This work
RL449	*flhC4*::Tn*5 fliA36*::Tn*5*B50 (Kan^r Tet^r)	This work
LT2 derivatives		
TBW19812	Δ*phoB1*::*cat* (Cam^r)	47
TR6583	*metE205 ara-9*	78
LS1860	TR6583 *fadR101*	78
TBW19912	*pst-4*::Tn*10*	47
EE251	*trpA8 hisC527 rpsL*	54
Plasmids		
pTrc99c	Amp^r, *tac* expression vector containing *lacI*^{q}	5
pSIIA1	Amp^r, pTrc99c with *S. enterica* serovar Typhimurium *fliA* under *tac* promoter	52
pN300	Amp^r, pACYC177 containing *E. coli fadD*	13
pSN507	Amp^r, pBR322 containing *E. coli pstSCAB-phoU* operon	6
pKK223-3	Amp^r, pBR322-derived *tac* expression vector	62
pDSM29	Amp^r, pKK223-3 with *E. coli fliZ* under *tac* promoter	62
pDSM30	Amp^r, pKK223-3 with *E. coli fliY* under *tac* promoter	62
pDSM32	Amp^r, pKK223-3 with *E. coli fliZY* under *tac* promoter	62
pAID325	Cam^r, pACYC184 containing *lacI*^{q}	26

AAGCATAACGGTG). PCR products were TA cloned into DH5α and sequenced with primers M13F and M13R (CAGGAAACAGCTATGAC). Sequencing was performed with the PRISM ready reaction dideoxy terminator cycle sequencing kit and the model 373A DNA sequencing system (Applied Biosystems).

Isolation of *fliA36*::Tn*5*B50. Pools of EE251::Tn*5*B50 mutants were generated as previously described (54). Tn*5*B50 mutations were transduced into CL87, and transductants were selected on LB agar supplemented with 10 μg of tetracycline per ml and 10 mM EGTA. Mutants were screened on motility agar supplemented with 10 μg of tetracycline per ml and 10 mM EGTA. Nonmotile mutants were examined for flagella and tested for β-galactosidase activity. Tn*5*B50 insertions in nonmotile mutants with decreased *iagB87*::*lacZY* expression were tested for linkage to *fliA51*::Tn*5* by P22 transduction. RL241 was confirmed to have a Tn*5*B50 insertion in *fliA* by PCR with primers at the 5′ and 3′ ends of *fliA*, *fliA1* (GGCGCTACAGGTTACATAAG) and *fliA2* (TAGTCTATACGTTGTGCGGC), respectively.

RESULTS

Reporter fusion strains. To monitor the effects of mutations on *hilA* and invasion gene expression, we utilized the *iagB87*::*lacZY* fusion strain CL87 and the *sipA44*::Tn*5lacZY* fusion strain EE633. *iagB* is a gene downstream of *hilA* encoding a product of unknown function, and *sipA* encodes a secreted factor whose expression is dependent on HilA (Fig. 1). *iagB* is thought to be cotranscribed with *hilA*, since *hilA339*::*kan* abol-

ishes *iagB87*::*lacZY* expression, even in the presence of plasmids expressing *hilA* or *hilA* and *iagB* (data not shown). Thus, *iagB87*::*lacZY* and *sipA4*::Tn*5lacZY* are used as reporters of *hilA* and *hilA*-dependent invasion gene expression, respectively (9, 48). Both CL87 and EE633 are able to invade HEp-2 cells as efficiently as their wild-type parent, SL1344 (C. A. Lee, unpublished observations).

Isolation of mutants. To isolate mutants with reduced *hilA* and invasion gene expression, we transduced Tn*5* insertions from our pools of Tn*5*-mutagenized EE251 into CL87 and EE633. Among approximately 9,000 transductants, we chose 36 exhibiting a lactose-negative (pink or white) phenotype on MacLac agar. Of these, we confirmed that 3 CL87::Tn*5* mutants had decreased *iagB87*::*lacZY* expression, and 25 EE633::Tn*5* mutants had decreased *sipA4*::Tn*5lacZY* expression by β-galactosidase assays. Two mutants completely lacking β-galactosidase activity had lost the *sipA4*::Tn*5lacZY* reporter fusion during transduction of the Tn*5* mutations into EE633 due to linkage between the Tn*5* and *sipA*. We examined the remaining 26 mutants for their ability to invade HEp-2 cells, and, since loss of motility results in decreased invasiveness (50), we tested noninvasive mutants for motility by microscopy and motility agar assays. To determine linkage of the mutations to SPI1 and to test their effects on other invasion genes, we transduced the kanamycin-resistant Tn*5* insertions into strains carrying various invasion gene Tn*5lacZY* reporter fusions encoding tetracycline resistance. In two cases, the phenotypes of the mutants did not cotransduce with the Tn*5* insertions, suggesting that the Tn*5* mutations are not responsible for decreased *sipA4*::Tn*5lacZY* expression in those mutants.

Based on these data, we have classified 3 CL87::Tn*5* mutants with decreased *iagB* expression and 16 EE633::Tn*5* mutants with decreased *sipA* expression into three different groups: (i) 7 motile mutants with Tn*5* insertions linked to SPI1, (ii) 7 motile mutants containing Tn*5* insertions unlinked to SPI1, and (iii) 5 nonmotile mutants with Tn*5* insertions unlinked to SPI1.

Characterization of SPI1-linked Tn*5* insertions. Seven EE633::Tn*5* mutants exhibiting 2- to 7-fold decreases in *sipA4*::Tn*5lacZY* expression and 50- to >100-fold reductions in invasion of HEp-2 cells contained Tn*5* insertions linked to SPI1. One of these insertions was closely linked to *sipA4*::Tn*5lacZY* and was not further characterized. Six other insertions were found to be >69% linked to *invG*, a gene immediately downstream of *invF*. One of them, *invF-29*::Tn*5*, was also tested for linkage to *invF11-5*::Tn*5lacZY* and was found to be 100% linked. Since disruptions in *invF* are known to cause strong defects in invasion and reduced expression of certain *hilA*-dependent genes (23, 29), the *invG*-linked Tn*5* insertions isolated in our screen are likely to decrease expression of *sipA* by reducing or abolishing *invF* expression. Such mutations are expected to decrease expression of *sipC*, but not *hilA* or other *hilA*-dependent genes (Fig. 1). In support of this, we found that *invF-29*::Tn*5* reduced expression of *sipA* and *sipC*, but not *hilA*, *orgA*, *prgH*, or *prgK* (data not shown). Other *invG*-linked insertions were not further analyzed.

Identification of motile mutants with Tn*5* insertions unlinked to SPI1. One motile CL87::Tn*5* mutant with a fivefold decrease in *iagB87*::*lacZY* expression and six motile EE633::Tn*5* mutants with two- to fourfold decreases in *sipA4*::Tn*5lacZY* expression exhibited less-than-sevenfold reductions in invasion of HEp-2 cells and contained Tn*5* insertions unlinked to SPI1. Based on sequencing of the Tn*5* insertion site junctions, two of these appear to be in the *S. enterica* serovar Typhimurium *pstS* and *fadD* genes. The region flanking *pstS55*::Tn*5* is predicted to encode amino acids that are 93% identical to PstS from *E. coli*. The insertion site corresponds to a location 798 bp downstream of the translational start site for *E. coli pstS*. The region flanking *fadD1*::Tn*5* is predicted to encode amino acids that are 75% identical to *E. coli* FadD. Its insertion site corresponds to a location 125 bp downstream of the translational start site for *E. coli fadD*. The locations of the other five Tn*5* insertions in this group have not yet been determined.

Identification of nonmotile mutants. Two CL87::Tn*5* mutants with 2- to 4-fold decreases in *iagB87*::*lacZY* expression and three EE633::Tn*5* mutants with 2- to 4-fold decreases in *sipA4*::Tn*5lacZY* expression exhibited 50- to 100-fold reductions in invasion of HEp-2 cells and had Tn*5* insertions unlinked to SPI1. These mutants were nonmotile, as determined by swarming phenotypes on motility agar plates, and staining results indicated that they lacked flagella (data not shown). Thus, the strong invasion defects of these mutants are most likely due to loss of motility. Based on DNA sequencing, the *fliA51*::Tn*5* mutant has an insertion in *S. enterica* serovar Typhimurium *fliA* 63 bp downstream of the translational start site.

The four remaining nonmotile mutants had Tn*5* insertions >57% linked to *tar*::Tn*10*. For three of these mutants, the regions between *tar* and Tn*5* were amplified by PCR, cloned, and sequenced. Two lie in *flhC*, and one lies in *flhD*. The *flhC4*::Tn*5* and *flhC36*::Tn*5* insertions lie 119 and 101 bp downstream of the *S. enterica* serovar Typhimurium *flhC* translational start site, respectively. The *flhD37*::Tn*5* insertion lies 303 bp downstream of the *S. enterica* serovar Typhimurium *flhD* translational start site. *zec57*::Tn*5* is 57.5% linked to *tar*, but its insertion site has not yet been identified.

Characterization of *pstS55*::Tn*5* mutant. The *pstS55*::Tn*5* mutation reduced expression of *hilA* and invasion genes two- to threefold, and this defect was complemented by pSN507, a pBR322 derivative containing the *E. coli pstSCAB-phoU* operon (Fig. 2A). pBR322 had no effect on *hilA* or invasion gene expression (data not shown). In *E. coli*, the *pstSCAB-phoU* operon encodes a high-affinity inorganic phosphate (P_i) uptake system (83). In addition to its role in importing P_i, the Pst system is required for negative control of the PhoR-PhoB two-component regulatory system. When extracellular P_i levels are low (<4 μM), PhoR phosphorylates the transcriptional regulator PhoB, activating expression of the phosphate (Pho) regulon (see Fig. 6). When P_i levels are high (>4 μM), PhoR acts as a phosphatase and dephosphorylates PhoB~P, inhibiting expression of the Pho regulon. In the absence of the Pst system, PhoB~P accumulates even in the presence of high P_i levels. Thus, the *pstS55*::Tn*5* mutation is expected both to abolish the high-affinity Pst system and lead to accumulation of PhoB~P.

To distinguish whether loss of the Pst transporter or accumulation of PhoB~P is responsible for reduced *hilA* expression in the *pstS55*::Tn*5* mutant, we tested the effect of Δ*phoB1*::*cat*. In the presence of *pstS55*::Tn*5*, Δ*phoB1*::*cat* restored *hilA* and invasion gene expression to wild-type levels (Fig. 2B). These data suggest that repression of *hilA* and invasion genes by *pstS55*::Tn*5* is mediated by PhoB and that the PhoR-PhoB two-component regulatory system has the ability to directly or indirectly regulate *hilA* and invasion gene expression. As expected, Δ*phoB1*::*cat* had no effect on *hilA* or invasion gene expression when the Pst system was intact (Fig. 2B). Under our growth conditions (i.e., rich medium), P_i levels are not limiting and PhoR is able to dephosphorylate PhoB in the presence of an intact Pst system, preventing any accumulation of PhoB~P.

Characterization of *fadD1*::Tn*5* mutant. *fadD1*::Tn*5* reduced expression of *hilA* and invasion genes three- to fivefold, and

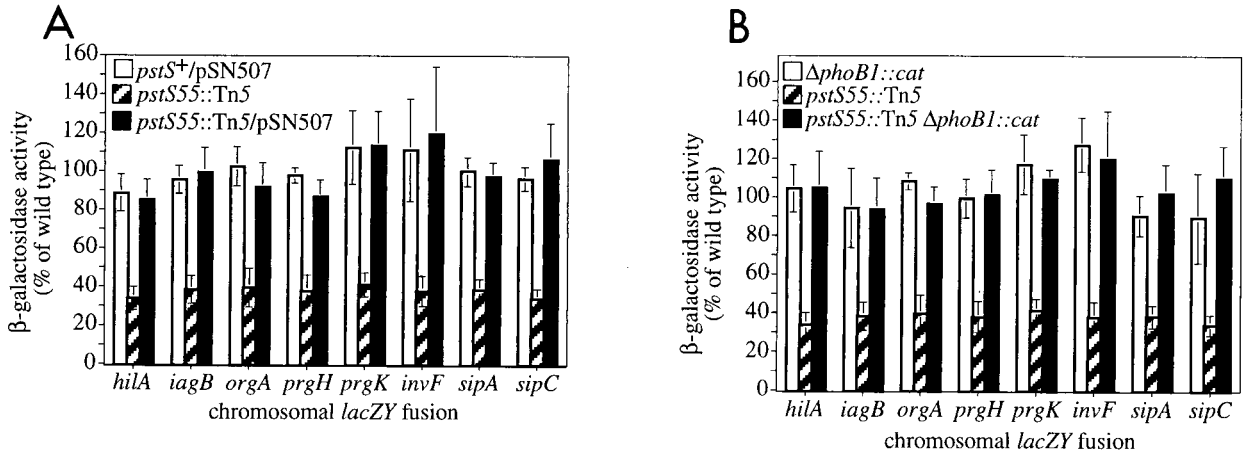

FIG. 2. *pstS55*::Tn*5* reduces *hilA* and invasion gene expression in a *phoB*-dependent manner. (A) Complementation of *pstS55*::Tn*5* by pSN507, a plasmid containing *E. coli pstSCAB-phoU*. (B) Δ*phoB1*::*cat* suppresses effects of *pstS55*::Tn*5* on *hilA* and invasion gene expression. β-Galactosidase activity for each fusion is expressed as a percentage of its activity in a wild-type SL1344 background. Average percentages were calculated by using three or more values from at least two different experiments. Error bars represent the standard deviation of normalized values. Typical β-galactosidase activities (Miller units [U]) for fusions in a wild-type background were as follows: *hilA080*::Tn*5lacZY*, 710 U; *iagB87*::*lacZY*, 637 U; *orgA*::Tn*5lacZY*, 2,331 U; *prgH020*::Tn*5lacZY*, 1,577 U; *prgK100*::Tn*5lacZY*, 3,157 U; *invF11-5*::Tn*5lacZY*, 2,663 U; *sipA4*::Tn*5lacZY*, 1,836 U; *sipC11-6*::Tn*5lacZY*, 5,233 U.

this defect was complemented by pN300, a pACYC177 derivative containing *E. coli fadD* (Fig. 3A). pACYC177 had no effect on invasion gene expression (data not shown). In *E. coli*, *fadD* encodes acyl coenzyme A (CoA) synthetase, a protein required for import of long-chain fatty acids (LCFA) and for activation of LCFA with CoA prior to the first step in β-oxidation (13). As expected, *fadD1*::Tn*5* mutants were unable to grow on LCFA as the sole carbon source (data not shown).

In *E. coli*, acyl-CoA modulates the activity of the transcriptional regulator, FadR (27, 28). In the absence of LCFA, FadR activates expression of fatty acid biosynthesis (*fab*) genes and represses expression of fatty acid degradation (*fad*) genes. In the presence of LCFA, however, long-chain fatty acyl-CoA (LCFACoA) is produced in a *fadD*-dependent manner and binds to FadR, preventing it from binding to the *fab* and *fad* promoters (Fig. 6). In *fadD* mutants, LCFA cannot be imported and LCFACoA is no longer produced, allowing FadR to bind to the *fab* and *fad* promoters even in the presence of exogenous LCFA.

Recent studies suggest that FadR activity is also inhibited by LCFACoA in *S. enterica* serovar Typhimurium (78). Since loss of *fadD* abolishes production of LCFACoA, we speculated that decreased *hilA* and invasion gene expression in the *fadD* mutant might be due to repression of *hilA* by FadR. If so, a mutation in *fadR* should suppress the effect of the *fadD* mutation on *hilA* expression. To test this, we transduced *hilA080*::Tn*5lacZY* and *fadD1*::Tn*5* into wild-type or *fadR101* LT2 strains. As a control, we used *fadF103*::Mu*dJ*, a *lacZY* fusion known to be repressed by FadR (78). *fadF* was repressed under our conditions, and, as expected, this repression was alleviated by the mutation in *fadR* (Fig. 3B). In contrast, *hilA* expression was relatively high under the same conditions.

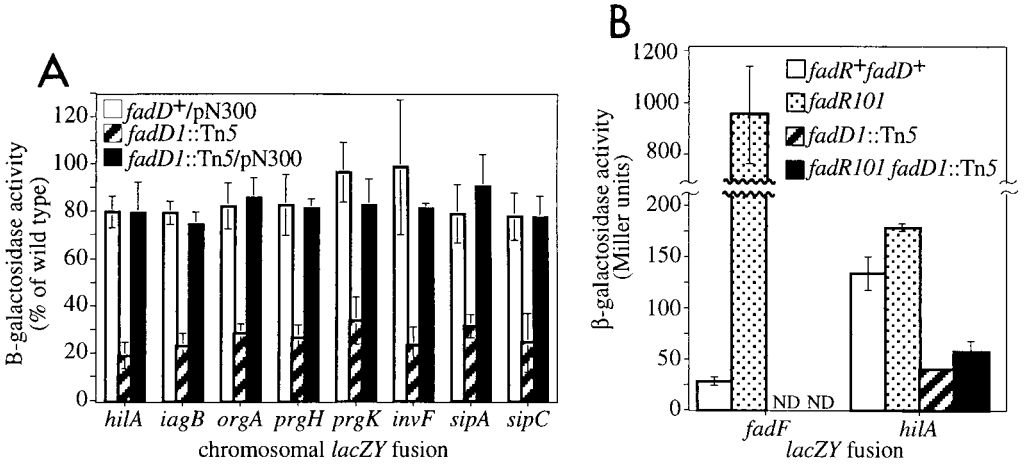

FIG. 3. *fadD1*::Tn*5* reduces *hilA* and invasion gene expression in a *fadR*-independent manner. (A) Complementation of *fadD1*::Tn*5* by pN300, a plasmid containing *E. coli fadD.* β-Galactosidase activity for each fusion is expressed as a percentage of its activity in a wild-type SL1344 background. Average percentages were calculated by using three or more values from at least two different experiments. Error bars represent the standard deviation of normalized values. (B) *fadR101* allows expression of *fadF103*::Mu*dJ* but does not suppress the effect of *fadD1*::Tn*5* on *hilA080*::Tn*5lacZY* expression. Reporter fusions and *fadD1*::Tn*5* were P22 transduced into TR6583 and LS1860. β-Galactosidase assays were performed on cultures grown in LB medium under oxygen-limiting conditions. β-Galactosidase activity is expressed as Miller units. Averages were calculated by using four or more values from at least two different experiments. Error bars represent the standard deviations. ND, not determined.

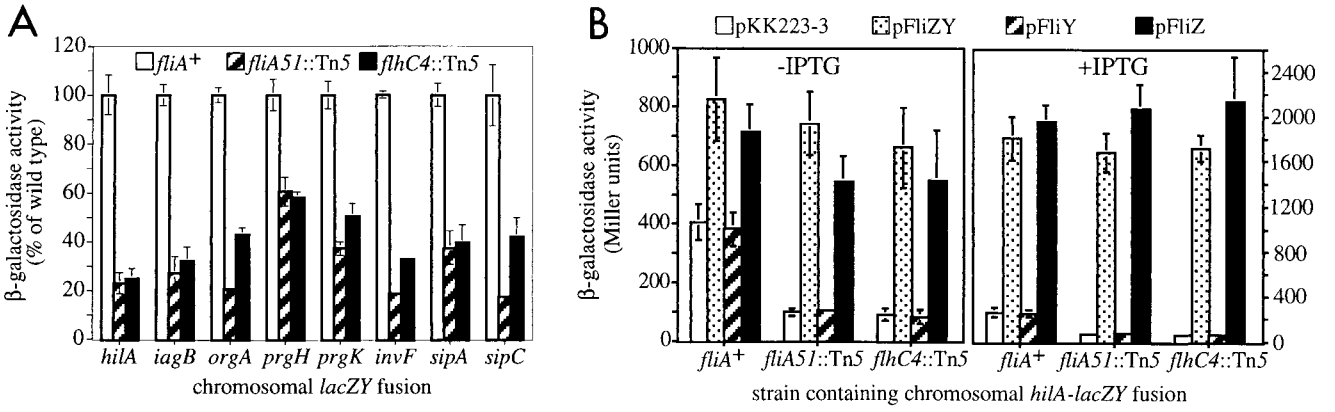

FIG. 4. (A) *fliA51*::Tn*5* and *flhC4*::Tn*5* reduce *hilA* and invasion gene expression. β-Galactosidase activity for each fusion is expressed as a percentage of its activity in a wild-type SL1344 background. Average percentages were calculated by using at least two values. Error bars represent the standard deviation of normalized values. (B) Effects of *fliA51*::Tn*5* and *flhC4*::Tn*5* on *hilA* expression are complemented by expression of *E. coli fliZ*. pFliZ is pDSM29, pFliY is pDSM30, and pFliZY is pDSM32. *fliZ*, *fliY*, or *fliZY* expression was induced by growing cultures in LB medium containing 10 mM IPTG under oxygen-limiting conditions. β-Galactosidase activity is expressed as Miller units. Averages were calculated by using four or more values from at least two different experiments. Error bars represent the standard deviations.

fadD1::Tn*5* reduced *hilA* expression in the LT2 strain, but this decrease was not suppressed by the *fadR* mutation. These results indicate that *fadD1*::Tn*5* decreases *hilA* and invasion gene expression by a *fadR*-independent mechanism.

Characterization of *fliA51*::Tn*5* and *flhC4*::Tn*5*. In *E. coli* and *S. enterica* serovar Typhimurium *flhC* and *flhD* encode master regulators of flagellar genes. FlhC and FlhD form heterotetramers to activate expression of operons encoding the flagellar basal body and FliA (56). FliA is an alternate sigma factor required for the expression of operons encoding flagellin, chemotaxis machinery, and the flagellar motor (64). *flhC4*::Tn*5* and *fliA51*::Tn*5* had comparable effects on invasion gene expression (Fig. 4A), and both abolished swarming phenotypes on motility agar plates (Table 2). As expected, addition of pSIIA1, a plasmid expressing *fliA* from the *tac* promoter, restored motility to the *fliA51*::Tn*5* mutant but not the *flhC4*::Tn*5* mutant in the presence of IPTG (Table 2). However, pSIIA1 did not complement the effect of *fliA51*::Tn*5* on *hilA* expression (data not shown), suggesting that loss of FliA itself is not responsible for decreased *hilA* and invasion gene expression in this mutant.

In both *E. coli* and *S. typhimurium*, *fliA* is in an operon with two downstream genes of unknown function, *fliZ* and *fliY* (46, 62). Since *flhDC* is required for expression of *fliA* and since *fliZ* and *fliY* are cotranscribed with *fliA* (Fig. 6), we postulated that the effects of *flhC*, *flhD*, and *fliA* mutations on *hilA* and invasion gene expression could be due to reduced or abolished expression of *fliZ* and/or *fliY*. We therefore examined the effects of *E. coli fliZY*, *fliZ*, or *fliY* on *hilA* expression. Even in the absence of IPTG, addition of plasmids expressing *E. coli fliZY* (pDSM32) or *fliZ* (pDSM29) from the *tac* promoter increased expression of *hilA* in a wild-type background and complemented the effects of *fliA51*::Tn*5* and *flhC4*::Tn*5* on *hilA* expression (Fig. 4B). In the presence of IPTG, these plasmids induced high-level expression of *hilA*, even in the *fliA* and *flhC* mutants. In contrast, the parent plasmid (pKK223-3) and a plasmid expressing *E. coli fliY* from the *tac* promoter (pDSM30) did not increase *hilA* expression in wild-type or mutant strains in the presence or absence of IPTG. As expected, the plasmid expressing *E. coli fliZ* from the *tac* promoter (pDSM29) did not restore motility to the *fliA* or *flhC* mutant in the presence of IPTG (Table 2). These results suggest that the effects of *flhC4*::Tn*5* and *fliA51*::Tn*5* on motility are due to reduced or abolished *fliA* expression, while their effects on *hilA* and invasion gene expression are due to decreased expression of *fliZ*.

TABLE 2. Swarming phenotypes on motility agar

Plasmid	Plasmid genotype	Strain genotype		
		fliA$^+$	*fliA51*::Tn*5*	*flhC4*::Tn*5*
pKK223-3	Vector	+	−	−
pDSM29	*fliZ*$^+$	+	−	−
pTrc99c	Vector	+	−	−
pSIIA1	*fliA*$^+$	+	+	−

Effects of double mutations on *iagB87*::*lacZY* expression and invasion of HEp-2 cells. The results presented above show that multiple regulators appear to control *hilA* and invasion gene expression. We therefore tested whether they operate via common or different regulatory pathways. We predicted that mutations repressing *hilA* through the same pathway would not reduce *hilA* expression more when combined than when acting alone. In contrast, we expected that mutations reducing *hilA* expression by distinct regulatory pathways would decrease *hilA* expression much more when combined than when acting alone. We therefore used P22 transduction to combine mutations that reduce *hilA* expression and compared the effects of single and double mutations on *iagB87*::*lacZY* expression and invasion of HEp-2 cells.

In addition to the mutations identified in our screen, low osmolarity (9) and disruptions in *sirA* (1, 48) are known to reduce *hilA* expression. In *E. coli* and *S. enterica* serovar Typhimurium, the EnvZ-OmpR two-component regulatory system is thought to modulate expression of certain genes in response to changes in osmolarity (67). Thus, we also tested mutants containing *envZ182*::*cam* or *sirA2*::*kan* alone and in combination with mutations identified by our screen. Mutants with insertions in *fliA* and *flhC* were not tested in invasion assays, since nonmotility per se is known to greatly reduce invasiveness, even in strains that express *hilA* (50).

In order to construct certain double mutants, we obtained mutations with alternate antibiotic resistance markers in the *pstSCAB-phoU* operon and *fliA*. *pst-4*::Tn*10* disrupts the

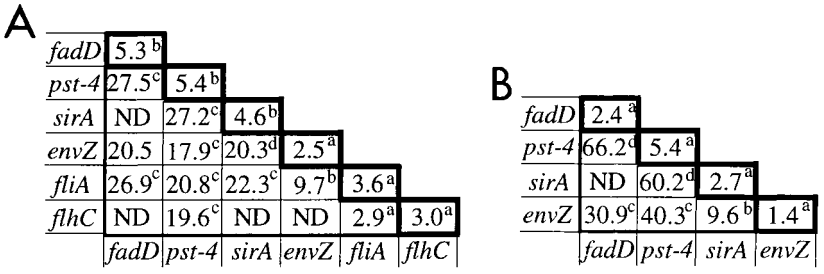

FIG. 5. Effects of single and double mutations on *iagB87*::*lacZY* expression and invasion of HEp-2 cells. The following mutations were used in these experiments: *fadD1*::Tn*5*, *pst-4*::Tn*10*, *sirA2*::*kan*, *envZ182*::*cam*, and *flhC4*::Tn*5*. *fliA51*::Tn*5* was used in combination with the *pst-4*::Tn*10* mutation, but all other *fliA* mutants contain *fliA36*::Tn*5*B50. (A) Fold reduction in *iagB87*::*lacZY* expression. Each value was calculated by dividing the β-galactosidase activity of CL87 (613 ± 100 Miller units) by the β-galactosidase activity (Miller units) of each mutant ($n \geq 4$). a, standard deviation (SD) ≤ 0.6; b, SD < 2; c, SD ≤ 3.4; d, SD = 4.9. (B) Fold reduction in invasion indices. Values were calculated by dividing the invasion index of CL87 by the invasion index of each mutant ($n \geq 3$). The invasion index for each strain was determined by measuring the percentage of bacterial inoculum surviving gentamicin treatment. a, SD ≤ 1.5; b, SD = 2.8; c, SD ≤ 18.1; d, SD ≤ 31.5. ND, not determined.

pstSCAB-phoU operon and encodes tetracycline resistance (47). To obtain a mutation in *fliA* with a different marker, we transduced Tn*5*B50 insertions encoding tetracycline resistance into CL87 and screened for nonmotile mutants with decreased *iagB87*::*lacZY* expression. We then mapped these mutations relative to *fliA51*::Tn*5* and identified a Tn*5*B50 insertion in *fliA* (data not shown).

Compared to *pstS55*::Tn*5* (Fig. 2A), *pst-4*::Tn*10* had a somewhat stronger effect on *iagB* expression (Fig. 5A). *fliA36*::Tn*5*B50's effect on *iagB* expression was comparable to that of *fliA51*::Tn*5* and was complemented by pDSM29 (data not shown). Other single mutations, including *envZ182*::*cam*, mildly reduced *iagB* expression. However, with the exception of the *fliA36*::Tn*5*B50 *flhC4*::Tn*5* mutant, double mutants exhibited much greater reductions in expression of *iagB* (Fig. 5A) than their single-mutant parents. Similarly, the single mutants invaded HEp-2 cells almost as well as CL87, while the double mutants exhibited strong invasion defects (Fig. 5B). Invasion assay results were confirmed by coverslip assays as described in Materials and Methods (data not shown). These data indicate that most of the mutations act independently of each other to reduce *hilA* expression and invasion of HEp-2 cells. For example, the mutation in *sirA* does not appear to reduce *hilA* expression by the same pathway as the mutation in *envZ*. In contrast, *fliA36*::Tn*5*B50 and *flhC4*::Tn*5* appear to work in the same pathway to reduce *hilA* expression. The latter result is consistent with our model, in which *flhDC* is required for expression of the *fliAZY* operon and *fliZ* expression is required for maximal *hilA* and invasion gene expression.

DISCUSSION

S. enterica serovar Typhimurium encounters many different environmental conditions and barriers at various sites during infection (73). After being ingested, the bacteria must survive the acidity of the stomach to reach the low-oxygen, hyperosmotic environment of the small intestine. There they must overcome the deleterious effects of defensins, secretory immunoglobulin A, bile salts, and pancreatic enzymes. To cause systemic infection, the bacteria must penetrate the intestinal epithelium and withstand the host's immune system. The latter process may be achieved in part by *S. enterica* serovar Typhimurium's ability to grow inside macrophages, where the bacteria must overcome exposure to cationic peptides, acid pH, and nutrient deprivation.

Although some genes may be important for *S. enterica* serovar Typhimurium's survival throughout infection, particular genes seem to be required to overcome unique challenges that bacteria face at specific sites. For example, SPI1 invasion genes are thought to play an important role in colonization and persistence in the intestine (R. A. Murray, unpublished observations), while certain genes on SPI2 (19, 43, 63) and SPI3 (14) appear to be important for survival in macrophages. In order to correlate expression of appropriate sets of genes with specific locations, *S. enterica* serovar Typhimurium may regulate expression of virulence genes in response to environmental signals found at critical sites during infection.

Fatty acids and virulence. Genes involved in uptake and degradation of fatty acids may be important at various stages of infection, and many of these genes seem to be induced in vivo (42). For example, in a screen for in vivo-induced genes, Mahan et al. recovered a *fadB* fusion from the spleens of mice infected intraperitoneally with *S. enterica* serovar Typhimurium (57). *fadB* encodes an enzyme required for β-oxidation of LCFA (20). In a similar screen, a *fadL* fusion was isolated from a mouse intestine 24 h after intragastric inoculation with *S. enterica* serovar Typhimurium (D. M. Heithoff, C. P. Conner, and M. J. Mahan, unpublished results). *fadL* is an outer membrane transporter required for uptake of LCFA (11, 12). In addition, a fusion to *fadF*, a gene required for metabolism of medium-chain fatty acids and LCFA (20), was induced in cultured epithelial cells (78). Finally, a Fad^- mutant has been shown to exhibit reduced virulence after oral inoculation in mice (80). Together, these results suggest that fatty acid degradation may be important for *S. enterica* serovar Typhimurium's survival throughout infection. However, the availability of fatty acids and the effects of these fatty acids on the bacteria's nutritional status, membrane composition, and levels of fatty acid intermediates may vary at different sites during infection. In turn, *S. enterica* serovar Typhimurium may utilize these variations to evaluate its environment and alter virulence gene expression accordingly.

Our studies with the *fadD* mutant suggest that loss of acyl-CoA synthetase represses *hilA* expression by a FadR-independent mechanism (Fig. 6). One explanation may be that LCFA-CoA regulates the activity or expression of some other transcriptional regulator that, in turn, modulates *hilA* expression. Another fatty acid-responsive transcriptional regulator called FarR has been identified in *E. coli* (68). FarR represses its own expression and the expression of genes encoding enzymes of the tricarboxylic acid cycle. Its ability to bind DNA in vitro is inhibited by saturated LCFA and their CoA derivatives. However, an *S. enterica* serovar Typhimurium FarR homolog is probably not responsible for repression of *hilA* in the *fadD* mutant. In our experiments, we found that *fadF* was repressed in a *fadR*-dependent manner, implying that LCFACoA levels

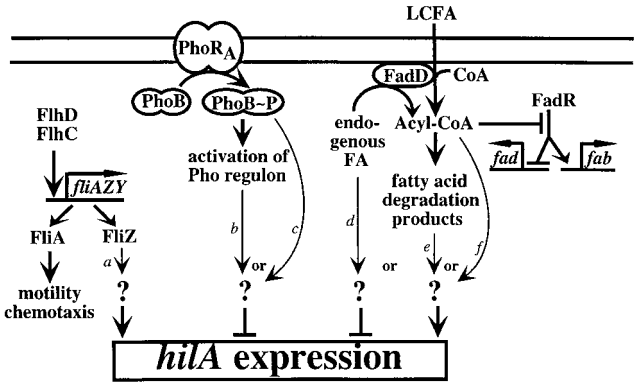

FIG. 6. Model for regulation of *hilA* expression by three distinct pathways identified in this study. Thin lines represent potential mechanisms for regulation of *hilA* by each pathway: *a*, FliZ induces the expression or activity of an activator of *hilA* expression; *b* or *c*, a gene product in the Pho regulon (*b*) or PhoB~P (*c*) activates expression or activity of a repressor of *hilA* expression; *d*, accumulation of an endogenous precursor of fatty acid metabolism activates expression or activity of a repressor of *hilA* expression; *e* or *f*, alternatively, a fatty acid degradation product (*e*) or acyl-CoA (*f*) activates the expression or activity of an activator of *hilA* expression. Other models for regulation of *hilA* by these pathways are possible.

were quite low under our conditions. In fact, no LCFA were added to the medium. *hilA* expression, however, was relatively high under these conditions in a *fadD*$^+$ strain, suggesting that very small amounts of LCFACoA are sufficient for *hilA* expression. If the reduced expression of *hilA* in the *fadD* mutant is due to loss of LCFACoA itself, any fatty acid-responsive transcriptional regulator(s) involved would have to be more sensitive to changes in minute quantities of LCFACoA than FadR. This would seem to eliminate a FarR homolog as a candidate, since *E. coli* FarR is thought to be much less sensitive to LCFACoA than FadR (27, 28, 68). No fatty acid-responsive regulator with greater sensitivity to LCFACoA than FadR has yet been identified in *E. coli* or *S. enterica* serovar Typhimurium. However, it remains possible that another fatty acid-responsive regulator is expressed or activated only in the presence of our low-oxygen, hyperosmotic conditions and that this regulator is responsible for *fadD*-dependent regulation of *hilA*.

Another possibility is that some endogenous precursor or product of fatty acid metabolism regulates expression of *hilA*. Endogenous fatty acids produced by membrane turnover and fatty acid biosynthesis in *E. coli* are thought to be converted to acyl-CoA for degradation (27). Thus, even without exogenous LCFA, a low level of β-oxidation may occur under the growth conditions used in our study. The *fadD* mutation blocks degradation of fatty acids and may lead to accumulation of endogenous precursors that might somehow repress *hilA* expression. One candidate for such an activity is long-chain acyl-acyl carrier protein (acyl-ACP), an important intermediate of fatty acid biosynthesis responsible for feedback inhibition of fatty acid biosynthetic enzymes (27). Long-chain acyl-ACP is a substrate for synthesis of lipid A and phospholipids, and changes in its levels may affect the phospholipid composition of the cellular membrane. Such alterations are known to affect expression of several genes (27). Acyl-ACP has also been implicated in production of autoinducers (71), suggesting another link between this intermediate and gene regulation. Alternatively, a product of β-oxidation may activate *hilA* expression. However, in the absence of exogenous LCFA, the levels of intermediates in this pathway are extremely low (27). Thus, any transcriptional regulator modulating *hilA* expression in response to fatty acid degradation products would have to be extremely sensitive to such metabolites.

Finally, it may be that FadD produces short- or medium-chain fatty acyl-CoA derivatives which do not affect FadR but modulate a regulator of *hilA*. Significant quantities of short-chain fatty acids have been found in the intestinal lumen (22), and their CoA derivatives may act as signals inducing *hilA* expression. Thus, production of short-chain acyl-CoA may help to activate *hilA* and invasion gene expression in the intestinal lumen. Whatever the mechanism, our results link fatty acid metabolism with regulation of *hilA*, suggesting that *S. enterica* serovar Typhimurium may somehow monitor this pathway to coordinate expression of invasion genes during infection.

Motility and regulation of invasion genes. Our results indicate that *hilA* and invasion genes are also regulated by expression of *fliZ* (Fig. 6). In addition, Eichelberg et al. have reported that *invF* expression is reduced by a *fliA*::Tn*10* insertion in *Salmonella enterica* serovar Typhi (K. Eichelberg, K. Kaniga, and J. E. Galan, Abstr. 95th Gen. Meet. Am. Soc. Microbiol. 1995, abstr. B-319, p. 221, 1995), and our results would suggest that this effect is due to reduced or abolished expression of *fliZ*. Although an in-frame deletion of *fliZ* is reported to have a mild effect on motility (46), *fliZ*'s function is unknown, and its predicted product bears no significant homology to any known protein or structural motif. It seems unlikely that FliZ itself acts as a transcriptional regulator, since it contains no apparent DNA binding domain. A mutation in *fliA* that presumably abolishes *fliZ* expression does not significantly affect *S. enterica* serovar Typhimurium's ability to cause disease in mice (75), suggesting that FliZ is not required for virulence.

Because *fliZ* is cotranscribed with *fliA* in *S. enterica* serovar Typhimurium (46), the regulation of *hilA* by FliZ implies a link between motility and invasion gene expression. Interestingly, many other pathogenic organisms also appear to coordinate motility with expression of virulence factors. In *Bordetella bronchiseptica*, BvgAS activates expression of certain virulence factors, while inhibiting expression of flagella and motility (2, 3), and in *Yersinia enterocolitica*, both motility and expression of *inv*, which encodes an invasion factor, are maximal at 23°C (51). Temperature-dependent expression of *inv* and flagellin genes seems to be coordinately regulated at the level of transcription (7). In *Vibrio cholerae*, mutations resulting in nonmotile or hypermotile phenotypes also affect expression of virulence factors, including the toxin-coregulated pilus and cholera toxin (35). Our results indicate that *S. enterica* serovar Typhimurium motility per se is not required for *hilA* expression, and *fliZ* is not necessary for motility. However, environmental signals modulating *fliAZY* expression may coordinately regulate motility via FliA and expression of invasion genes via FliZ. The significance of this link between motility and invasion gene expression in *S. enterica* serovar Typhimurium virulence is unknown.

PhoPQ and PhoR-PhoB: differential regulation of virulence genes. While this study implicates *fadD* and *fliZ* in regulating expression of only one set of virulence factors (i.e., invasion genes), evidence suggests that some regulatory pathways modulate expression of more than one set of virulence factors. One example of this is the model for regulation of virulence genes by PhoPQ. When extracellular cation levels are low, PhoPQ activates expression of PhoP-activated genes (*pag*), including genes on SPI2 (24) and SPI3 (14). Although it is unclear whether cation levels are the true signals for PhoPQ activation in vivo, many PhoP-activated genes are known to be induced in macrophages (81), implying that PhoPQ is active in that envi-

ronment. However, PhoPQ is thought to inhibit *hilA* and invasion gene expression (9, 65), suggesting that these genes are repressed in macrophages. In turn, when exposed to conditions that inactivate PhoPQ, *S. enterica* serovar Typhimurium presumably expresses invasion genes while repressing PhoP-activated genes. Thus, PhoPQ may be inactive in the small intestine where invasion gene expression is thought to be important. PhoPQ's differential regulation of PhoP-activated gene and invasion gene expression evokes a model in which *S. enterica* serovar Typhimurium uses the same regulatory system to activate the expression of genes whose products are required at a particular site (such as the intestinal lumen or inside macrophages) while simultaneously turning off expression of genes whose products are not needed at that specific location.

An interesting parallel can be drawn between this model and the regulation of virulence genes by the PhoR-PhoB two-component regulatory system. Our results indicate that PhoB~P directly or indirectly represses *hilA* and invasion genes, suggesting that low extracellular P_i levels may be capable of repressing *hilA* and invasion genes via PhoR-PhoB (Fig. 6). This leads to speculation that P_i levels are high in the intestinal lumen, allowing expression of invasion genes at that site. In contrast, P_i levels may be low in the macrophage. *gfp* fusion studies indicate that the *pstSCAB-phoU* operon is induced in macrophages (81), implying that P_i uptake and P assimilation are important for survival of *S. enterica* serovar Typhimurium in this environment. In addition, the *pstSCAB-phoU* operon is a member of the Pho regulon, and its induction in the macrophage is therefore probably mediated by PhoR-PhoB (83). This implies that P_i levels in the macrophage are low enough (<4 μM) to activate the PhoR sensor kinase, resulting in the accumulation of PhoB~P. Thus, in response to low levels of P_i in the macrophage, PhoR-PhoB may reduce expression of genes whose products are no longer needed, including invasion genes, while turning on expression of genes that help *S. enterica* serovar Typhimurium survive the harsh conditions inside macrophages, including *pstSCAB-phoU*. In support of this model, Deiwick et al. have shown that SPI2 genes are activated by low P_i levels in vitro (24).

As with PhoPQ, it is unclear how PhoR-PhoB might regulate *hilA* expression. The *E. coli* Pho regulon consists of at least 31 genes involved in P_i uptake and P assimilation (83). PhoB~P might repress *hilA* by modulating the expression of any one of these genes. Also, PhoB~P may regulate SPI2 genes in response to P_i levels, and since mutations in certain SPI2 genes reduce *hilA* expression, the regulation of *hilA* by PhoB~P may be an indirect effect resulting from a change in expression of those SPI2 genes. Alternatively, PhoB~P might repress *hilA* expression through some unknown gene not yet identified as part of the Pho regulon, or it may repress *hilA* directly. Finally, it could be that an increase in PhoB~P affects accumulation of an intracellular signal such as ppGpp, which may somehow reduce *hilA* expression (16, 77). Future studies will aim to distinguish between these possibilities.

Restricted gene expression and complex regulation. The activation and repression of different virulence genes by PhoPQ and PhoR-PhoB raise an important point about *S. enterica* serovar Typhimurium pathogenesis. While expression of a particular set of genes at a specific site may be crucial for virulence, repression of a different set of genes at the same site may be equally important for survival of bacteria in the host. Mutations causing ectopic expression of virulence genes can greatly reduce *S. enterica* serovar Typhimurium's ability to cause disease in mice (41). The attenuation of such mutants may be due in part to premature host immune responses to antigens from inappropriately expressed virulence factors. Some ectopically produced proteins may interfere with other processes whose functions are required at certain sites during infection. Also, it may be important for *S. enterica* serovar Typhimurium's survival in the host to minimize the expenditure of energy and resources required for producing certain virulence determinants. For example, type III secretion systems are relatively complex structures, and it may be energetically unfavorable to produce them continuously. Thus, it may be necessary to restrict virulence gene expression to one or a few specific locations during infection.

Our results suggest that FliZ, FadD, PhoB, EnvZ, and SirA each affect *hilA* expression independently and act through distinct pathways. PhoPQ, SPI2 genes, and CsrA-CsrB may represent other independent pathways regulating *hilA* expression, and many more such pathways may await discovery. The regulation of *hilA* expression by multiple pathways may be necessary to ensure that SPI1-mediated type III secretion occurs only in the presence of specific environmental conditions representing the precise location(s) where this process is necessary. Several candidate regulators that may integrate signals from various regulatory pathways include HilC, HilD, the unknown repressor, and/or HilA itself.

Prior studies have demonstrated that *hilA* and invasion genes are severely repressed by high oxygen, low osmolarity, or the *pho-24* mutation (9), suggesting that the expression of *hilA* (and therefore the expression of the secretion apparatus and its secreted effectors) is similar to an all-or-nothing response. However, mutations identified in this study have milder effects than the previously identified repressing environmental signals, and these mutations result in intermediate expression levels of *hilA* and invasion genes. This suggests that instead of simply being turned on or off, *hilA* can be modulated incrementally by different regulatory inputs. However, it is possible that the pathways implicated by these mutations really have much stronger effects on *hilA* expression under certain appropriate conditions.

Another possibility is that the strong repression of *hilA* by certain conditions involves two or more regulatory pathways. For example, the effect of the *envZ* mutation on *hilA* expression is fairly mild, suggesting that this regulatory system cannot fully account for the strong repression of *hilA* by low osmolarity. However, changes in osmolarity might regulate *hilA* expression through both EnvZ and some other independent pathway, and it may be necessary to abolish both pathways in order to observe the level of repression seen under low-osmolarity conditions. Thus, the regulation of *hilA* and invasion genes in response to individual environmental conditions may be more complex than previously thought.

Finally, it may be that *hilA* and invasion genes are incrementally regulated at certain sites within the host, as suggested by the effects of mutations identified in this screen. This type of regulation may be important if particular environmental conditions are the same at several critical locations during infection. By requiring more than one inhibiting signal, *S. enterica* serovar Typhimurium may have more flexibility, permitting intermediate levels of *hilA* and invasion gene expression at sites where one inhibiting condition is present. At the same time, such regulation would allow for inhibition of *hilA* and invasion gene expression when particular combinations of repressing conditions are encountered. In contrast, signals like high oxygen or low osmolarity that completely repress *hilA* and invasion genes may represent a very unique environment encountered by the bacteria at a specific site during infection. As previously suggested, a condition at one site in the host may be precisely the opposite of a condition found at another site, allowing differential regulation of distinct sets of virulence

factors by a common pathway, such as PhoPQ. In such situations, an all-or-nothing response may be more efficient than incremental regulation. Whether incremental or absolute, the repression of *hilA* and invasion genes by mutations identified in this study adds to the growing list of regulatory pathways implicated in modulating *hilA* and invasion gene expression. Future studies will aim to determine the mechanisms whereby these pathways affect expression of *hilA* and may provide insights into how various signals are integrated to ultimately influence *S. enterica* serovar Typhimurium invasiveness.

ACKNOWLEDGMENTS

We are grateful to R. Langdon and S. Pardo-Reoyo for preliminary studies of the *fadD* and *envZ* mutants; K. Kutsukake, S. Lindgren, and B. Ahmer for sharing strains; and M. Mahan for sharing unpublished data. We also thank L. Schechter, R. Murray, and S. Akbar for critical reading of the manuscript.

This work was supported by American Heart Association grant-in-aid 96006780 (C.A.L.), the Albany Medical College Strategic Research Initiative (C.C.D.), NIH grant no. GM47628-01 (M.P.S.), and NIH Senior Fellowship F33 AI10093 (B.L.W.).

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