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Roles of *hilC* and *hilD* in Regulation of *hilA* Expression in *Salmonella enterica* Serovar Typhimurium

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Sequences between −332 and −39 upstream of the *hilA* promoter are required for repression of *hilA*. An unidentified repressor is thought to bind these upstream repressing sequences (URS) to inhibit *hilA* expression. Two AraC-like transcriptional regulators encoded on *Salmonella* pathogenicity island 1 (SPI1), HilC and HilD, bind to the URS to counteract the repression of *hilA*. The URS is required for regulation of *hilA* by osmolarity, oxygen, PhoP/PhoQ, and SirA/BarA. Here, we show that FadD, FliZ, PhoB, and EnvZ/OmpR also require the URS to regulate *hilA*. These environmental and regulatory factors may affect *hilA* expression by altering the expression or activity of HilC, HilD, or the unknown repressor. To begin investigating these possibilities, we tested the effects of environmental and regulatory factors on *hilC* and *hilD* expression. We also examined *hilA* regulation when *hilC* or *hilD* was disrupted or expressed to a high level. Although *hilC* is regulated by all environmental conditions and regulatory factors that modulate *hilA* expression, *hilC* is not required for the regulation of *hilA* by any conditions or factors except EnvZ/OmpR. In contrast, *hilD* is absolutely required for *hilA* expression, but environmental conditions and regulatory factors have little or no effect on *hilD* expression. We speculate that EnvZ/OmpR regulates *hilA* by altering the expression and/or activity of *hilC*, while all other regulatory conditions and mutations regulate *hilA* by modulating *hilD* posttranscriptionally. We also discuss models in which the regulation of *hilA* expression is mediated by modulation of the expression or activity of one or more repressors.

Salmonella enterica serovar Typhimurium is a gram-negative bacterium that causes various host-specific diseases. To do so, the pathogen must overcome barriers and manipulate host cells at specific sites along the course of infection. Following ingestion, the bacteria withstand the stomach's acid environment and subsequently colonize the small intestine. In calves and humans, *S. enterica* serovar Typhimurium induces cytokine production and neutrophil migration across the intestinal epithelium to elicit inflammatory diarrhea (69). In mice, however, the bacteria spread to systemic sites by traversing the intestinal epithelium to reach Peyer's patches, lymphatics, and the bloodstream. Before reaching the Peyer's patches, bacteria can also be intercepted by $CD18^+$ phagocytes, which shuttle the bacteria directly to the liver and spleen (67). During systemic infection, the pathogen evades the host's immune response by residing within macrophages, causing a typhoid-like disease (19).

To execute such activities, *S. enterica* serovar Typhimurium produces virulence factors, including those encoded on the 40-kb *Salmonella* pathogenicity island 1 (SPI1) at centisome 63 (53). SPI1 genes encode several effectors and a type III secretory apparatus that translocates the effectors directly into the cytosol of intestinal epithelial cells (13, 64). There, the effectors interact with host cell proteins to rearrange the actin cytoskeleton and induce morphological changes that ultimately cause these normally nonphagocytic cells to take up the bacteria in a process called invasion (11, 21, 22, 31, 35). In addition to their roles in invasion, SPI1 invasion genes are important for intestinal colonization (55), destruction of M cells in Peyer's patches (39, 57), activation of cytokine secretion (69), and induction of neutrophil migration (24, 42, 49). Furthermore, effectors secreted by the SPI1 secretion apparatus activate proinflammatory and cytotoxic signal transduction pathways in host cells (11, 34, 35, 42, 54). Thus, the SPI1 type III secretion apparatus and its effectors may function in several ways to promote *Salmonella* virulence.

Virulence genes are thought to be regulated in the host such that they are expressed only at those sites where their products are needed (47). Unregulated production of virulence factors at inappropriate sites may inhibit the bacteria's ability to cause disease (32). SPI1 invasion genes are regulated in vitro by several transcription factors that may help limit SPI1 invasion gene expression to appropriate sites in vivo. HilA, an OmpR/ToxR family member encoded on SPI1, controls the expression of genes on SPI1, SPI4, SPI5, and SopEΦ (1, 6, 12, 16). HilA directly binds to and activates promoters of SPI1 operons encoding the type III secretory apparatus, several secreted effectors, and InvF, an AraC-like transcriptional regulator (46). InvF promotes expression of HilA-activated effector genes on SPI1 by directly inducing their transcription from a second, HilA-independent promoter (12). InvF also appears to directly induce expression of effector genes outside of SPI1, including *sigD*(*sopB*) and *sopE* (12, 16). Because HilA directly modulates *invF* expression, InvF-dependent transcription of effector genes is regulated indirectly by HilA. Thus, HilA directly and/or indirectly activates the expression of genes encoding the SPI1 type III secretion apparatus and its secreted effectors, thereby playing a central role in the regulatory hierarchy controlling invasion-related gene expression.

Many two-component regulatory systems have been impli-

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cated in modulating *hilA* expression in vitro. One example is PhoP/PhoQ (7). PhoQ is a membrane sensor that phosphorylates its cognate transcriptional regulator PhoP only when extracellular cation levels are low (68). PhoP~P binds to and activates promoters of *pags* (PhoP-activated genes), including genes required for bacterial survival in macrophages (26, 27, 52). A point mutation in *phoQ* called *pho-24* renders the sensor hyperactive, resulting in net phosphorylation of PhoP and activation of *pag* expression even when extracellular cation levels are high (28). The *pho-24* mutation also greatly reduces *hilA* expression, suggesting that PhoP~P represses *hilA* (7). Interestingly, a disruption in the newly identified *pag* gene increases *hilA* expression, indicating that *pag* represses *hilA* (18). This suggests that the repression of *hilA* by PhoP/PhoQ may be mediated by *pag*. However, it has not yet been determined whether a disruption in *pag* can overcome the repression of *hilA* by a *pho-24* mutation.

The PhoR/PhoB two-component signal transduction system may also regulate *hilA* expression. The PhoR sensor kinase phosphorylates PhoB when extracellular P_i levels are low (70). PhoB~P then binds to and activates promoters in the Pho regulon. However, when extracellular P_i concentrations are high, PhoR dephosphorylates PhoB~P, thereby preventing the transcriptional regulator from binding promoters and modulating gene expression. For its phosphatase activity, PhoR requires the presence of the Pst high-affinity P_i uptake system. Disruptions in the *pstSCAB-phoU* operon, which encodes the Pst system, result in an accumulation of PhoB~P and activation of the Pho regulon even when extracellular P_i levels are high. Such mutations also reduce *hilA* expression in a *phoB*-dependent manner, suggesting that PhoB~P represses *hilA* (48).

Mutant analyses also imply that BarA/SirA and EnvZ/OmpR regulate *hilA*. BarA and SirA are homologs of the *Pseudomonas* two-component regulatory factors LemA and GacA, respectively. BarA is believed to activate the transcriptional regulator SirA in response to an unknown environmental signal. Disruptions in *sirA* and *barA* repress *hilA* and invasion gene expression, suggesting that this putative two-component system activates *hilA* expression (1, 4, 38). Similarly, the EnvZ/OmpR two-component system may activate *hilA* expression since disruptions in *envZ* and *ompR* repress *hilA* (48). The sensor EnvZ modulates the activity of its cognate transcriptional regulator OmpR in response to changes in osmolarity (60).

In addition to two-component regulatory systems, small nucleoid binding proteins H-NS, HU, and Fis may modulate *hilA* expression. Recent genetic evidence suggests that H-NS can repress *hilA* under certain conditions while HU and Fis help activate *hilA* expression (L. M. Schechter and C. A. Lee, unpublished results, and reference 72). H-NS is a homodimeric protein that preferentially binds to and condenses curved DNA, causing local as well as global transcriptional effects (5). HU is composed of two similar but nonidentical subunits encoded by *hupA* and *hupB*. It binds DNA nonspecifically with respect to sequence and influences expression of a number of genes (59). In contrast, Fis is a site-specific DNA binding protein that induces sharp bends (58) and can behave both as an activator and a repressor of gene expression (20). It is unknown whether these proteins modulate *hilA* expression directly or indirectly.

hilA expression also appears to be modulated in vitro by several other factors whose roles in transcriptional regulation are not fully understood. One such factor, called CsrB, is an RNA that sequesters CsrA, which is a protein that selectively destabilizes specific mRNAs (43, 74). A disruption in *csrB* or expression of *csrA* from a plasmid represses *hilA*, suggesting that one of CsrA's target mRNAs encodes an activator of *hilA* expression (4). Loss of *csrA* also decreases *hilA* expression, suggesting that CsrA affects a repressor of *hilA* as well (3). A disruption in *ams*, which encodes RNase E, increases *hilA* expression, suggesting that RNase E inhibits *hilA* expression (18). Like CsrA, RNase E may target an RNA that induces *hilA* expression. Another potential inhibitor of *hilA* expression is a protein of unknown function called HilE. A disruption in *hilE* increases *hilA* expression, suggesting that HilE somehow represses *hilA* (18).

FliZ, whose function is also not well understood, appears to induce *hilA*. An enhancer of class II flagellar gene expression (40), FliZ is encoded in an operon with *fliA* (36), which encodes the alternative sigma factor required for class III flagellar gene expression (56). The *fliAZY* operon requires the master flagellar gene regulators, FlhD and FlhC, for expression (44). Disruptions in *flhDC* and *fliA* that reduce *fliZ* expression also repress *hilA*, and the effects of these mutations on *hilA* expression are complemented by a plasmid expressing *fliZ* from an inducible promoter (48). Furthermore, controlled expression of *fliZ* results in high-level expression of *hilA*, suggesting that FliZ somehow induces *hilA* expression.

Other factors promoting *hilA* expression may include FadD and SPI2 gene products, since disruptions in *fadD* and certain SPI2 genes repress *hilA* expression (14, 48). *fadD* encodes acyl coenzyme A synthetase, which is required for the uptake and degradation of long-chain fatty acids (15). SPI2 genes encode another type III secretion system required for *S. enterica* serovar Typhimurium's ability to survive in macrophages (33). The mechanisms whereby these factors influence *hilA* expression remain cryptic.

Several environmental conditions, including oxygen, osmolarity, and pH, also regulate *hilA* expression in vitro, but the sensors and transcription factors responsible for this environmental regulation have not yet been identified (7). Previous studies indicate that *phoP* is not required for regulation of *hilA* by any of these conditions (7). *pmrA*, an Fe^{3+}-responsive regulator (73) whose expression and activity are influenced by PhoP/PhoQ and pH (29, 65), is not required for pH regulation of *hilA* expression (unpublished observations). Furthermore, *arcA* and *oxrA*, two transcriptional regulators known to modulate expression of many genes in response to changes in redox states (8), are not required for oxygen-mediated repression of *hilA* (unpublished observations). However, it is unknown whether the environmental regulation of *hilA* expression is mediated by two-component signal transduction systems such as PhoR/PhoB, BarA/SirA, or EnvZ/OmpR, which are known to influence *hilA* expression.

It is also unclear how all of these environmental and regulatory inputs are integrated to modulate *hilA* expression. Recent evidence indicates that sequences −332 to −39 upstream of the *hilA* promoter are required for repression of *hilA* by low osmolarity, high oxygen, the *pho-24* mutation, or a disruption in *sirA* (63). This suggests that the upstream repressing se-

quences (URS) define a common regulatory node where all of these conditions and mutations converge to reduce *hilA* expression. It was proposed that an unknown repressor binds to the URS under repressing conditions to reduce *hilA* expression (63). Two AraC-like transcriptional regulators encoded on SPI1, HilC (also called SirC [61] or SprA [17]) and HilD, also bind to the URS (Schechter and Lee, unpublished results) and appear to derepress *hilA* expression (63). Thus, the environmental and regulatory inputs may affect *hilA* expression by altering the expression or activity of HilC, HilD, or the unknown repressor.

Previously, we provided evidence that FadD, FliZ, PhoB, SirA, and EnvZ regulate *hilA* expression by independent pathways (48). In this paper, we demonstrate that these pathways require the URS to regulate *hilA* expression. This finding suggests that these regulatory pathways ultimately modulate the repression-derepression mechanism at this site to alter expression of *hilA*. Our results suggest that the EnvZ/OmpR regulatory pathway modulates *hilA* expression primarily by altering the expression and/or activity of *hilC*. In contrast, our results favor models in which all other regulatory factors and environmental conditions tested affect *hilA* expression by transcriptional and/or posttranscriptional modulation of *hilD* or by altering the expression or activity of the repressor.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise specified, bacterial cultures were grown at 37°C in Luria-Bertani (LB) medium comprised of 0.5% Bacto yeast extract, 1% Bacto tryptone, and 1% NaCl. For "no salt" growth conditions, NaCl was omitted from the medium. When appropriate, the medium was supplemented with 10 μg of chloramphenicol per ml, 100 μg of ampicillin per ml, 50 μg of kanamycin per ml, or 10 μg of tetracycline per ml. To induce genes under P_{BAD} control, the medium was supplemented with 0.02% arabinose before bacterial inoculation. All strains containing plasmids that express *hilC* or *hilD* from P_{BAD} also carry a deletion in the chromosomal *araBAD* operon and therefore cannot metabolize arabinose. β-Galactosidase assays were performed on bacterial cultures grown under low-oxygen or high-oxygen conditions as previously described (7, 41), and activities were quantified by the Miller method (51).

For "extended high-oxygen" assays, the protocol for high-oxygen assays was slightly modified. Each strain was grown in LB from a single colony to saturation. Each saturated culture was then diluted 1:1,000 in fresh LB and dispensed into culture tubes such that there was 1 ml per tube. One-milliliter cultures derived from the same initial inoculum (i.e., sister cultures) were grown on a roller at 37°C for different lengths of time (2.5 to 4 h) and to varying optical densities at 600 nm (OD_{600}s). β-Galactosidase assays were performed on the cultures, and the β-galactosidase activity of each culture was plotted with respect to its OD_{600}. Data points from sister cultures grown to different OD_{600}s were combined to represent the effects of growth on the β-galactosidase activity of the strain from which the sister cultures were derived.

DNA methods. Restriction enzymes were obtained from New England Biolabs. PCR was done using Ex *Taq* polymerase from Takara Shuzo Co. Plasmid DNA was isolated using Qiagen columns, and chromosomal DNA was purified using the Easy-DNA kit from Invitrogen. Enzymes and kits were used according to the manufacturers' directions.

Plasmid construction. pSA4 was produced by cloning *hilD* downstream of P_{BAD} in pBAD33 (S. Akbar, unpublished results). pRL692 was constructed by inserting *lacZ* into the *hilD* open reading frame (ORF) on pSA4. *lacZ* was cut out of pCS3 (71) using *Bam*HI and *Bgl*II. This fragment was inserted into pSA4's unique *Bgl*II site 398 bp downstream of *hilD*'s translational start site, creating a *hilD*::*lacZ* transcriptional fusion under P_{BAD} control. To ensure that *lacZ* was in the proper orientation for a transcriptional *hilD*::*lacZ* fusion, candidates were tested for arabinose induction of *lacZ* expression and their plasmids were tested by restriction digests.

pLS106 was constructed by cloning the *hilD* promoter into pRW50 (45) upstream of *lacZ*. A region extending from 315 bp upstream to 13 bp downstream of *hilD*'s translational start site was amplified from SL1344 chromosomal DNA by PCR using primers PRG2 (CGGGATCCATATACTGTTAGCGATGTC) and LS48 (CCAAGCTTACATTTTCCATATTATCCC). The resulting PCR product has a *Bam*HI site added to its 5′ end and a *Hin*dIII site added to its 3′ end. It was first cloned into the *Bam*HI and *Hin*dIII sites in pBCKS to yield pLS103. The fragment was later cut out of pLS103 using *BamH*I and *Hin*dIII and ligated into the *Bam*HI and *Hin*dIII sites in pRW50, yielding pLS106.

Bacterial strain construction. Marked mutations were transduced into different strain backgrounds by using P22. Plasmids were passed through the $r^- m^+$ LT2 strain LB5000 (10, 62) before being electroporated into SL1344 derivatives.

RL696 was constructed by generating the chromosomal *hilD696*::*lacZ* fusion in SL1344. *hilD*::*lacZ* was cut out of pRL692 using *Bam*HI and *Pst*I. The gel-purified fragment was ligated into pLD55 (50), which had also been digested with *Bam*HI and *Pst*I. pLD55 contains the R6Kγ DNA replication origin and requires the π protein (encoded by *pir*) to be maintained (50). The ligation was therefore electroporated into DH5αλ*pir*, and Amp^r transformants were selected. These transformants were also tested for Tet^r, because pLD55 confers both Amp^r and Tet^r. Candidate plasmids were tested by restriction digests for insertion of *hilD*::*lacZ* into pLD55, and of these candidates, pRL693 was chosen for construction of RL696.

pRL693 was electroporated into the *Escherichia coli* strain SM10λ*pir*, and Amp^r transformants were then mated with SL1344. Because SL1344 lacks *pir*, Amp^r *Salmonella* conjugates contain pRL693 integrated into the chromosome. To select for these SL1344 integrants, conjugates were restreaked on M9 minimal medium supplemented with 0.1 mM histidine, 0.2% glucose, and 25 μg of ampicillin per ml. The integrants were subsequently restreaked on LB plates without selection to allow recombination. The resulting colonies were restreaked on TSS agar containing 7 μg of fusaric acid per ml to select for Tet^s bacteria that had lost the plasmid from the chromosome (9, 50). Large colonies were chosen and patched onto LB-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. Lac^+ bacteria were selected and checked for ampicillin and tetracycline sensitivity. Confirmation that RL696 contains the chromosomal *lacZ* insertion in *hilD* was obtained by using PCR with primers PRG2 and LS39 (GCGGATCCTGATAGAGCGTGTTAATG) as well as primers PRG2 and CL2 (CCAGGGTTTTCCCAGTC). LS39 hybridizes to sequences 69 to 91 bp downstream of the *hilD* translational stop site. CL2 hybridizes to the 5′ end of *lacZ*.

RESULTS

Multiple regulatory pathways act at a common node to modulate *hilA* expression. Because the URS is required for regulation of *hilA* expression by oxygen, osmolarity, PhoP/PhoQ, and SirA/BarA (63), we tested whether this *cis* element is also required for regulation of *hilA* expression by FadD, FliZ, PhoB, and EnvZ. For these experiments, we used two pRW50 reporter plasmids, pLS50 and pLS79, to measure the activity of the *hilA* promoter. In these plasmids, portions of the *hilA* promoter and the 5′ untranslated region of *hilA* are cloned upstream of a promoterless *lacZ* gene. pLS50 contains −332 to +416 of *hilA*, and pLS79 contains −39 to +416 of *hilA*.

As shown in Fig. 1, *lacZ* expression from pLS50 was reduced in *fadD, fliA, pstS*, and *envZ* mutants, compared to *lacZ* expression from this reporter in wild-type SL1344. The relative effects of these mutations on *lacZ* expression from pLS50 are comparable to their effects on the expression of the chromosomal *hilA080*::Tn*5lacZY* fusion (Fig. 2). In contrast, these mutations did not reduce *lacZ* expression from pLS79 (Fig. 1), indicating that the URS is required for repression of *hilA* by these mutations. Thus, the same regulatory node that is required for regulation of *hilA* expression by SirA, PhoP/PhoQ, oxygen, and osmolarity is also required by FadD, FliZ, PhoB, and EnvZ to modulate *hilA* expression.

Regulation of mutants by oxygen and osmolarity. The apparent convergence of multiple regulatory pathways at a common site upstream of the *hilA* promoter could be explained in part if regulation of *hilA* expression by oxygen or osmolarity is mediated by one of these regulatory factors. For example, if

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

Strain or plasmid	Genotype or relevant phenotype	Source or reference
Serovar Typhimurium SL1344 derivatives		
EE658	*hilA080*::Tn*5lacZY* (Tet^r)	7
RL21	*fadD1*::Tn*5 hilA080*::Tn*5lacZY* (Kan^r Tet^r)	48
RL119	*fliA51*::Tn*5 hilA080*::Tn*5lacZY* (Kan^r Tet^r)	48
RL147	*pstS55*::Tn*5 hilA080*::Tn*5lacZY* (Kan^r Tet^r)	48
EE711	*envZ182*::*cam hilA080*::Tn*5lacZY* (Cam^r Tet^r)	48; S. Lindgren and B. A. Ahmer, unpublished data
EE720	*sirA2*::*kan hilA080*::Tn*5lacZY* (Kan^r Tet^r)	48; B. A. Ahmer, unpublished data
LM401	*hilD1*::*kan hilA080*::Tn*5lacZY* (Kan^r Tet^r)	63
RL669	Δ*araBAD22 hilA080*::Tn*5lacZY* (Tet^r)	This work; S. Akbar, unpublished data
RL670	Δ*araBAD22 fadD1*::Tn*5 hilA080*::Tn*5lacZY* (Kan^r Tet^r)	This work and reference 48
RL672	Δ*araBAD22 fliA51*::Tn*5 hilA080*::Tn*5lacZY* (Kan^r Tet^r)	This work and reference 48
RL674	Δ*araBAD22 pstS55*::Tn*5 hilA080*::Tn*5lacZY* (Kan^r Tet^r)	This work and reference 48
RL829	Δ*araBAD22 envZ182*::*cam hilA080*::Tn*5lacZY* (Cam^r Tet^r)	This work; S. Lindgren and B. A. Ahmer, unpublished data
RL831	Δ*araBAD22 hilD1*::*kan hilA080*::Tn*5lacZY* (Kan^r Tet^r)	This work and reference 63
RL856	Δ*araBAD22 sirA2*::*kan hilA080*::Tn*5lacZY* (Kan^r Tet^r)	This work; B. A. Ahmer, unpublished data
CL87	*iagB87*::*lacZY*	48
EE724	*oxrA2*::Tn*10 iagB87*::*lacZY*(Tet^r)	This work and reference 66
LM70	*arcA201*::Tn*10dTc iagB87*::*lacZY*(Tet^r)	This work and reference 2
CL204	*pmrA1*::*cat iagB87*::*lacZY*(Cam^r)	This work and reference 65
RL353	*pst-4*::Tn*10 iagB87*::*lacZY* (Tet^r)	48, 37
RL291	*pstS55*::Tn*5 iagB87*::*lacZY* (Kan^r)	48
RL414	*fadD1*::Tn*5 iagB87*::*lacZY* (Kan^r)	48
RL415	*fliA51*::Tn*5 iagB87*::*lacZY* (Kan^r)	48
RL446	*fliA36*::Tn*5B50 iagB87*::*lacZY* (Tet^r)	48
EE710	*envZ182*::*cam iagB87*::*lacZY* (Cam^r)	48; S. Lindgren and B. A. Ahmer, unpublished data
EE719	*sirA2*::*kan iagB87*::*lacZY* (Kan^r)	48; B. A. Ahmer, unpublished data
EE725	*ompR1009*::Tn*10*Δ*16*Δ*17 iagB87*::*lacZY* (Tet^r)	This work and reference 25
RL661	*hilC1*::*cam iagB87*::*lacZY* (Cam^r)	This work; L. M. Schechter and C. A. Lee, unpublished data
RL663	*hilC1*::*cam fadD1*::Tn*5 iagB87*::*lacZY* (Cam^r Kan^r)	This work and reference 48
RL665	*hilC1*::*cam fliA51*::Tn*5 iagB87*::*lacZY* (Cam^r Kan^r)	This work and reference 48
RL667	*hilC1*::*cam pstS55*::Tn*5 iagB87*::*lacZY* (Cam^r Kan^r)	This work and reference 48
RL792	*hilC1*::*cam ompR1009*::Tn*10*Δ*16*Δ*17 iagB87*::*lacZY* (Cam^r Tet^r)	This work and reference 25
RL794	*hilC1*::*cam sirA2*::*kan iagB87*::*lacZY* (Cam^r Kan^r)	This work; B. A. Ahmer, unpublished data
RL739	*hilD1*::*kan iagB87*::*lacZY* (Kan^r)	This work and reference 63
RL850	*hilD1*::*kan envZ182*::*cam iagB87*::*lacZY* (Kan^r Cam^r)	This work; S. Lindgren and B. A. Ahmer, unpublished data
RL852	*hilD1*::*kan pst-4*::Tn*10 iagB87*::*lacZY* (Kan^r Tet^r)	This work and reference 37
RL854	*hilD1*::*kan fliA36*::Tn5B50 *iagB87*::*lacZY* (Kan^r Tet^r)	This work and reference 48
RL696	*hilD696*::*lacZ*	This work
RL699	*fliA51*::Tn*5 hilD696*::*lacZ* (Kan^r)	This work and reference 48
RL703	*sirA2*::*kan hilD696*::*lacZ* (Kan^r)	This work; B. A. Ahmer, unpublished data
RL705	*fadD1*::Tn*5 hilD696*::*lacZ* (Kan^r)	This work and reference 48
RL716	*hilC1*::*cam hilD696*::*lacZ* (Cam^r)	This work; L. M. Schechter and C. A. Lee, unpublished data
RL718	*envZ182*::*cam hilD696*::*lacZ* (Cam^r)	This work; S. Lindgren and B. A. Ahmer, unpublished data
RL752	*pstS55*::Tn*5 hilD696*::*lacZ* (Kan^r)	This work and reference 48
EE635	*hilC9*::Tn*5lacZY* (Tet^r)	63
RL707	*fadD1*::Tn*5 hilC9*::Tn*5lacZY* (Kan^r Tet^r)	This work and reference 48
RL709	*fliA51*::Tn*5 hilC9*::Tn*5lacZY* (Kan^r Tet^r)	This work and reference 48
RL711	*pstS55*::Tn*5 hilC9*::Tn*5lacZY* (Kan^r Tet^r)	This work and reference 48
RL713	*sirA2*::*kan hilC9*::Tn*5lacZY* (Kan^r Tet^r)	This work; B. A. Ahmer, unpublished data
RL715	*envZ182*::*cam hilC9*::Tn*5lacZY* (Cam^r Tet^r)	This work; S. Lindgren and B. A. Ahmer, unpublished data
RL756	*hilD1*::*kan hilC9*::Tn*5lacZY* (Kan^r Tet^r)	This work and reference 63
Plasmids		
pBAD33	Cam^r, arabinose-inducible expression vector	30
pSA4	Cam^r, pBAD33 with *hilD* under *ara* promoter	S. Akbar, unpublished data
pRL692	Cam^r, pSA4 with *lacZ* inserted in *hilD* ORF	This work
pBAD-Myc/HisC	Amp^r, arabinose-inducible expression vector	Invitrogen
pLS119	Amp^r, pBAD-Myc/HisC with *hilC* under *ara* promoter	L. M. Schechter and C. A. Lee, unpublished data
pBAD-Myc/HisC-*lacZ*	Amp^r, pBAD-Myc/HisC with *lacZ* under *ara* promoter	Invitrogen
pRW50	Tet^r, *lacZ* reporter vector	45
pLS50	Tet^r, pRW50 containing −332 to +420 of *hilA*	63
pLS79	Tet^r, pRW50 containing −39 to +420 of *hilA*	63
pLS106	Tet^r, pRW50 containing *hilD* promoter	This work

the EnvZ/OmpR two-component system is responsible for osmoregulation of *hilA* expression, this would explain why both low osmolarity and a disruption in *envZ* require the URS to reduce *hilA* expression.

To test whether FadD, FliZ, PhoB, EnvZ, or SirA is responsible for the environmental regulation of *hilA* expression, we examined the effects of oxygen and osmolarity on *hilA080*::Tn*5lacZY* expression in mutants containing disruptions in *fadD, fliA, pstS, envZ*, and *sirA*. As expected, these mutants exhibited reduced *hilA* expression under activating conditions compared to wild-type bacteria (Fig. 2). However, in the mutants, *hilA* expression was still reduced under high-oxygen or low-osmo-

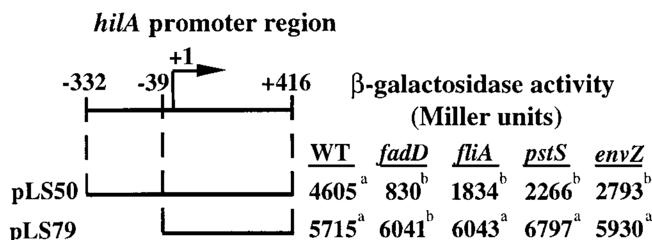

FIG. 1. Disruptions in *fadD, fliA, pstS*, and *envZ* require region −332 to −39 upstream of the *hilA* promoter to reduce *hilA* expression. The following mutations were used in this experiment: *fadD1*::Tn*5*, *fliA51*::Tn*5*, *pstS55*::Tn*5*, and *envZ182*::*cam*. β-Galactosidase assays were performed on cultures grown in high-salt LB medium (1% NaCl) under oxygen-limiting conditions (7). Averages were calculated using four or more values from at least two different experiments. a, standard deviation of ≤633; b, standard deviation of ≤263. WT, wild type.

larity (no-salt) conditions. This suggests that these regulatory factors are not required for repression of *hilA* by high oxygen or low osmolarity. In support of this conclusion, we found that a disruption in *phoB* has no effect on the environmental regulation of *hilA* expression (data not shown). Furthermore, a disruption in *ompR* reduces *hilA* expression similarly to the *envZ* mutation under activating conditions, and *hilA* is still repressed by low osmolarity in an *ompR* mutant (data not shown). Thus, the regulatory pathways mediating environmental regulation of *hilA* expression appear to be distinct from those affected by FadD, FliZ, PhoB, EnvZ, and SirA.

Effects of environmental conditions and regulatory mutations on *hilC* and *hilD* expression. Although the regulatory pathways modulating *hilA* expression appear to act independently of each other (48), their convergence at the URS suggests that they all ultimately influence a common mechanism that directly regulates *hilA* expression. HilC and HilD are thought to bind directly at this site to derepress the *hilA* promoter under activating conditions (Schechter and Lee, unpublished results). Thus, the environmental conditions and regulatory mutations that modulate *hilA* expression may do so by regulating expression of either *hilC* or *hilD*. HilC and HilD are encoded at different locations on SPI1 and are transcribed separately. Therefore, we examined the effects of these conditions and mutations on expression of *hilC9*::Tn*5lacZY* and *hilD696*::*lacZ* chromosomal fusions.

As shown in Fig. 3A, high-oxygen and low-osmolarity conditions mildly repressed *hilD* expression. Similar results were obtained with pLS106, a pRW50 reporter with *lacZ* expression under control of the *hilD* promoter (data not shown). *hilC* expression was also modestly reduced by low osmolarity but strongly repressed under high-oxygen conditions. Thus, it is possible that the repression of *hilA* expression under high-oxygen and low-osmolarity conditions is due to decreased *hilD* and *hilC* expression under these conditions.

Disruptions in *pstS* and *sirA* reduced *hilD* expression less than twofold, while mutations in *fadD, fliA*, and *envZ* had no effect (Fig. 3B). Similar results were obtained for pLS106 (data not shown). The regulation of *hilD* expression by SirA and PhoB may help mediate the regulation of *hilA* expression by these factors. However, it seems unlikely that the mild effects of the *sirA* and *pstS* mutations on *hilD* expression are entirely responsible for the effects of these mutations on *hilA* expression. The other mutations have no effect on *hilD* expression, suggesting that FadD, FliZ, and EnvZ do not regulate *hilA* by modulating *hilD* expression.

hilC expression was modestly repressed by all mutations tested (Fig. 3B). Thus, all of these regulatory factors may help modulate *hilA* expression by altering the expression of *hilC*. However, as with *hilD*, it seems unlikely that the mild effects of the mutations on *hilC* expression are entirely responsible for the effects of these mutations on *hilA* expression. The *sirA* mutation had the strongest repressing effect on *hilC* expression (approximately twofold). This effect is much milder than that reported by Rakeman et al. (61). The discrepancy may be explained by differences in growth conditions, reporter fusions, and *sirA* mutations used.

Although both HilC and HilD have been implicated in derepressing *hilA* expression, their effects on each other's expression are unknown. Such effects might help to explain how *hilA* expression is regulated by each of the derepressors. For example, if HilC regulates *hilD* expression, this might partially account for the effects of HilC on *hilA* expression. To test this, we examined the effect of *hilC1*::*cam* or *hilD1*::*kan* on *hilD* or *hilC* expression, respectively. As shown in Fig. 3B, the disruption in *hilC* had no effect on *hilD* expression. Similar results were obtained for pLS106 (data not shown). However, a disruption in *hilD* reduced *hilC* expression nearly twofold, indicating that HilD regulates *hilC* expression (Fig. 3B). Thus, the regulation of *hilA* expression by HilD may be mediated in part by the effects of HilD on *hilC* expression. Interestingly, *lacZ* expression from pLS106 is unaffected by a disruption in chromosomal *hilD*, suggesting that *hilD* is not autoregulated (data not shown).

Roles of *hilC* and *hilD* in regulation of *hilA* expression by environmental conditions and regulatory mutations. The mod-

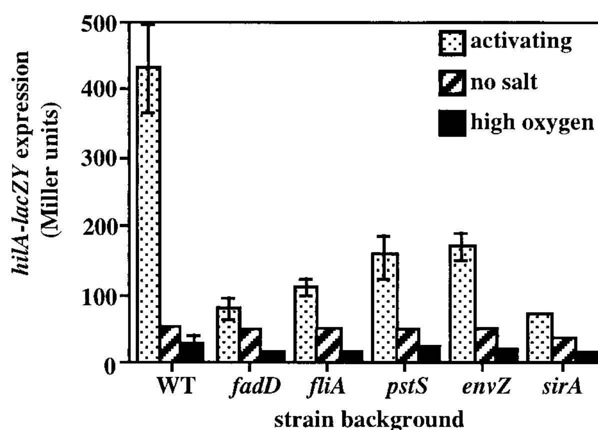

FIG. 2. Disruptions in *fadD, fliA, pstS, envZ*, and *sirA* do not abolish regulation of *hilA080*::Tn*5lacZY* expression by oxygen and osmolarity. The following mutations were used in this experiment: *fadD1*::Tn*5*, *fliA51*::Tn*5*, *pstS55*::Tn*5*, *envZ182*::*cam*, and *sirA2*::*kan*. β-Galactosidase assays were performed on cultures grown as indicated. Cultures exposed to activating conditions were grown in high-salt LB medium (1% NaCl) under oxygen-limiting conditions. No-salt cultures were grown in LB medium lacking NaCl under oxygen-limiting conditions. High-oxygen cultures were grown in high-salt LB medium (1% NaCl) under high-oxygen conditions to OD_{600}s of approximately 0.2 to 0.3 (7, 41). Averages were calculated using six or more values from at least three different experiments. Error bars represent standard deviations. WT, wild type.

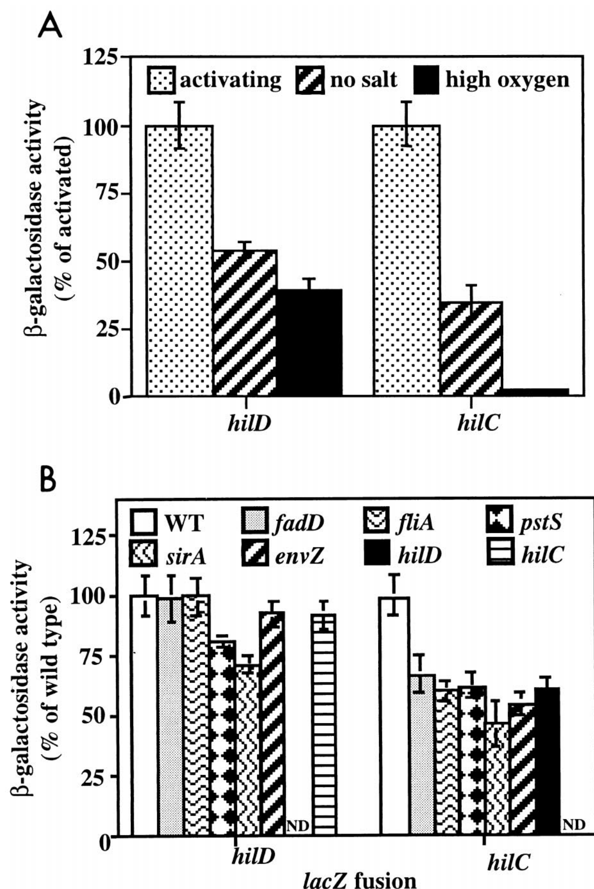

FIG. 3. Regulation of *hilC* and *hilD* expression by environmental conditions and regulatory mutations. β-Galactosidase activity for each fusion is expressed as a percentage of its activity in a wild-type (WT) background under activating conditions. Error bars represent the standard deviation of normalized values. (A) No-salt and high-oxygen conditions reduce expression of *hilC9*::Tn*5lacZY* and *hilD696*::*lacZ*. β-Galactosidase assays were performed on cultures grown as described for Fig. 2. Average percentages were calculated by using 10 or more values from at least three different experiments. (B) Effects of mutations on *hilC9*::Tn*5lacZY* and *hilD696*::*lacZ* expression. The mutations used in these experiments were as follows: *fadD1*::Tn*5*, *fliA51*::Tn*5*, *pstS55*::Tn*5*, *sirA2*::*kan*, *envZ182*::*cam*, *hilD1*::*kan*, and *hilC1*::*kan*. β-Galactosidase assays were performed on cultures grown in high-salt LB medium (1% NaCl) under oxygen-limiting conditions. Average percentages were calculated by using four or more values from at least two different experiments. Typical β-galactosidase activities (Miller units) for fusions in a wild-type background under activating environmental conditions were as follows: *hilC9*::Tn*5lacZY*, 1,081 units; *hilD696*::*lacZ*, 1,456 units.

ulation of *hilC* and *hilD* expression by oxygen and osmolarity might account for the regulation of *hilA* expression under these conditions. Furthermore, although the repression of *hilC* by *fadD, fliA, pstS, envZ*, and *sirA* mutations is mild, it is still possible that the modest effects of these mutations on *hilC* expression may help to cause the repression of *hilA* in these mutants. Similarly, although the repression of *hilD* in *sirA* and *pstS* mutants is very modest, it is still possible that this mild reduction in *hilD* expression helps mediate the repression of *hilA* in these mutants. Alternatively, these conditions and mutations may modulate *hilC* or *hilD* posttranscriptionally, thereby affecting *hilA* expression.

If environmental conditions and regulatory mutations alter *hilA* expression by modulating *hilC* or *hilD* transcriptionally or posttranscriptionally, we would expect that *hilC* or *hilD* would be required for regulation of *hilA* expression by these conditions and mutations. Therefore, we examined the effects of disruptions in *hilC* and *hilD* on the regulation of *iagB87*::*lacZY* expression by oxygen, osmolarity, and regulatory mutations. *iagB* is a gene downstream of and in the same operon with *hilA*, and the chromosomal *iagB87*::*lacZY* fusion is used as a reporter of *hilA* expression (48).

As shown in Fig. 4A, a disruption in *hilC* reduced *hilA* expression approximately twofold under activating conditions. However, *hilA* expression was further repressed under high-oxygen or low-osmolarity conditions in this mutant, indicating that *hilC* is not required for the environmental regulation of *hilA* expression. Thus, the reduction in *hilC* expression under high-oxygen and low-osmolarity conditions cannot fully account for the repression of *hilA* expression under these same conditions. Furthermore, environmental regulation of *hilA* expression is apparently not mediated by posttranscriptional modulation of *hilC*, since *hilA* expression is still regulated by environmental conditions when *hilC* is absent.

In contrast, a disruption in *hilD* strongly represses *hilA* expression. Indeed, the level of *hilA* expression in the *hilD* mutant is comparable to that observed under high-oxygen and low-osmolarity conditions in $hilD^+$ bacteria. These data are consistent with a model in which the environmental regulation of *hilA* expression is mediated by modulation of *hilD* transcriptionally or posttranscriptionally. However, these results could also be explained if *hilD* is absolutely required for *hilA* expression, such that *hilD*-independent mechanisms of regulating *hilA* expression cannot be easily observed in a *hilD* mutant. Thus, these results cannot rule out the possibility that environmental regulation of *hilA* expression occurs by a *hilD*-independent mechanism, such as modulating the expression or activity of the repressor.

We also examined the effects of regulatory mutations on *hilA* expression in *hilC* and *hilD* mutants. In Fig. 4B, we show that while *hilA* expression was reduced by a disruption in *hilC*, expression was reduced even further in a *hilC* mutant when a disruption in *fadD, fliA, pstS*, or *sirA* was also present. Conversely, while a *fadD, fliA, pstS*, or *sirA* mutant exhibits a reduced level of *hilA* expression compared to wild-type bacteria, expression is even further reduced in these mutants when *hilC* is also disrupted. Our result with the *sirA hilC* double mutant differs from that of Rakeman et al. (61), who found that *hilA* expression in a *sirA* mutant is not further repressed by a disruption in *sirC* (*hilC*). This conflict may be explained by differences in strain backgrounds and *sirA* mutations used (see Discussion). In contrast to all other mutations tested, *hilA* expression in a *hilC* mutant does not appear to be further repressed when *ompR* is disrupted, and an *ompR* mutant does not exhibit significantly greater repression when *hilC* is disrupted than when *hilC* is intact. Thus, it appears that FadD, FliZ, PhoB, and SirA act independently of *hilC* to reduce *hilA* expression. However, because EnvZ/OmpR appears to affect *hilA* expression in a *hilC*-dependent manner, the EnvZ/OmpR two-component system may modulate *hilA* expression by transcriptional and/or posttranscriptional effects on *hilC*.

In Fig. 4C, we show that *iagB87*::*lacZY* expression was se-

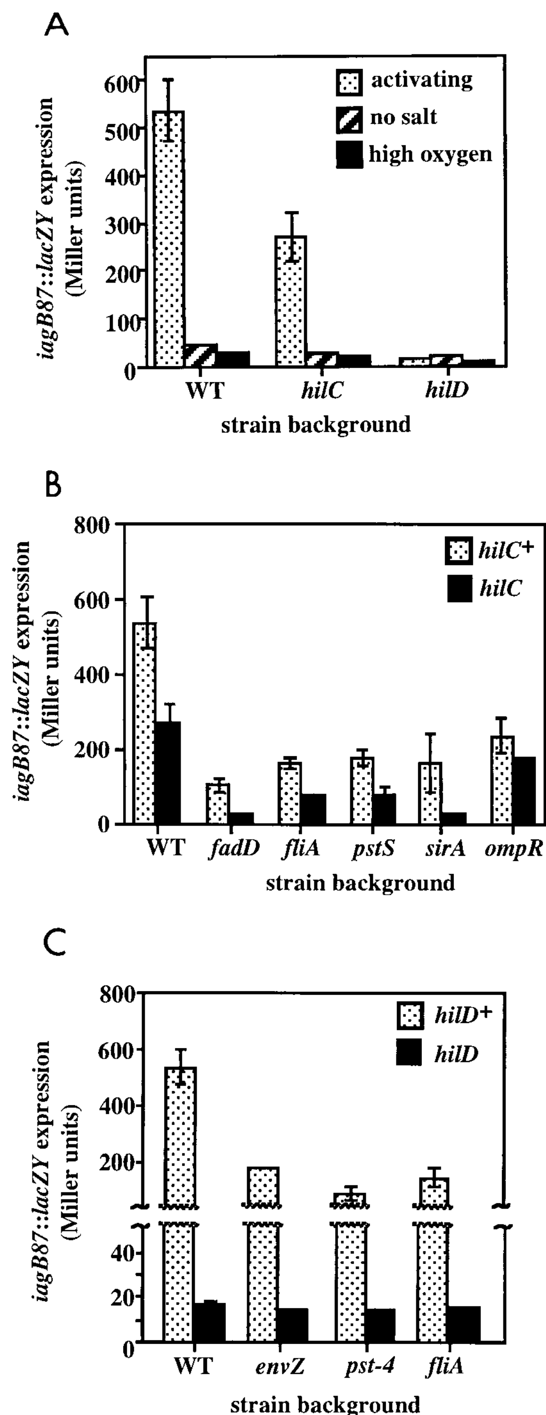

FIG. 4. Roles of *hilC* and *hilD* in regulation of *hilA* expression by environmental conditions and regulatory mutations. (A) Regulation of *iagB87*::*lacZY* expression by oxygen and osmolarity is *hilC* independent and *hilD* dependent. β-Galactosidase assays were performed on cultures grown as described for Fig. 2. Averages were calculated using four or more values from at least two different experiments. (B) The effects of *fadD, fliA, pstS*, and *sirA* disruptions on *iagB87*::*lacZY* expression are *hilC* independent. The following mutations were used in this experiment: *fadD1*::Tn*5*, *fliA51*::Tn*5*, *pstS55*::Tn*5*, *sirA2*::*kan*, *ompR1009*::Tn*10*Δ*16*Δ*17*, and *hilC1*::*cam*. β-Galactosidase assays were performed on cultures grown in high-salt LB medium (1% NaCl) under oxygen-limiting conditions. Averages were calculated using three or more values from at least two different experiments. (C) The effects of disruptions in *envZ, pst*, and *fliA* on *iagB87*::*lacZY* expression are *hilD* dependent. The following mutations were used in this experiment: *envZ182*::*cam*, *pst-4*::Tn*10*, *fliA36*::Tn*5*B50, and *hilD1*::*kan*. β-Galactosidase assays were performed on cultures grown in high-salt LB medium (1% NaCl) under oxygen-limiting conditions. Averages were calculated using six or more values from at least two different experiments. Error bars represent standard deviations. WT, wild type.

verely reduced by a disruption in *hilD* and was not further repressed by disruptions in *envZ, pstSCAB-phoU*, or *fliA*. *hilA080*::Tn*5lacZY* expression is also severely reduced when *hilD* is disrupted and is not further repressed by a disruption in *hilC* (data not shown). This suggests that these mutations may reduce *hilA* expression by transcriptional or posttranscriptional effects on *hilD*. However, the results from the *hilD envZ* double mutant suggest another interpretation of these results. Because the EnvZ/OmpR regulatory pathway appears to regulate *hilA* expression by a *hilC*-dependent mechanism (Fig. 4B), we expected that a disruption in *envZ* would repress *hilA* expression even further in a *hilD* mutant. However, *hilA* expression was no lower in the *envZ hilD* double mutant than it was in the *hilD* single mutant (Fig. 4C). This could be explained if *hilA* expression is so low in a *hilD* mutant that further repression by other mechanisms (such as reduced *hilC* expression or activity) is not observable. Thus, our results cannot rule out the possibility that the regulatory mutations affect *hilA* expression by *hilD*-independent mechanisms.

Controlled expression of *hilC* or *hilD* abolishes regulation of *hilA* expression by environmental conditions and regulatory mutations. Another way to investigate whether the regulation of *hilC* or *hilD* expression plays any role in the regulation of *hilA* expression by environmental conditions and regulatory factors is to induce *hilC* or *hilD* expression under repressing conditions. If repression by a particular regulatory pathway is specifically overcome by expressing either *hilC* or *hilD*, it might suggest that this regulatory pathway affects *hilA* expression primarily by controlling *hilC* or *hilD* expression. Because certain environmental conditions and regulatory mutations affect *hilC* or *hilD* expression and appear to regulate *hilA* expression in a *hilC*- or *hilD*-dependent manner, we expected that controlled expression of *hilC* or *hilD* would abolish the effects of some conditions and mutations on *hilA* expression but not others.

For example, we expected controlled expression of *hilC* to overcome regulation of *hilA* expression by EnvZ/OmpR because *hilC* expression appears to be regulated by this pathway and *hilC* is required for EnvZ/OmpR regulation of *hilA* expression. However, because *hilC* is not required for regulation of *hilA* expression by any other environmental condition or regulatory mutation tested, we expected that controlled expression of *hilC* would not abolish the regulation of *hilA* expression by these other conditions and mutations. Similarly, we expected that controlled expression of *hilD* would not overcome the effects of the *fadD, fliA*, or *envZ* mutations of *hilA* expression because *hilD* expression is not affected by these mutations. In contrast, we expected that controlled expression of *hilD* might overcome regulation of *hilA* expression by oxygen and osmolarity because *hilD* expression is regulated under these conditions.

To control *hilD* and *hilC* expression, we used pSA4 and pLS119, respectively. pSA4, referred to as philD, is pBAD33

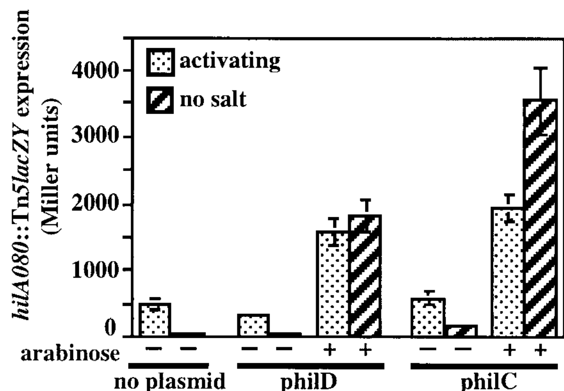

FIG. 5. Repression of *hilA080*::Tn*5lacZY* expression by low osmolarity is suppressed by arabinose-induced expression of *hilC* or *hilD*. β-Galactosidase assays were performed on cultures grown as described for Fig. 2. Media were supplemented with 0.02% arabinose prior to inoculation as indicated. Averages were calculated using six or more values from at least three different experiments. Error bars represent standard deviations.

with *hilD* cloned downstream of P_{BAD}. pLS119, referred to as philC, is pBAD-Myc/His with *hilC* cloned downstream of P_{BAD}. In the presence or absence of arabinose, the parent plasmids, pBAD33 and pBAD-Myc/His, have no effect on the regulation of *hilA* expression by oxygen, osmolarity, or a disruption in *fadD, fliA, pstS, sirA, envZ*, or *hilD* (data not shown). Furthermore, as shown in Fig. 5, philD and philC have no effect on osmoregulation of *hilA* expression in the absence of arabinose. However, arabinose-induced expression of *hilC* or *hilD* induces high levels of *hilA* expression under both activating and low osmolarity conditions. This suggests that controlled expression of *hilC* or *hilD* can abolish osmoregulation of *hilA* expression.

Similar results show that the repression of *hilA* expression under high-oxygen conditions is overcome by arabinose-induced expression of *hilC* or *hilD* (Fig. 6A). For these experiments, we performed extended high-oxygen assays in which we measured *hilA* expression of highly aerated cultures grown to various OD_{600}s. In the absence of plasmids, *hilA* expression remains low throughout growth, presumably due to oxygen repression. In contrast, *hilA* is expressed under low-oxygen (activating) conditions (Fig. 5). philD and philC have no effect on *hilA* expression under high-oxygen conditions when arabinose is omitted from the medium (Fig. 6A). However, arabinose-induced expression of *hilD* or *hilC* from these plasmids yields high levels of *hilA* expression under both activating (Fig. 5) and high-oxygen (Fig. 6A) conditions. This suggests that controlled expression of either *hilC* or *hilD* abolishes oxygen regulation of *hilA* expression.

Curiously, the repression of *hilA* expression under high-oxygen conditions is overcome by controlled *hilC* or *hilD* expression only at higher OD_{600}s. One interpretation of this result might be that *hilC* and *hilD* are posttranscriptionally modulated by oxygen at lower OD_{600}s, thereby repressing *hilA* expression. However, subsequent results revealed that this lag in *hilA* induction is probably due to a lag in the arabinose induction of *hilC* and *hilD* expression from philC and philD, respectively.

To investigate this possibility, we used pRL692 (philD with *lacZ* cloned into the *hilD* ORF) and pBAD-Myc/His-*lacZ*. In Fig. 6B, we show that *lacZ* was not expressed from pRL692 under our conditions until the cultures reached an OD_{600} of 0.25 to 0.3. This suggests that there is a delay in arabinose induction of P_{BAD} under our conditions. There is a similar delay in arabinose-induced *lacZ* expression from pBAD-Myc/His-*lacZ* (data not shown). Thus, the lag in *hilA* derepression after arabinose induction of philC or philD appears to be due to a delay in arabinose induction of P_{BAD} under our conditions rather than posttranscriptional modulation of *hilC* or *hilD*. The reason for this delay in P_{BAD} induction is unknown.

In addition to abolishing environmental regulation of *hilA* expression, controlled expression of *hilC* or *hilD* overcomes

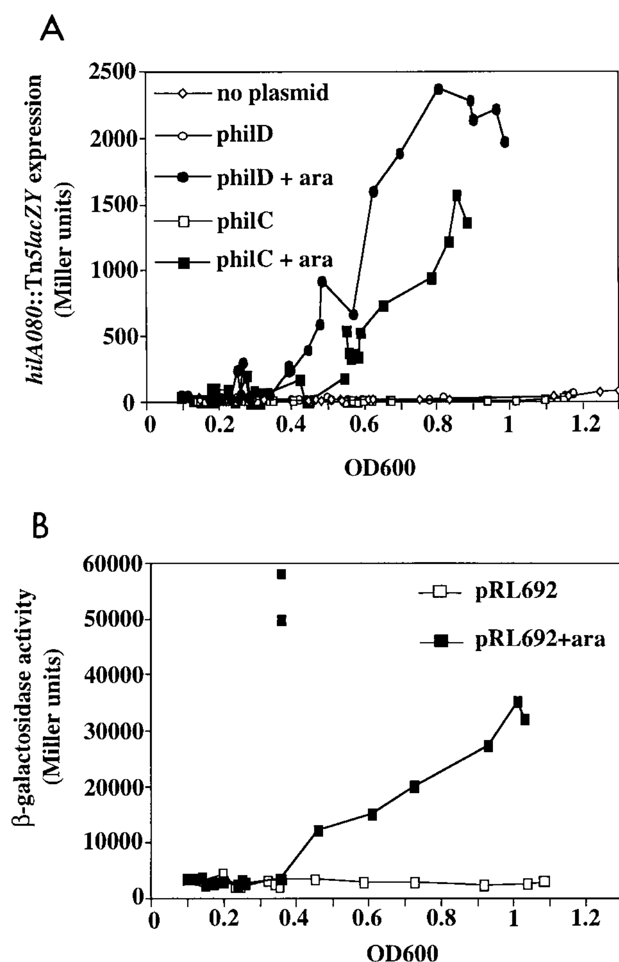

FIG. 6. Repression of *hilA080*::Tn*5lacZY* expression by high-oxygen conditions is overcome by arabinose-induced expression of *hilC* or *hilD*. β-Galactosidase assays were performed on cultures grown in high-salt LB medium (1% NaCl) with or without 0.02% arabinose under extended high-oxygen conditions as described in Materials and Methods. (A) After an initial lag, arabinose induction of *hilC* or *hilD* results in high-level expression of *hilA080*::Tn*5lacZY* under high-oxygen conditions. philC is pLS119, and philD is pSA4. Each curve is comprised of data points from at least four different experiments. (B) The delay in philD-mediated derepression of *hilA* expression is due to a lag in P_{BAD} induction. pRL692 is philD (pSA4) with *lacZ* cloned into the *hilD* ORF. Each curve is comprised of data points from three different experiments.

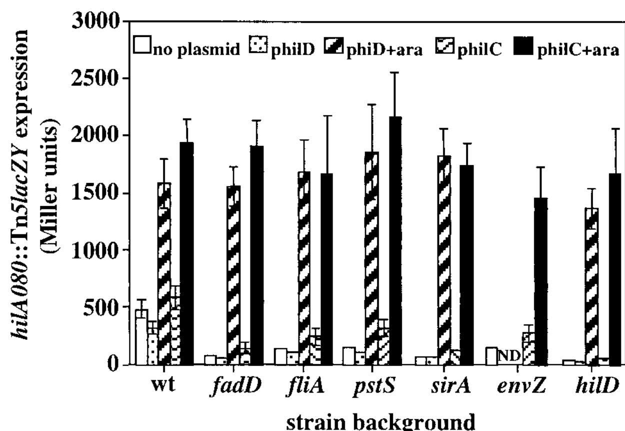

FIG. 7. Repression of *hilA080*::Tn*5lacZY* expression by disruptions in *fadD, fliA, pstS, sirA, envZ*, and *hilD* is overcome by arabinose-induced expression of *hilC* or *hilD*. The mutations used in these experiments were as follows: *fadD1*::Tn*5*, *fliA51*::Tn*5*, *pstS55*::Tn*5*, *sirA2*::*kan*, *envZ182*::*cam*, and *hilD1*::*kan*. philC is pLS119, and philD is pSA4. β-Galactosidase assays were performed on cultures grown under oxygen-limiting conditions in high-salt LB medium (1% NaCl) with or without 0.02% arabinose as indicated. Averages were calculated using four or more values from at least two different experiments. wt, wild type.

repression of *hilA* expression by all regulatory mutations tested, including a disruption in chromosomal *hilD* (Fig. 7). The latter finding confirms previous results suggesting that *hilC* expressed to high levels can substitute functionally for chromosomal *hilD* to promote *hilA* expression (63). In the absence of arabinose, philD and philC have no effect on the modulation of *hilA* expression by these regulatory mutations.

Taken together, our data demonstrate that controlled expression of either *hilC* or *hilD* abolishes regulation of *hilA* expression by all environmental conditions and regulatory mutations tested. Because *hilC* expressed from P_{BAD} can substitute functionally for chromosomal *hilD*, we cannot use these data to differentiate which regulatory pathways might act through which derepressor to modulate *hilA* expression. Furthermore, it is important to note that in wild-type bacteria under activating conditions, arabinose induction of *hilC* or *hilD* expression yields three- to fourfold higher *hilA* expression than that observed in bacteria not expressing *hilC* or *hilD* from a controlled promoter (Fig. 7). This implies that arabinose induction of *hilC* or *hilD* expression results in abnormally high levels of HilC or HilD, respectively, and may produce artificial situations that permit high-level expression of *hilA* regardless of any existing repression mechanisms (see Discussion). Thus, our findings should not be interpreted to mean that all repressing conditions and mutations affect *hilA* expression by modulating *hilC* or *hilD* expression. In fact, as previously discussed, results from some of our earlier experiments preclude this possibility for certain conditions and mutations.

DISCUSSION

Previously, Schechter et al. demonstrated that *hilA* expression is repressed in the absence of *hilD* (63). Because the URS is required for repression, an unidentified repressor is thought to bind to this region, thereby inhibiting *hilA* expression. When present, HilC or HilD binds to this same site and promotes *hilA* expression (63; Schechter and Lee, unpublished results). However, when this site is removed, the *hilA* promoter is no longer repressed even when *hilD* and *hilC* are absent. These results suggest a model in which HilC and HilD, unlike most other AraC-like transcriptional regulators (23), are not activators but instead behave as derepressors of *hilA* expression (Fig. 8). In this model, the repressor is always present but cannot inhibit *hilA* expression under activating conditions because HilD (and, to a lesser extent, HilC) displaces it from the URS. However, when *hilD* is absent, the repressor can bind to this site to inhibit *hilA* expression even under activating environmental conditions.

Previous results as well as data from this study demonstrate that the URS is also required for modulation of *hilA* expression by oxygen, osmolarity, PhoP/PhoQ, FadD, FliZ, PhoB, SirA, and EnvZ (63). None of the regulatory factors affecting *hilA* expression appears to be responsible for regulating the expression of *hilA* in response to oxygen or osmolarity. Also, previous results indicate that many of these regulatory factors act independently of each other to modulate *hilA* expression (48). Thus, multiple independent regulatory pathways converge at the URS, suggesting that they may all modulate the repression-derepression mechanism to regulate *hilA* expression. Such modulation might include altering the expression or activity of HilD, HilC, or the unknown repressor.

HilD. Mutations in *fadD, fliA, envZ*, and *hilC* have no effect on *hilD* expression. This suggests that these mutations do not repress *hilA* by modulating *hilD* expression. However, the finding that the *fliA, envZ*, and *hilC* mutations no longer affect *hilA* expression in a *hilD* mutant suggests that they may be modulating *hilA* expression by posttranscriptional effects on *hilD*. The *fadD* mutation may also affect *hilA* expression by modulating *hilD* posttranscriptionally. Alternatively, regulation by all of these mutations may be mediated by modulation of the repressor (see below).

In contrast, mutations in *pstS* and *sirA* mildly repress *hilD*.

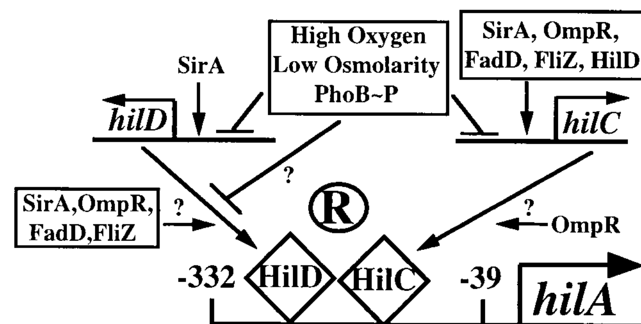

FIG. 8. Model for regulation of *hilA* expression. *hilA* expression may be regulated by modulating the expression or activity of *hilC* or *hilD*. R is an unknown repressor. Under activating conditions, HilC and HilD bind to region −332 to −39 upstream of the *hilA* promoter, displacing the repressor from this site. This allows derepression of *hilA* expression. High oxygen, low osmolarity, and PhoB~P repress expression of *hilC* and *hilD* and may modulate *hilD* posttranscriptionally. SirA, OmpR, FadD, FliZ, and HilD promote *hilC* expression, and OmpR may also modulate *hilC* posttranscriptionally. SirA, OmpR, FadD, and FliZ may also modulate *hilD* posttranscriptionally to regulate *hilA* expression.

Furthermore, *hilD* expression is repressed under high-oxygen and low-osmolarity conditions just as *hilA* expression is. This suggests that the regulation of *hilA* expression by these conditions and mutations might be mediated by modulating *hilD* expression. Consistent with this model, *hilA* expression is strongly repressed by a disruption in *hilD*, and a mutation in *pstSCAB-phoU* has no effect on *hilA* expression in a *hilD* mutant. However, such results are also consistent with models in which these conditions and mutations regulate *hilA* expression by modulating *hilD* posttranscriptionally. In an attempt to further investigate which pathways regulate *hilA* expression by modulating *hilD* expression, we examined the effect of controlled *hilD* expression on the modulation of *hilA* expression by environmental conditions and regulatory mutations.

Unexpectedly, controlled expression of *hilD* overcomes the effects of all repressing conditions and mutations on *hilA* expression. This finding should be interpreted with caution, because many of these mutations do not affect *hilD* expression and presumably regulate *hilA* expression either by posttranscriptional effects on *hilD* or by *hilD*-independent mechanisms. Thus, arabinose-induced expression of *hilD* appears to do more than just compensate for decreased *hilD* expression under repressing conditions. Indeed, *hilA* expression is much higher when *hilD* expression is driven from the P_{BAD} promoter than it is in bacteria expressing chromosomal *hilD* from its own promoter. This implies that an artificially high level of HilD is present when *hilD* is expressed from the P_{BAD} promoter. Such high-level expression of *hilD* may increase *hilA* expression under repressing conditions by compensating for posttranscriptional effects on *hilD*.

There are several ways in which *hilD* might be regulated posttranscriptionally, including modulation of *hilD* mRNA stability. A mechanism for this type of regulation may involve CsrA, which selectively destabilizes mRNAs (43). As previously discussed, both high-level expression and loss of CsrA repress *hilA*, suggesting that CsrA may destabilize different mRNAs that promote and inhibit *hilA* expression (3, 4). Alternatively, CsrA levels may directly affect the stability of *hilD* mRNA. In support of this idea, recent evidence demonstrates that *hilD* transcript levels are reduced in strains lacking or overexpressing CsrA (3). RNase E, which appears to somehow repress *hilA* expression (18), may affect *hilD* transcript stability in conjunction with CsrA. If environmental conditions and regulatory factors modulate *hilA* expression by affecting *hilD* transcript stability, high-level expression of HilD from the P_{BAD} promoter may help compensate for *hilD* transcript instability, allowing expression of *hilA* under repressing conditions. More experiments must be done to determine whether specific environmental conditions or regulatory mutations ultimately modulate *hilA* expression by these mechanisms.

Another possibility is that HilD protein activity is affected by particular environmental conditions or regulatory mutations. For example, HilD may interact with a ligand that is present under specific conditions, and the binding of this ligand may affect HilD's ability to bind DNA. Alternatively, HilD may be phosphorylated or otherwise modified such that its activity is modulated in response to particular conditions or mutations. If repression is mediated by modulating HilD activity, high-level expression of *hilD* might somehow overcome the effects of such modulation. One example of an AraC-like transcriptional regulator whose activity can be affected in this way is XylS from *Pseudomonas putida*.

XylS is thought to exist in a dynamic equilibrium between an active and inactive state, and binding of certain effectors favors the transition from the inactive to active form (23). Normally, XylS requires the binding of these effectors to activate transcription of its target genes. However, when XylS is overproduced, XylS-dependent transcription is induced even in the absence of effectors. It is thought that when the total amount of XylS in the cell is high, the amount of active XylS in equilibrium with inactive XylS is high enough to activate transcription. HilD may also exist in an equilibrium between an active and inactive state, similar to XylS. In such a model, overproduction of HilD could yield enough active HilD in equilibrium with inactive HilD to derepress *hilA* expression under repressing conditions.

Because arabinose-induced expression of *hilD* results in artificially high levels of HilD that might compensate for posttranscriptional regulation of *hilD*, our results cannot determine which environmental and regulatory pathways regulate *hilA* expression by modulating *hilD* expression. However, the effects of the *pstS* and *sirA* mutations on *hilD* expression are extremely mild, and it seems unlikely that such effects would significantly reduce *hilA* expression. Also, the reduction in *hilA* expression under high-oxygen or low-osmolarity conditions is much more dramatic than the repression of *hilD* expression by these conditions. Therefore, it seems unlikely that environmental regulation of *hilD* expression can fully account for the regulation of *hilA* expression by these environmental conditions. For these reasons, we favor models in which the regulation of *hilA* expression by environmental conditions and regulatory mutations is mediated by posttranscriptional effects on *hilD* or by modulating the expression or activity of a repressor (see below).

HilC. Interpreting the impact of controlled *hilD* expression on the modulation of *hilA* expression was made even more complicated by the fact that *hilC*, when expressed to high levels, can substitute functionally for *hilD*. Schechter et al. found that high-level expression of *hilC* could derepress the *hilA* promoter even in the absence of *hilD* (63). Furthermore, we found that controlled expression of *hilC* overcomes repression of chromosomal *hilA* expression by high oxygen levels, low osmolarity, and all mutations tested (Fig. 5, 6, and 7). Arabinose-induced expression of *hilC* overcomes repressing conditions and mutations even when *hilD* is disrupted (data not shown), suggesting that HilC is directly derepressing *hilA* expression. In support of this hypothesis, HilC binds to the URS in vitro (Schechter and Lee, unpublished results). Thus *hilC*, which is also a member of the AraC family, can behave as a derepressor of *hilA* expression when produced at high levels.

However, Schechter et al. found that a disruption in *hilC* has only modest effects on *S. enterica* serovar Typhimurium's ability to invade HEp-2 cells, while a disruption in *hilD* has profound effects on invasion (63). This suggests that *hilC* plays a minor role in vitro compared to *hilD*. Our results seem to confirm this prediction. A disruption in *hilC* reduced *iagB87*::*lacZY* expression only 2-fold, compared with the 32-fold repression seen in a *hilD* mutant. Furthermore, *hilC* is not required for the regulation of *hilA* expression by oxygen, osmolarity, FadD, FliZ, PhoB, or SirA. Only EnvZ/OmpR seems to require *hilC* to regulate *hilA* expression. Thus, while EnvZ/

OmpR may regulate *hilA* expression by modulating *hilC* expression and/or activity, all other conditions and regulatory factors affect *hilA* expression by a *hilC*-independent mechanism.

We have concluded that SirA and HilC affect *hilA* expression independently, based on results which show that a *hilC sirA* double mutant yields much lower *hilA* expression than either a *sirA* or *hilC* single mutant. This conflicts with data from Rakeman et al., who found that in a *sirA* mutant, *hilA* expression was not further reduced by a disruption in *sirC* (*hilC*) (61). We suspect that the conflict between our results and those of Rakeman et al. can be explained by differences in strain backgrounds and *sirA* mutations used.

We observed over 500 Miller units of β-galactosidase activity from the *iagB87*::*lacZY* fusion in SL1344 under our conditions. However, Rakeman et al. observed less than 200 Miller units of β-galactosidase activity from the same fusion in their strain background, 14028s, indicating that *hilA* expression is already somewhat repressed in their strain background (61). Furthermore, their *sirA* mutation has a stronger effect on *iagB87*::*lacZY* expression than our *sirA* mutation does (sixfold repression and threefold repression, respectively). The end result of these differences is that Rakeman et al. observed only approximately 30 Miller units of β-galactosidase activity from *iagB87*::*lacZY* in a *sirA* mutant. In this situation, the *hilA* promoter may already be as repressed as it can be. Thus, the effect of the *hilC* mutation on *hilA* expression may not be observable in the *sirA* mutant, which could explain why their *sirA hilC* double mutant did not exhibit lower *hilA* expression than a *sirA* single mutant. However, since we still have considerable *hilA* expression in our *sirA* mutant (168 Miller units of β-galactosidase activity from *iagB87*::*lacZY*), we can observe the combined effects of the *sirA* and *hilC* mutations on *hilA* expression in the double mutant (*iagB87*::*lacZY* expression dropped to 29 Miller units).

Parallels between regulation of *hilC* and *hilA* expression. Rakeman et al. observed that *hilC* expression is reduced by a disruption in *sirA* (61). Our results confirm this observation, though our *sirA* mutation has a milder effect on *hilC* expression than that reported by Rakeman et al. In addition, we found that *hilC* expression is repressed by high-oxygen conditions, low osmolarity, and all regulatory mutations tested. Thus, although *hilC* is not required for the regulation of *hilA* expression by any of these conditions or mutations, *hilC* expression is regulated in a manner that parallels the regulation of *hilA* expression. *hilC* expression is unaffected by a disruption in *hilA* (61), but it is mildly reduced by a disruption in *hilD*. This suggests that the same repression-derepression mechanism that regulates *hilA* expression may also affect *hilC* expression. If so, future studies on the regulation of *hilC* expression by all of these environmental conditions and regulatory mutations may provide clues about how *hilA* expression is regulated. Interestingly, preliminary results indicate that in a *hilD* mutant, *hilC* expression is even further repressed by low-osmolarity conditions, suggesting that osmoregulation of *hilC* expression is *hilD* independent (data not shown). The same may be true for osmoregulation of *hilA* expression.

Repression. Although this study focused on the roles of *hilC* and *hilD* in regulating *hilA* expression, this regulation could also be mediated by affecting the expression or activity of a repressor. The finding that arabinose-induced expression of *hilD* overcomes the repression of *hilA* by all environmental conditions and regulatory mutations would seem to argue against this possibility. However, controlled expression of *hilD* from the P_{BAD} promoter may result in such high levels of HilD that the repressor is completely outcompeted for binding at the URS. If repression by a particular condition or mutation is mediated by increasing the expression or activity of the repressor, this effect could be counteracted by flooding the system with so much HilD that the repressor is unable to bind to the URS at all. In such a situation, the abundance and activity of the repressor would become irrelevant, such that environmental conditions and regulatory mutations acting through the repressor would no longer affect *hilA* expression. Similarly, if *hilD* is absolutely required for expression of *hilA*, increased expression or activity of the repressor might have no effect in a *hilD* mutant. This might explain why *hilA* expression is not strongly affected by environmental conditions or regulatory mutations in the absence of *hilD*. Thus, our data do not rule out models in which environmental conditions and regulatory mutations modulate *hilA* expression by altering the expression or activity of a repressor.

Future directions. Our results have excluded *hilC* from playing a major role in the in vitro regulation of *hilA* expression by all environmental conditions and regulatory factors tested (except EnvZ/OmpR). However, regulation of *hilC* expression by these conditions and regulatory factors parallels the regulation of *hilA* expression. This implies that studies on the regulation of *hilC* expression may provide more clues about how *hilA* is regulated. In fact, something equivalent to the URS may be present upstream of the *hilC* promoter, and future studies must explore this possibility.

We have also shown that FadD, FliZ, and EnvZ do not regulate *hilA* expression by modulating *hilD* expression. Furthermore, we suspect that the mild effects of oxygen, osmolarity, SirA, and PhoB on *hilD* expression do not fully account for the effects of these conditions and mutations on *hilA* expression. We are left with a model in which these conditions and mutations regulate *hilA* expression by modulating *hilD* posttranscriptionally (Fig. 8). Future molecular and biochemical studies must focus on determining how posttranscriptional regulation of *hilD* might be achieved.

Our data are also consistent with a model in which the regulation of *hilA* expression is mediated by modulating the expression or activity of the repressor. Future experiments to test such a model await the identification of the repressor. In fact, more than one repressor may be involved in mediating inhibition of *hilA* expression by different environmental conditions and regulatory mutations. One likely candidate, *hns*, appears to be required for repression of *hilA* under low-osmolarity conditions, even in the absence of *hilC* and *hilD* (Schechter and Lee, unpublished results). Another potential repressor is HilE. Fahlen et al. demonstrated that some mutations (such as *hilE1*, which contains a disruption in *hilE*) result in increased *hilA* expression under activating conditions (18). Thus, HilE may act as a repressor under certain circumstances. Experiments must be done to determine whether H-NS and HilE interact directly with the URS, as we would expect if they are repressors.

It is unlikely that any one mechanism of regulation can account for the changes in *hilA* expression in the presence of various environmental conditions and regulatory mutations.

Transcriptional and posttranscriptional regulation of *hilD* as well as modulation of the expression and activity of one or more repressors may all contribute to the regulation of *hilA* expression. Thus, future studies must focus on measuring how much each mechanism contributes to the regulation of *hilA* expression by each environmental condition and regulatory factor.

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ADDENDUM IN PROOF

Iyoda et al. (S. Iyoda, T. Kamidoi, K. Hirose, K. Katsukake, and H. Watanabe, Microb. Pathog. **30**:81–90, 2001) have shown that a disruption in *fliZ* represses *hilA*, confirming that FliZ positively regulates *hilA* expression.

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