Two-Component Regulators Control hilA Expression by Controlling fimZ and hilE Expression within Salmonella enterica Serovar Typhimurium

M. Aaron Baxter, Bradley D. Jones

Salmonellae initiate disease through the invasion of host cells within the intestine. This ability to invade requires the coordinated action of numerous genes, many of which are found within Salmonella pathogenicity island 1 (SPI-1). The key to this process is the ability of the bacteria to respond to the environment, thereby upregulating the necessary genes under optimal conditions. Central to the control of SPI-1 is the transcriptional activator hilA. Work has identified at least 10 different activators and 8 different repressors responsible for the control of hilA. We have previously shown that hilE is a Salmonella-specific negative regulator that is able to repress hilA expression and invasion. Additionally, fimZ, a transcriptional activator responsible for the expression of type I fimbriae as well as flagellar genes, has also been implicated in this process. fimZ is homologous to response regulators from other two-component regulatory systems, although a sensor for the system has not been identified. The phoPQ and phoBR regulons are both two-component systems that negatively affect hilA expression, although the mechanism of action has not been determined. Our results show that PhoBR is capable of inducing fimZ expression, whereas PhoPQ does not affect fimZ expression but does upregulate hilE in an FimZ-dependent manner. Therefore, phosphate (sensed by PhoBR) and magnesium (sensed by PhoPQ) levels are important in controlling hilA expression levels when Salmonella is in the intestinal environment.

Salmonellae have caused disease for many years. These Gram-negative bacteria can be transmitted through meat, dairy products, or eggs, from animals through the fecal-oral route, and indirectly via fecally contaminated water (1). The CDC tracks two forms of the disease, salmonellosis and typhoid fever. Salmonellosis is a mild form of disease that is typically confined to the gastrointestinal tract. It produces symptoms of fever, abdominal cramps, nausea, and diarrhea (2). A common feature of gastroenteritis or typhoid fever is the ability of Salmonella to invade host cells.

Salmonella contains an island known as pathogenicity island 1 (SPI-1). This island is responsible for encoding both the structural proteins necessary for creating a type III secretion needle complex as well as some of the secreted effectors responsible for the manipulation of host cells (3, 4). Central to the control of this island is the transcriptional activator hilA, which needs to be upregulated for invasion to occur. This upregulation leads to the increased expression of all the other genes contained within SPI-1 (5, 6). Many different activators and repressors of hilA have been identified. These activators respond to a myriad of environmental signals, specifically, osmolarity, oxygen, pH, growth state, short-chain fatty acids, bile, and temperature (6–12), leading to precise control of hilA expression. The transcriptional activators HilD and HilC play an important role in controlling hilA expression. Work has shown that both genes are encoded within SPI-1 (13–15), bind directly to promoter sequences upstream of hilA, and are required for hilA induction even in the absence of multiple repressors (16–19). In addition, RtsA, a transcriptional activator encoded outside SPI-1, works in conjunction with HilD and HilC in a feed-forward loop (20, 21). The interactions of these three activators lead to the upregulation of hilA. Many other transcriptional activators have also been identified as being involved in this process. These include the genes csrAB, sirA-barA, fis, fliZ, fadD, fur, mle, dsbA, and ompR-envZ (22–31).

Studies of hilA regulation have also identified many different repressors of hilA expression. Some of these genes include hha, lon, hilE, ams, rtsB, and pag (21, 32–34). In addition, two-component regulators have been shown to impact hilA expression as well. These regulators are typically composed of a histidine kinase that responds to specific extracellular signals by being autophosphorylated. The phosphorylation of the sensor initiates a phosphorelay in which phosphate is transferred to its cognate response regulator. This phosphorylation causes the response regulator to activate multiple genes (35).

The phoPQ two-component system is an important regulator of hilA expression (36, 37). The sensor protein PhoQ resides in the membrane of the bacterial cell and stops dephosphorylating the response regulator PhoP when magnesium levels drop to micromolar levels. When PhoP is constitutively expressed and phosphorylated, hilA expression is reduced by 9-fold, which correlated to a 63-fold decrease in HEP-2 cell invasion (36, 37). The molec

Received 14 August 2014 Returned for modification 7 September 2014 Accepted 19 December 2014 Accepted manuscript posted online 29 December 2014 Citation Baxter MA, Jones BD. 2015. Two-component regulators control hilA expression by controlling fimZ and hilE expression within Salmonella enterica serovar Typhimurium. Infect Immun 83:978–985. doi:10.1128/IAI.02506-14.

Editor: A. J. Baumler
Address correspondence to Bradley D. Jones, bradley-jones@uiowa.edu
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The phoBR two-component system also represses hilA expression. This system detects the levels of phosphate in the extracellular environment. When phosphate levels are low, the system is activated by the autophosphorylation of the sensor PhoR followed by the activation of the response regulator PhoB. The activation of PhoB leads to the induction of more than 21 genes within Salmonella enterica serovar Typhimurium. Many of these genes are involved in transporting phosphate from the environment into the bacterial cell (38). PstS is a protein that represses the PhoR sensor under conditions of high environmental phosphate. When PstS was mutated, it led to a 5-fold decrease in hilA expression, which subsequently reduced HEp-2 cell invasion by 5-fold (29). As is the case with phoPQ, the nature of how the PhoB signal leads to hilA repression is not understood.

Previous work by our research group has shown that HilE interacts with HilD, which prevents the activation of hilA by HilD (39). Due to the importance of HilE in mediating repression of Salmonella invasion genes, we undertook a search for genes that activate hilE expression. This search identified the transcriptional activator fimZ, which has been shown to be responsible for the activation of type I fimbriae and whether bacteria adhere to a surface or are motile (40–42). In our studies, we showed that FimZ upregulates hilE expression, thereby playing a significant role in whether hilA is expressed or not. The fimZ gene is homologous to other response regulators found within two-component systems, yet a specific sensor has not been identified (43). We therefore hypothesized that the signals from the PhoPQ and PhoBR two-component regulators are processed through FimZ, leading to the repression of hilA. The following studies show that PhoPQ and PhoBR regulate hilE expression via the fimZ gene.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are shown in Table 1. Bacteria were routinely grown in Lennox broth (LB; Gibco-BRL) containing the appropriate antibiotics added at the following concentrations: ampicillin at 100 μg/ml, tetracycline at 25 μg/ml, kanamycin at 25 μg/ml, and chloramphenicol at 25 μg/ml. For the β-galactosidase analysis, S. enterica serovar Typhimurium strains were grown in LB overnight shaken at 225 rpm at 37°C. For conditions in which the levels of magnesium were manipulated, the bacterial cultures were grown in an N-salts minimal medium following a previously established protocol, except for the changes indicated by Hmiel et al. and Nelson and Kennedy (44, 45). For bacterial growth in medium that induces hilA expression via the increase in acetate, we followed the protocol outlined by Lawhon et al. (9). Plasmid purifications were performed utilizing Qiagen DNA purification kits, and all other molecular manipulations were conducted using previously established protocols (46).

**Creation of defined chromosomal mutations within the hilE and fimZ genes.** In an effort to create defined chromosomal mutations within the S. enterica serovar Typhimurium LT2 phoQ’ strain TA2367, we utilized the linear transformation procedure (47). Briefly, PCR primers were synthesized with 50 bp of homology to the 5’ and 3’ ends of the hilE gene. In addition, the hilE5W’ primer (5’-TTATAGCAGATTGTCGGTATTT-3’) was synthesized so that it carried priming site 2 of pkD3 (47), and the hilE3W’ primer (5’-ATTTCGCTATACGATCGCCACCACCAGCGCACATG-3’) was synthesized so that it carried priming site 1 of pkD3. PCR amplification was performed with these primers using plasmid pkD3 as the template, and the expected 1.1-kb fragment was obtained. The linear PCR fragment was purified and electroporated into SL1344 carrying pkD46, and mutants were selected on L-CAM plates at 37°C. Several Cam’ Amp’ colonies were purified and found by PCR to have the transformed fragment recombined into the hilE gene on the chromosome. The procedure for creating the defined fimZ:cam mutations followed the above-described protocol utilizing the primers fimZ5W’ (5’-
FIG 1 The effects of constitutive phoQ expression on hilA are reduced by deletion of the Salmonella hilE gene. Strains were grown with shaking in LB broth to late stationary phase. The wild-type strain is S. enterica serovar Typhimurium LT2 strain EE251 carrying the hilA::lacZY plasmid reporter pLS31. The TA2367 strain contains the phoQ:: mutation and the hilA::lacZY reporter plasmid pLS31. BJ3106 is the TA2367 strain containing a defined hilE::cam mutation and the hilA::lacZY reporter plasmid pLS31. Expression levels were determined by lacZ output as measured by β-galactosidase activity. The experiment is representative of an assay which was repeated in triplicate on three separate days.

FIG 2 The effect of constitutive phoQ expression on hilE and hilA expression is mediated through fimZ. Strains were grown with shaking in LB broth to late stationary phase. The wild-type strain is S. enterica serovar Typhimurium LT2 strain EE251 carrying either the hilA::lacZY plasmid reporter pLS31 or the hilE::lacZY plasmid reporter pMAB69. The strain TA2367 contains the phoQ:: mutation and either the hilA::lacZY reporter plasmid pLS31 or the hilE::lacZY plasmid reporter pMAB69. BJ3179 is the TA2367 strain containing a defined fimZ::cam mutation and carries the hilE::lacZY reporter pMAB69. BJ3185 is the TA2367 strain containing a defined fimZ::cam mutation and carries the hilA::lacZY reporter pLS31. Expression levels were determined by lacZ output as measured by β-galactosidase activity. The experiment is representative of an assay which was repeated in triplicate on three separate days.

β-Galactosidase assays. β-Galactosidase assays were conducted on bacterial cultures using the standard method described by Miller (48).

P22-mediated transductions. The P22 HT int phage was used to move mutations marked by antibiotic-resistant genes between strains as described previously (49). Transductants were selected on LB agar containing the necessary antibiotic and 10 mM EGTA to prevent P22 reinfection. Transductants were purified twice on LB EGTA agar prior to use of the colonies.

RESULTS
The effects of a phoQ:: mutation on hilA can be alleviated by the deletion of hilE. The phoQ:: mutant has been demonstrated to exert a powerful influence on the expression of hilA (37). However, the means by which phoQ:: exerts its effect on hilA have not been characterized. In our previous studies, we identified hilE as a Salmonella-specific repressor of hilA (39). To determine the effects of HilE on the regulation of hilA via PhoPQ, we conducted a β-galactosidase assay examining the effect of an hilE:: mutation on hilA::lacZY expression when phoQ:: is constitutively expressed. As seen in Fig. 1, normal expression of hilA::lacZY from pLS31 within the S. Typhimurium EE251 strain was at 577.9 ± 19.0 Miller units. When the constitutive phoQ:: mutant is introduced, hilA::lacZY expression is reduced 7.5-fold to 76.4 ± 3.5 Miller units. The introduction of a defined hilE::cam mutation within the chromosome to the constitutive phoQ:: mutation increased hilA::lacZY expression by 4-fold to 305.7 ± 12.4 Miller units. Although repression of hilA::lacZY within the phoQ:: strain was not completely eliminated, most of the hilA expression could be restored by deletion of hilE. This indicates that HilE mediates a substantial portion of the repressing activity that a phoQ:: mutation has on hilA expression.

The signal from the phoPQ regulon is transmitted through the transcriptional activator fimZ. We have previously shown that hilE is regulated by the transcriptional activator FimZ (50). FimZ is a transcriptional activator of type 1 fimbiae genes (41, 43) and also exerts regulatory effects on motility (40), invasion gene expression, and biofilm formation (50). Analysis of the FimZ amino acid sequences reveals that this activator has substantial similarity to response regulators from two-component signaling systems, yet a sensor partner for FimZ has not been identified (43). In this work, we hypothesize that FimZ is responsible for responding to signals from the PhoPQ regulon, which causes an increase in hilE expression. We conducted β-galactosidase assays measuring the levels of hilA::lacZY and hilE::lacZY expression in the presence or absence of a functional fimZ gene. As shown in Fig. 2, the hilE::lacZY reporter expressed at 197.3 ± 5.9 Miller units. When phoQ:: is constitutively expressed, hilE::lacZY expression increased by 4.4-fold to 873.2 ± 28.8 Miller units. When an fimZ::cam mutation was present in this strain and tested under the same conditions, hilE::lacZY expression was reduced 7.4-fold to 117.3 ± 3.9 Miller units. This indicated that the effect of a phoQ:: mutation on an hilE::lacZY reporter was being mediated by FimZ. This result was confirmed by measuring the effects an fimZ:: mutation has on hilA::lacZY expression when the phoQ:: mutation is also present. Control levels of hilA::lacZY were at 1,017.1 ± 20.8 Miller units. Upon introduction of a phoQ:: mutation, hilA::lacZY expression was reduced 23-fold to 44.2 ± 3.5 Miller units. When the fimZ::cam mutation was introduced, hilA::lacZY expression increased by 11.8-fold to 522.3 ± 19.7 Miller units. These results confirmed our hypothesis that the effect of PhoPQ on hilA follows a signaling pathway through FimZ and HilE. Since complete alleviation of hilA::lacZY repression by the phoQ:: mutation did not occur with deletion of fimZ, it seems likely that the signal from the phoQ:: mutation is also being processed by other pathways.

Expression of fimZ is not affected by phoQP. Since the PhoPQ signal processes through fimZ to regulate hilA, we wanted to determine whether PhoQP regulates fimZ transcription. To do so, we measured the expression of an fimZ::lacZY reporter in the wild-type S. Typhimurium LT2 strain and in an LT2 strain in which the phoQ:: mutation was introduced. Wild-type S. Typhimurium ex-
pressed fimZ::lacZY at 334.8 ± 8.0 Miller units, whereas a phoQ′
strain expressed the fimZ::lacZY reporter at 297.8 ± 6.9 Miller
units. Examination of the FimZ primary sequence indicates that
the protein contains motifs that are homologous to proteins that
are phosphorylated, although phosphorylation of specific residues
has not been demonstrated for FimZ (43). From these results, we
hypothesize that FimZ is activated in response to the PhoPQ signal
(likely by phosphorylation), which leads to induction of hilE ex-
pression.

hilA::lacZY expression can be altered by various magnesium
concentrations. As our work has demonstrated that the overex-
pression of phoQ′ strongly represses hilA using a signaling pathway
that includes FimZ and HilE, we next determined whether hilA::
lacZY expression could be altered within S. Typhimurium solely
by altering magnesium levels, the primary signal for the PhoPQ
two-component regulator. This was done by measuring hilA::
lacZY expression in N-salts minimal medium that was either in-
ducing for PhoPQ signaling (8 μM magnesium) or repressing for
PhoPQ signaling (10 mM magnesium). Analysis of wild-type S.
Typhimurium strain SL1344 containing the hilA::lacZY reporter plasmid pLS31 was compared to the BJ2462 strain, which is an SL1344 strain containing a hilE::cam deletion and carries the same reporter plasmid. Expression levels were determined by lacZ output as measured by β-galactosidase activity. The experiment is repre-
sentative of an assay which was repeated in triplicate on three separate days. High magnesium was at a concentration of 10 mM, whereas low magnesium was at 8 μM.

hilE, under other conditions that do not induce invasion-associ-
ated genes.

hilE::lacZY expression can be induced upon the induction of
phoB. A mutation in the pstS gene was previously identified as
causing a reduction in hilA expression (29). Previous work has
shown that PstS is responsible for the repression of the two-com-
ponent regulator PhoBR, which is responsible for the activation of
scavenger genes that move phosphate into the cell under low
phosphate conditions (38). Since our studies indicated that PhoPQ
plays a role in inducing hilE expression, we examined
whether the phoBR regulon also regulates hilA via HilE. A β-ga-
lactosidase assay was conducted measuring the amount of hilE::
lacZY expression in wild-type S. Typhimurium SL1344 and the
mutant strain RL291 (an SL1344 derivative with a pstS mutation).
Utilizing the hilE::lacZY plasmid reporter pMAB69, we measured
wild-type hilE expression at 56.6 ± 0.2 Miller units (Fig. 4). When
the pstS gene was disrupted, increasing PhoB activation, expres-
sion of hilE::lacZY increased to 169.1 ± 1.0 Miller units. This
3-fold increase in hilE expression indicated that hilE responds
to signals from both PhoPQ and PhoBR.

The effects of a pstS mutation on hilA are alleviated by the
deletion of hilE. Since the deletion of hilE partially reverses the
effects of a phoQ′ mutation on hilA, we also examined the effect an
hilE deletion has on hilA expression when PhoBR is activated by
the pstS mutation. Utilizing the hilE::lacZY plasmid reporter
plasmid pLS31, hilA expression levels were measured in wild-type S.
Typhimurium SL1344, the pstS mutant RL291, and BJ3100, a pstS
mutant containing a defined hilE::cam insertion. Wild-type
SL1344 expressed hilA::lacZY at 711.4 ± 25.2 Miller units (Fig. 5).
Deletion of pstS decreased hilA expression by 5.6-fold to 127.2 ±
8.6 Miller units. When BJ3100 (ΔpstS hilE::cam) was assayed, hi-
la::lacZY expression increased 6.2-fold to 793 ± 24.0 Miller units.
The deletion of hilE completely reversed the effects of a pstS mu-
sion on hilA expression, indicating that the pstS mutation, which
activates PhoBR signaling, exerts its effect via hilE, to regulate hilA
transcription.

The activation of phoB increases fimZ expression. Since
the PhoBR signal regulates hilA via hilE, it was logical to examine

FIG 3 Various magnesium concentrations will alter the levels of hilA expression independently of hilE. Strains were grown with shaking in LB broth to late
stationary phase. The wild-type S. enterica serovar Typhimurium strain SL1344 containing the hilA::lacZY reporter plasmid pLS31 was compared to the BJ2462 strain, which is an SL1344 strain containing a hilE::cam deletion and carries the same reporter plasmid. Expression levels were determined by lacZ output as measured by β-galactosidase activity. The experiment is representative of an assay which was repeated in triplicate on three separate days.

FIG 4 Overexpression of phoB leads to the activation of hilE. The wild-type strain is S. enterica serovar Typhimurium SL1344. The mutant tested is the
SL1344 strain RL291, which contains a pstS deletion leading to the constitutive activation of phoB. Each strain contained the hilE::lacZY reporter plasmid
pMAB69. The strains were grown in LB overnight with shaking, and expres-
sion levels were determined by measuring lacZ output as measured by β-ga-
lactosidase activity. The experiment is representative of an assay which was repeated in triplicate on three separate days.
BJ3100 is an RL291 strain containing a defined phoB-expressing strain. The wild-type strain is S. enterica serovar Typhimurium SL1344. BJ3100 is an RL291 derivative that contains a defined hilE::cam mutation within the chromosome. Each strain contained the hilA::lacZY reporter pLS31. The strains were grown in LB overnight with shaking, and expression levels were determined by measuring lacZ output as measured by β-galactosidase activity.

Whether the regulatory signal was transmitted through fimZ similar to that seen with PhoPQ. Utilizing the fimZ::lacZY reporter pISF239, fimZ expression levels were measured in wild-type S. Typhimurium SL1344 and in a pstS mutant. In wild-type SL1344, fimZ expressed at 208.8 ± 0.8 Miller units (Fig. 6). A pstS mutation increased fimZ expression 4-fold to 853.2 ± 25.4 Miller units. One possible explanation of this finding is that the pstS mutation increases protein levels of FimZ, potentially activating transcription of its own gene, consistent with previous work by Yeh et al. (43). Therefore, to rule out the possibility that the induction of fimZ transcription was solely due to the self-induction of the transcriptional activator, we constructed strain BJ3184, which is an SL1344 derivative containing the pstS mutation and a defined fimZ::cam insertion. Within this strain that lacks functional FimZ protein, fimZ::lacZY expression was 555.1 ± 10.6 Miller units, which is still a 2.7-fold increase in fimZ expression compared to that of the wild type (Fig. 6). These results demonstrate that the activation of the PhoB response regulator leads to increased fimZ expression, in the absence of functional FimZ, although the presence of FimZ further increases fimZ expression due to autoactivation. This increase in FimZ activates hilE expression, leading to the repression of hilA.

**DISCUSSION**

The process of invasion in *Salmonella* requires the coordinated control of many different genes responding to a myriad of environmental signals. For invasion to occur, the bacteria must induce the expression of genes within SPI-1 as well as genes encoding the effectors that are secreted by the SPI-1 type III secretion system. The combined functions of these gene products cause the mammalian host cell cytoskeleton to ruffle outward around the invading organism so that it is internalized into the host cells via macropinocytosis (3, 4, 26, 51). *Salmonella* species have developed a complex regulatory network that determines whether the bacterium has entered an environment that is conducive for invasion. If conditions are not optimal, invasion gene expression is repressed, whereas entry into a more conducive environment leads to activation of the invasion genes. Currently, many different environmental signals have been identified that impact invasion gene expression. These activating signals include oxygen-limiting conditions, high osmolality, temperature, and growth in a near-neutral pH (6–8, 11). In addition, the bacteria downregulate invasion gene expression as the organisms reaches the stationary phase of growth (52). Additional signals, such as the concentrations of short-chain fatty acids (i.e., acetate, propionate, and butyrate), as well as the presence of bile salts, impact gene expression (9, 12, 53), with recent evidence showing that propionyl coenzyme A (propionyl-CoA) specifically regulates HiiD posttranslationally, possibly by propionylation of the HiiD protein (54).

Induction of SPI-1 requires the expression of hilA and invF, two transcriptional activators found within SPI-1. In the absence of these regulators, the proteins required for formation of the type III secretion system and the secreted effectors will not be produced (5–55). Work in many different laboratories has identified additional genes that regulate expression of hilA and invF. Currently, csrAB, sirA-barA, fis, fliZ, fadD, ompR-envZ, fur, mle, dbhA, rtsA, hilC, and hilD have all been shown to positively upregulate hilA expression (13–15, 22–31, 56). In addition, a number of repressors have also been identified that are important in controlling hilA expression. These repressors include lon, hha, amn, pad, phoQ, phoB, rtsB, and hilE (21, 29, 32–35, 37, 59). Our group has characterized the negative regulatory hilE gene and its impact on hilA expression. We have shown by two-hybrid analysis that HilE interacts with HiiD to repress hilA transcription (39). Other work has shown that hilE is a *Salmonella*-specific gene that is not expressed by *Escherichia coli* (39). Work from several groups has identified factors that regulate *Salmonella* invasion gene expression through the HilE repressor. The Mlc global regulator has been shown to downregulate an hilE promoter (27). The small noncoding RNA icasM targets the hilE transcript to reduce the repressing activity of hilE (57). The LysR-type regulator LeuO has been shown to activate hilE transcription to repress HilD activity (58).

In an effort to contribute to our understanding of *Salmonella* SPI-1 virulence gene regulation, we conducted a search for genes that induce hilE::lacZY expression. This search identified fimZ, an important transcriptional activator of type 1 fimbriae (42). FimZ

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**FIG 5** The deletion of hilE reverses the repression of hilA in a constitutive phoB-expressing strain. The wild-type strain is *S. enterica* serovar Typhimurium SL1344. RL291 is an SL1344 derivative that contains a pstS mutation. BJ3100 is an RL291 strain containing a defined hilE::cam mutation within the chromosome. Each strain contained the hilA::lacZY reporter pLS31. The strains were grown in LB overnight with shaking, and expression levels were determined by measuring lacZ output as measured by β-galactosidase activity.

**FIG 6** Overexpression of phoB increases the level of fimZ expression. The strains were shaken overnight in LB growing at 37°C. The wild-type bacterium is *S. enterica* serovar Typhimurium strain SL1344. RL291 is an SL1344 derivative that contains a pstS mutation that causes the overexpression of phoB. The BJ3184 strain is the RL291 strain containing a defined fimZ::cam mutation. Each strain tested contained the fimZ::lacZY reporter plasmid pLS31. The strains were grown in LB overnight with shaking, and expression levels were determined by measuring lacZ output as measured by β-galactosidase activity. The experiment is representative of an assay which was repeated in triplicate on three separate days.
has been implicated in the control of other regulatory systems in *Salmonella*. Prior to our demonstration of the involvement of *fimZ* in *Salmonella* invasion gene regulation, work was published showing that the overexpression or deletion of *fimZ* inversely controlled motility and fimbrial gene expression (40). Recent work has shown that these three *Salmonella* properties (i.e., adherence [type 1 fimbriae], motility [flagella], and invasion [SPI-1 gene expression]) are all dynamically regulated via a cross talk mechanism utilizing posttranslationally modified FliZ, which is proposed to monitor the bacterial growth state (28, 59–61). Additional work has demonstrated that the *flhDC* genes, the master operon of the flagellar hierarchy, activate transcription of the *hilD* gene at early stages of growth, while the HilD regulator activates promoter 5 of the *flhDC* genes at later stages of growth (62). FimZ is of interest, as it has homology to response regulators (highest homology to *bvgrA* of *Bordetella*), yet no sensor kinase has been identified as its partner (43). Analysis of mutations that resulted in repression of *hilA* showed that two of these regulators were either part of a two-component regulatory system (*phoPQ*) or involved in the function of a two-component system (*pstS* and *phoBR*). In this work, we have explored how these mutations impact *hilA* expression by examining their interactions with FimZ and HilE.

In this work, we have studied how PhoPQ, HilE, and FimZ function together to regulate the S. Typhimurium SPI-1 transcriptional activator *hilA*. We have employed strains with mutations in genes of interest as well as manipulation of magnesium concentrations in the growth medium to ask experimental questions. We have found that most, but not all, of the PhoPQ effects are mediated by FimZ and HilE. The exception was that deletion of *hilE* did not completely reverse the effects of *phoQ* on *hilA* expression. Consistent with our data, recent work has shown that the positive *hilA* activator DsbA reduces *phoPQ* expression (63). Our results contribute to the evolving story that multiple environmental signals are processed by various *Salmonella* two-component regulators to increase or decrease invasion gene expression. Since *phoPQ* seems to exert its effect at FimZ posttranslationally, a likely mechanism is via a phosphorylation mechanism from PhoPQ to FimZ, which would fit the established mechanism of activation of two-component regulatory systems. The likelihood that FimZ functions by receiving phosphorylation signals from multiple two-component signals provide a model to understand how this gene can regulate genes involved in motility, biofilm formation, invasion gene repression, and type I fimbrial gene expression (64). Recent work by Golubeva et al. suggests that PhoPQ is a class III regulator and exerts its regulatory influence by acting directly on *hilA* (65, 66). This is possible, since the *hilE* deletion does not completely repress expression of *hilA* by the *phoQ* mutation. Future efforts will be required to resolve these points.

Additionally, we investigated the effects of the PhoBR regulon on *fimZ* and *hilE* expression. Unlike what was observed with PhoPQ, we found that PhoBR directly affects *fimZ* expression but that its ability to control *hilA* expression is mediated entirely by HilE. Accordingly, these two-component regulators (PhoPQ and PhoBR) alter *hilE* and *hilA* expression by different mechanisms in that one is via a posttranscriptional mechanism and the other is via transcriptional control of *hilE*.

The concept that multiple two-component regulatory systems interact in an overlapping fashion to control a biological pathway is not new. Previous work has shown that *Salmonella* has three different two-component regulators that control the synthesis of the *ugd* gene, which is involved in both polymyxin B resistance and capsule biosynthesis (67). An overlapping regulatory network has also been described for the *pho regulon* of *Bacillus subtilis* (68). A study analyzing the regulons of known two-component systems in *E. coli* concluded that there are three possible regulatory schemes that can occur. One, a single sensor can directly interact with a single response regulator. Two, a single sensor can interact with or activate multiple response regulators of DNA-binding activators. Three, multiple sensor proteins can converge onto a single response regulator and the genes that it controls (69). We have contributed data here that we believe help to further define the regulatory hierarchy of regulation of the *Salmonella* invasion genes and have shown how two different two-component sensing systems interact with other activators and repressors to control expression of *Salmonella* virulence (Fig. 7). The involvement of *fimZ* in this process demonstrates that *Salmonella* has evolved to coordinate the expression or repression of the invasion phenotype.
with expression of type 1 fimbriae and motility. Future efforts will be aimed at determining the molecular details of this highly coordinated network of gene expression in this important bacterial pathogen.

ACKNOWLEDGMENTS

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

B.D.J. is supported by NIH grant 2PO1 AI044642.

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