IAI Journals.ASM.org

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

M. Aaron Baxter,[a,c] Bradley D. Jones[a,b]

Genetics Program[a] and Department of Microbiology,[b] University of Iowa Roy J. and Lucille A. Carver School of Medicine, Iowa City, Iowa, USA; Department of Biomedical Sciences, Grand Valley State University, Allendale, Michigan, USA[c]

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*, *mlc*, *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. Bäumler

Address correspondence to Bradley D. Jones, bradley-jones@uiowa.edu.

doi:10.1128/IAI.02506-14

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Reference
Salmonella enterica serovar Typhimurium strains		
BJ644	SL1344 *phoP* mutant Tet^r	70
BJ704	SL1344 *phoP* mutant	This work
BJ2462	SL1344 *hilE-cam* Cam^r	39
BJ3100	SL1344 *pstS55*::Tn*5 hilE-cam* pLS31 (*hilA*::*lacZY*) Kan^r Cam^r Tet^r	This work
BJ3106	LT2 *pho-24 hilE-cam* pLS31 (*hilA*::*lacZY*) Cam^r Tet^r	This work
BJ3179	LT2 *pho-24 fimZ-cam* pMAB69 (*hilE*::*lacZY*) Cam^r Tet^r	This work
BJ3184	SL1344 *pstS55*::Tn*5* Δ*fimZ* Kan^r	This work
BJ3185	LT2 *pho-24 fimZ*::*cam* Cam^r	This work
BJ3371	SL1344 *phoB*::*cam* Cam^r	This work
BJ3372	SL1344 *phoP* mutant *phoB*::*cam* Cam^r	This work
EE251	Invasive LT2 derivative, Δ*rpsL*	55
RL291	SL1344 *pstS55*::Tn*5* Kan^r	29
SL1344	Wild-type virulent strain	71
SL1344 *fimZ*::*kan*	Kan^r	50
TA2367	LT2 *pho-24*	72
TBW19812	LT2 *phoB1*::*cat* Cam^r	73
Plasmids		
pISF239	pMC1403 vector containing an *fimZ*::*lacZY* reporter, Amp^r	43
pLS31	Low-copy-number vector pRW50 containing an *hilA*::*lacZY* reporter, Tet^r	15
pMAB69	Low-copy-number vector pRW50 containing an *hilE*::*lacZY* reporter, Tet^r	50

[a] Tet^r, tetracycline resistance; Amp^r, ampicillin resistance; Cam^r, chloramphenicol resistance; Kan^r, kanamycin resistance.

ular mechanism for *hilA* repression by PhoPQ has not been characterized.

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*^{c} 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′-TTATAGCAGATTGTCGGTATTTAATCTGGTATACAGAGACACCAACGAACATATGAATATCCTCCTTA-3′) was synthesized so that it carried priming site 2 of pKD3 (47), and the hilE3W′ primer (5′-ATTTCGCTATACAGCATCGCCCACTGCGAGTCCGCAAGCTTGTTTTGTCCGTGTAGGCTGGAGCTGCTTC-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^r Amp^s 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′-

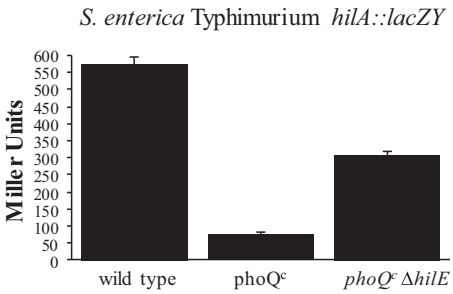

FIG 1 The effects of constitutive *phoQ* expression on *hilA* are reduced by deletion of the *Salmonella hilE* gene. Strains were grown with shaking in LB broth to late stationary phase. The wild-type strain is *S. enterica* serovar Typhimurium LT2 strain EE251 carrying the *hilA*::*lacZY* plasmid reporter pLS31. The TA2367 strain contains the *phoQ*^{c} 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.

TGACGCTTATTATAAAACGAAGGACGCATAACAGTCTGAGG CATACAACACATATGAATATCCTCCTTA-3′) and fimZ3W (5′-AT TAGTGTCCGTTATTGTGGCTCCCGAACGATAATTCGCC GGGAGTACATGTGTAGGCTGGAGCTGCTTC-3′).

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*^{c} mutation on *hilA* can be alleviated by the deletion of *hilE*. The *phoPQ* regulon has been demonstrated to exert a powerful influence on the expression of *hilA* (37). However, the means by which *phoPQ* 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* mutation 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*^{c} 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*^{c} 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 fimbriae 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 *hilE*::*lacZY* and *hilA*::*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*^{c} 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*^{c} mutation is also present. Control levels of *hilA*::*lacZY* were at 1,017.1 ± 20.8 Miller units. Upon introduction of a *phoQ*^{c} 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*^{c} mutation did not occur with deletion of *fimZ*, it seems likely that the signal from the *phoQ*^{c} mutation is also being processed by other pathways.

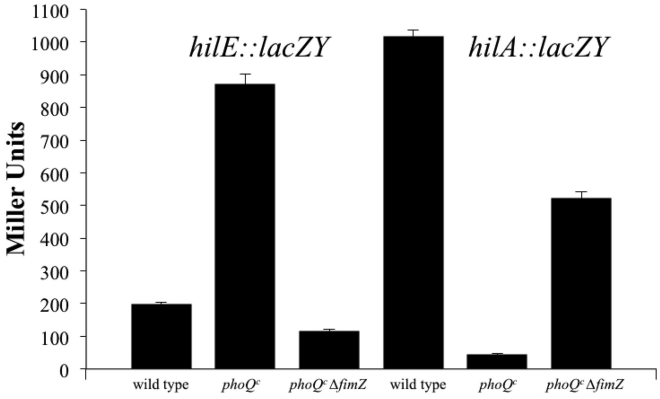

FIG 2 The effect of constitutive *phoQ* expression on *hilE* and *hilA* expression is mediated through *fimZ*. Strains were grown with shaking in LB broth to late stationary phase. The wild-type strain is *S. enterica* serovar Typhimurium LT2 strain EE251 carrying either the *hilA*::*lacZY* plasmid reporter pLS31 or the *hilE*::*lacZY* plasmid reporter pMAB69. The strain TA2367 contains the *phoQ*^{c} 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.

Expression of *fimZ* is not affected by *phoPQ*. Since the PhoPQ signal processes through *fimZ* to regulate *hilA*, we wanted to determine whether PhoPQ 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*^{c} mutation was introduced. Wild-type *S.* Typhimurium ex-

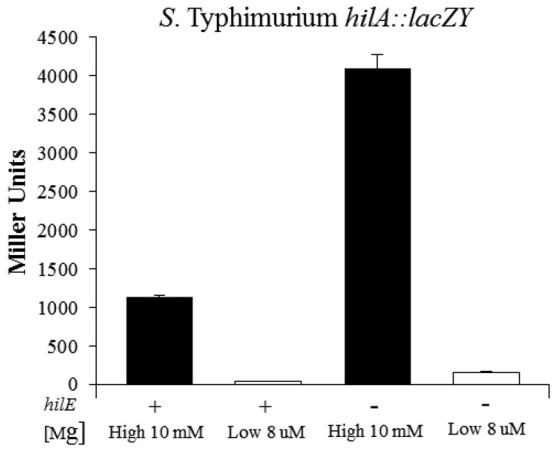

FIG 3 Various magnesium concentrations will alter the levels of *hilA* expression independently of *hilE*. Strains were grown with shaking in LB broth to late stationary phase. The wild-type *S. enterica* serovar Typhimurium strain SL1344 containing the *hilA*::*lacZY* reporter plasmid pLS31 was compared to the BJ2462 strain, which is an SL1344 strain containing an *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. High magnesium was at a concentration of 10 mM, whereas low magnesium was at 8 μM.

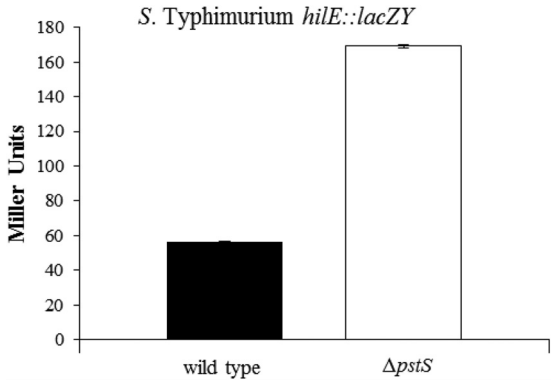

FIG 4 Overexpression of *phoB* leads to the activation of *hilE*. The wild-type strain is *S. enterica* serovar Typhimurium SL1344. The mutant tested is the SL1344 strain RL291, which contains a *pstS* deletion leading to the constitutive activation of *phoB*. Each strain contained the *hilE*::*lacZY* reporter plasmid pMAB69. The strains were grown in LB overnight with shaking, and 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.

pressed *fimZ*::*lacZY* at 334.8 ± 8.0 Miller units, whereas a *phoQ*^{c} 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* expression.

***hilA*::*lacZY* expression can be altered by various magnesium concentrations.** As our work has demonstrated that the overexpression 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 inducing for PhoPQ signaling (8 μM magnesium) or repressing for PhoPQ signaling (10 mM magnesium). Analysis of wild-type *S.* Typhimurium strain SL1344 with the *hilA*::*lacZY* plasmid reporter pLS31 showed that inducing levels of magnesium for *phoPQ* expression reduced *hilA*::*lacZY* expression 28.2-fold from 1,139.7 ± 20.1 to 40.4 ± 0.8 Miller units (Fig. 3). Next, the effects of an *hilE*::*cam* mutation on *hilA*::*lacZY* expression when magnesium was either inducing or repressing for PhoPQ were measured. Even in the absence of *hilE*, *hilA*::*lacZY* plasmid reporter levels still showed a 25.9-fold reduction (4,098.7 ± 173.4 to 158.3 ± 5.6 Miller units) when the PhoPQ regulators were activated by magnesium (Fig. 3). These results suggest that the effects of magnesium on *hilA* are not solely mediated through the FimZ/HilE signaling pathway. It is possible that another regulatory system within *Salmonella* responds to magnesium levels and that these secondary pathways affect *hilA* expression levels, independent of *hilE*, under other conditions that do not induce invasion-associated 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-component 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-galactosidase 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, expression 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*^{c} 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 *hilA*::*lacZY* plasmid reporter 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 (Δ*pstS hilE*::*cam*) was assayed, *hilA*::*lacZY* expression increased 6.2-fold to 793 ± 24.0 Miller units. The deletion of *hilE* completely reversed the effects of a *pstS* mutation 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

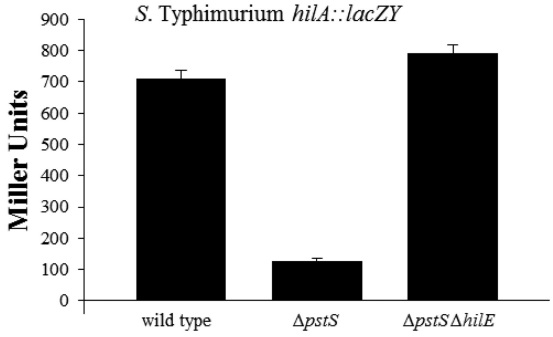

FIG 5 The deletion of *hilE* reverses the repression of *hilA* in a constitutive *phoB*-expressing strain. The wild-type strain is *S. enterica* serovar Typhimurium SL1344. RL291 is an SL1344 derivative that contains a *pstS* mutation. BJ3100 is an RL291 strain containing a defined *hilE*::*cam* mutation 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.

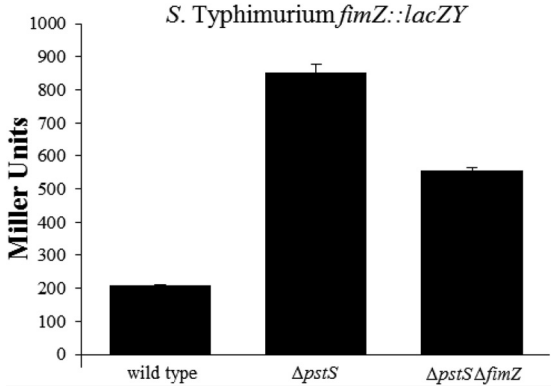

FIG 6 Overexpression of *phoB* increases the level of *fimZ* expression. The strains were shaken overnight in LB growing at 37°C. The wild-type bacterium is *S. enterica* serovar Typhimurium strain SL1344. RL291 is an SL1344 derivative that contains a *pstS* mutation that causes the overexpression of *phoB*. The BJ3184 strain is the RL291 strain containing a defined *fimZ*::*cam* mutation. Each strain tested contained the *fimZ*::*lacZY* reporter plasmid pISF239. 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.

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 osmolarity, 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 HilD posttranslationally, possibly by propionylation of the HilD 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*, *mlc*, *dsbA*, *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*, *ams*, *pag*, *phoQ*^{c}, *phoB*, *rtsB*, and *hilE* (21, 29, 32–35, 37, 39). 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 HilD 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 *isrM* 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

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 posttranscriptionally 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 *bvgA* 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 (*phoQ*^{c}) 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*^{c} 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 posttranscriptionally, a likely mechanism is via a phosphorelay 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 provides 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 eliminate repression of *hilA* by the *phoQ*^{c} 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 effects *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

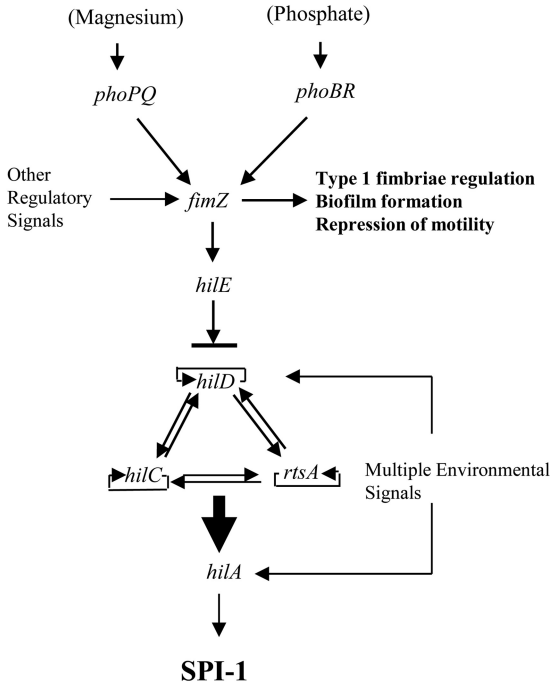

FIG 7 Model of the regulatory cascade that transfers environmental signals into changes in *Salmonella* gene expression. Environmental signals such as magnesium or phosphate concentration increase or decrease *fimZ* expression. Under conditions of low magnesium concentration, the PhoPQ regulon is activated, leading to the phosphorylation of FimZ with the subsequent increase in *hilE* expression. Under conditions of low phosphate, PhoBR is activated, which increases *fimZ* expression, which upregulates *hilE* expression. A FimZ-activating signal additionally leads to increased type 1 fimbrial expression by direct binding of the FimZ activator to the type 1 fimbrial gene operon. Increased *fimZ* expression also decreases expression of the *flhDC* master regulatory proteins for the flagellar regulon by an unknown mechanism. Other regulatory signals generated from the cross talk between the flagellar system or other environmental signals may further influence the expression of *fimZ*. The increase in FimZ leads to the subsequent increase in HilE protein levels, which limits the availability of HilD to activate the *hilA* promoter due to HilE-HilD binding. A FimZ-deactivating signal would have the opposite effects. Collectively, these regulatory pathways control *hilA* expression and downstream expression of SPI-1 (22). In addition, it is likely that there are FimZ-independent signals that affect the expression and cross talk between all the systems described (motility, adherence, and invasion), which allows *Salmonella* to dynamically control when these various systems respond (60).

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.

REFERENCES

1. **Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV.** 1999. Food-related illness and death in the United States. Emerg Infect Dis **5:**607–625. http://dx.doi.org/10.3201/eid0505.990502.
2. **CDC.** 2010. Salmonella. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/salmonella/index.html.
3. **Collazo CM, Galan JE.** 1997. The invasion-associated type III system of *Salmonella* Typhimurium directs the translocation of Sip proteins into the host cell. Mol Microbiol **24:**747–756. http://dx.doi.org/10.1046/j.1365-2958.1997.3781740.x.
4. **Darwin KH, Miller VL.** 1999. Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. Clin Microbiol Rev **12:**405–428.
5. **Bajaj V, Hwang C, Lee CA.** 1995. *hilA* is a novel *ompR*/*toxR* family member that activates the expression of *Salmonella* Typhimurium invasion genes. Mol Microbiol **18:**715–727. http://dx.doi.org/10.1111/j.1365-2958.1995.mmi_18040715.x.
6. **Bajaj V, Lucas RL, Hwang C, Lee CA.** 1996. Co-ordinate regulation of *Salmonella* Typhimurium invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol Microbiol **22:**703–714. http://dx.doi.org/10.1046/j.1365-2958.1996.d01-1718.x.
7. **Ernst RK, Dombroski DM, Merrick JM.** 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella* Typhimurium. Infect Immun **58:**2014–2016.
8. **Galan JE, Curtiss R, III.** 1990. Expression of *Salmonella* Typhimurium genes required for invasion is regulated by changes in DNA supercoiling. Infect Immun **58:**1879–1885.
9. **Lawhon SD, Maurer R, Suyemoto M, Altier C.** 2002. Intestinal short-chain fatty acids alter *Salmonella* Typhimurium invasion gene expression and virulence through BarA/SirA. Mol Microbiol **46:**1451–1464. http://dx.doi.org/10.1046/j.1365-2958.2002.03268.x.
10. **Olekhnovich IN, Kadner RJ.** 2007. Role of nucleoid-associated proteins Hha and H-NS in expression of *Salmonella enterica* activators HilD, HilC, and RtsA required for cell invasion. J Bacteriol **189:**6882–6890. http://dx.doi.org/10.1128/JB.00905-07.
11. **Ono S, Goldberg MD, Olsson T, Esposito D, Hinton JC, Ladbury JE.** 2005. H-NS is a part of a thermally controlled mechanism for bacterial gene regulation. Biochem J **391:**203–213. http://dx.doi.org/10.1042/BJ20050453.
12. **Prouty AM, Gunn JS.** 2000. *Salmonella enterica* serovar Typhimurium invasion is repressed in the presence of bile. Infect Immun **68:**6763–6769. http://dx.doi.org/10.1128/IAI.68.12.6763-6769.2000.
13. **Eichelberg K, Hardt WD, Galan JE.** 1999. Characterization of SprA, an AraC-like transcriptional regulator encoded within the *Salmonella* Typhimurium pathogenicity island 1. Mol Microbiol **33:**139–152. http://dx.doi.org/10.1046/j.1365-2958.1999.01458.x.
14. **Rakeman JL, Bonifield HR, Miller SI.** 1999. A HilA-independent pathway to *Salmonella* Typhimurium invasion gene transcription. J Bacteriol **181:**3096–3104.
15. **Schechter LM, Damrauer SM, Lee CA.** 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the *hilA* promoter. Mol Microbiol **32:**629–642. http://dx.doi.org/10.1046/j.1365-2958.1999.01381.x.
16. **Boddicker JD, Knosp BM, Jones BD.** 2003. Transcription of the *Salmonella* invasion gene activator, *hilA*, requires HilD activation in the absence of negative regulators. J Bacteriol **185:**525–533. http://dx.doi.org/10.1128/JB.185.2.525-533.2003.
17. **Lucas RL, Lee CA.** 2001. Roles of *hilC* and *hilD* in regulation of *hilA* expression in *Salmonella enterica* serovar Typhimurium. J Bacteriol **183:**2733–2745. http://dx.doi.org/10.1128/JB.183.9.2733-2745.2001.
18. **Olekhnovich IN, Kadner RJ.** 2002. DNA-binding activities of the HilC and HilD virulence regulatory proteins of *Salmonella enterica* serovar Typhimurium. J Bacteriol **184:**4148–4160. http://dx.doi.org/10.1128/JB.184.15.4148-4160.2002.
19. **Schechter LM, Lee CA.** 2001. AraC/XylS family members, HilC and HilD, directly bind and derepress the *Salmonella* Typhimurium *hilA* promoter. Mol Microbiol **40:**1289–1299. http://dx.doi.org/10.1046/j.1365-2958.2001.02462.x.
20. **Ellermeier CD, Ellermeier JR, Slauch JM.** 2005. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator *hilA* in *Salmonella enterica* serovar Typhimurium. Mol Microbiol **57:**691–705. http://dx.doi.org/10.1111/j.1365-2958.2005.04737.x.
21. **Ellermeier CD, Slauch JM.** 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in *Salmonella enterica* serovar Typhimurium. J Bacteriol **185:**5096–5108. http://dx.doi.org/10.1128/JB.185.17.5096-5108.2003.
22. **Ahmer BM, van Reeuwijk J, Watson PR, Wallis TS, Heffron F.** 1999. Salmonella SirA is a global regulator of genes mediating enteropathogenesis. Mol Microbiol **31:**971–982. http://dx.doi.org/10.1046/j.1365-2958.1999.01244.x.
23. **Altier C, Suyemoto M, Lawhon SD.** 2000. Regulation of *Salmonella enterica* serovar Typhimurium invasion genes by *csrA*. Infect Immun **68:**6790–6797. http://dx.doi.org/10.1128/IAI.68.12.6790-6797.2000.
24. **Altier C, Suyemoto M, Ruiz AI, Burnham KD, Maurer R.** 2000. Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. Mol Microbiol **35:**635–646.
25. **Ellermeier JR, Slauch JM.** 2008. Fur regulates expression of the *Salmonella* pathogenicity island 1 type III secretion system through HilD. J Bacteriol **190:**476–486. http://dx.doi.org/10.1128/JB.00926-07.
26. **Johnston C, Pegues DA, Hueck CJ, Lee A, Miller SI.** 1996. Transcriptional activation of *Salmonella* Typhimurium invasion genes by a member of the phosphorylated response-regulator superfamily. Mol Microbiol **22:**715–727. http://dx.doi.org/10.1046/j.1365-2958.1996.d01-1719.x.
27. **Lim S, Yun J, Yoon H, Park C, Kim B, Jeon B, Kim D, Ryu S.** 2007. Mlc regulation of *Salmonella* pathogenicity island I gene expression via *hilE* repression. Nucleic Acids Res **35:**1822–1832. http://dx.doi.org/10.1093/nar/gkm060.
28. **Lin D, Rao CV, Slauch JM.** 2008. The *Salmonella* SPI1 type three secretion system responds to periplasmic disulfide bond status via the flagellar apparatus and the RcsCDB system. J Bacteriol **190:**87–97. http://dx.doi.org/10.1128/JB.01323-07.
29. **Lucas RL, Lostroh CP, DiRusso CC, Spector MP, Wanner BL, Lee CA.** 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar Typhimurium. J Bacteriol **182:**1872–1882. http://dx.doi.org/10.1128/JB.182.7.1872-1882.2000.
30. **Troxell B, Sikes ML, Fink RC, Vazquez-Torres A, Jones-Carson J, Hassan HM.** 2011. Fur negatively regulates *hns* and is required for the expression of HilA and virulence in *Salmonella enterica* serovar Typhimurium. J Bacteriol **193:**497–505. http://dx.doi.org/10.1128/JB.00942-10.
31. **Wilson RL, Libby SJ, Freet AM, Boddicker JD, Fahlen TF, Jones BD.** 2001. Fis, a DNA nucleoid-associated protein, is involved in *Salmonella* Typhimurium SPI-1 invasion gene expression. Mol Microbiol **39:**79–88. http://dx.doi.org/10.1046/j.1365-2958.2001.02192.x.
32. **Fahlen TF, Mathur N, Jones BD.** 2000. Identification and characterization of mutants with increased expression of *hilA*, the invasion gene transcriptional activator of *Salmonella* Typhimurium. FEMS Immunol Med Microbiol **28:**25–35. http://dx.doi.org/10.1111/j.1574-695X.2000.tb01453.x.
33. **Fahlen TF, Wilson RL, Boddicker JD, Jones BD.** 2001. Hha is a negative modulator of transcription of *hilA*, the *Salmonella enterica* serovar Typhimurium invasion gene transcriptional activator. J Bacteriol **183:**6620–6629. http://dx.doi.org/10.1128/JB.183.22.6620-6629.2001.
34. **Takaya A, Tomoyasu T, Tokumitsu A, Morioka M, Yamamoto T.** 2002. The ATP-dependent *lon* protease of *Salmonella enterica* serovar Typhimurium regulates invasion and expression of genes carried on *Salmonella* pathogenicity island 1. J Bacteriol **184:**224–232. http://dx.doi.org/10.1128/JB.184.1.224-232.2002.
35. **Robinson VL, Buckler DR, Stock AM.** 2000. A tale of two components: a novel kinase and a regulatory switch. Nat Struct Biol **7:**626–633. http://dx.doi.org/10.1038/77915.
36. **Behlau I, Miller SI.** 1993. A PhoP-repressed gene promotes *Salmonella* Typhimurium invasion of epithelial cells. J Bacteriol **175:**4475–4484.
37. **Pegues DA, Hantman MJ, Behlau I, Miller SI.** 1995. PhoP/PhoQ transcriptional repression of *Salmonella* Typhimurium invasion genes: evi-

dence for a role in protein secretion. Mol Microbiol **17:**169–181. http://dx.doi.org/10.1111/j.1365-2958.1995.mmi_17010169.x.
38. **Wanner BL.** 1996. Phosphorus assimilation and control of the phosphate regulon. *Escherichia coli* and *Salmonella* Typhimurium: cellular and molecular biology, 2nd ed. ASM Press, American Society for Microbiology, Washington, DC.
39. **Baxter MA, Fahlen TF, Wilson RL, Jones BD.** 2003. HilE interacts with HilD and negatively regulates *hilA* transcription and expression of the *Salmonella enterica* serovar Typhimurium invasive phenotype. Infect Immun **71:**1295–1305. http://dx.doi.org/10.1128/IAI.71.3.1295-1305.2003.
40. **Clegg S, Hughes KT.** 2002. FimZ is a molecular link between sticking and swimming in *Salmonella enterica* serovar Typhimurium. J Bacteriol **184:**1209–1213. http://dx.doi.org/10.1128/jb.184.4.1209-1213.2002.
41. **Swenson DL, Clegg S.** 1992. Identification of ancillary *fim* genes affecting *fimA* expression in *Salmonella* Typhimurium. J Bacteriol **174:**7697–7704.
42. **Yeh KS, Hancox LS, Clegg S.** 1995. Construction and characterization of a *fimZ* mutant of *Salmonella* Typhimurium. J Bacteriol **177:**6861–6865.
43. **Yeh KS, Tinker JK, Clegg S.** 2002. FimZ binds the *Salmonella* Typhimurium *fimA* promoter region and may regulate its own expression with FimY. Microbiol Immunol **46:**1–10. http://dx.doi.org/10.1111/j.1348-0421.2002.tb02670.x.
44. **Hmiel SP, Snavely MD, Miller CG, Maguire ME.** 1986. Magnesium transport in *Salmonella* Typhimurium: characterization of magnesium influx and cloning of a transport gene. J Bacteriol **168:**1444–1450.
45. **Nelson DL, Kennedy EP.** 1971. Magnesium transport in *Escherichia coli*. Inhibition by cobaltous ion. J Biol Chem **246:**3042–3049.
46. **Maniatis T, Fritsch EF, Sambrook J.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
47. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
48. **Miller JH.** 1972. Experiments in molecular genetics. Cold Spring Harbor Press, Cold Spring Harbor, NY.
49. **Davis RW, Botstein D, Roth JR.** 1980. Advanced bacterial genetics: a manual for genetic engineering. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
50. **Baxter MA, Jones BD.** 2005. The *fimYZ* genes regulate *Salmonella enterica* serovar Typhimurium invasion in addition to type 1 fimbrial expression and bacterial motility. Infect Immun **73:**1377–1385. http://dx.doi.org/10.1128/IAI.73.3.1377-1385.2005.
51. **Francis CL, Ryan TA, Jones BD, Smith SJ, Falkow S.** 1993. Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. Nature **364:**639–642. http://dx.doi.org/10.1038/364639a0.
52. **Lee CA, Falkow S.** 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc Natl Acad Sci U S A **87:**4304–4308. http://dx.doi.org/10.1073/pnas.87.11.4304.
53. **Gantois I, Ducatelle R, Pasmans F, Haesebrouck F, Hautefort I, Thompson A, Hinton JC, Van Immerseel F.** 2006. Butyrate specifically down-regulates *Salmonella* pathogenicity island 1 gene expression. Appl Environ Microbiol **72:**946–949. http://dx.doi.org/10.1128/AEM.72.1.946-949.2006.
54. **Hung CC, Garner CD, Slauch JM, Dwyer ZW, Lawhon SD, Frye JG, McClelland M, Ahmer BM, Altier C.** 2013. The intestinal fatty acid propionate inhibits *Salmonella* invasion through the posttranslational control of HilD. Mol Microbiol **87:**1045–1060. http://dx.doi.org/10.1111/mmi.12149.
55. **Lee CA, Jones BD, Falkow S.** 1992. Identification of a *Salmonella* Typhimurium invasion locus by selection for hyperinvasive mutants. Proc Natl Acad Sci U S A **89:**1847–1851. http://dx.doi.org/10.1073/pnas.89.5.1847.
56. **Wang H, Liu B, Wang Q, Wang L.** 2013. Genome-wide analysis of the *Salmonella* Fis regulon and its regulatory mechanism on pathogenicity islands. PLoS One **8:**e64688. http://dx.doi.org/10.1371/journal.pone.0064688.
57. **Gong H, Vu GP, Bai Y, Chan E, Wu R, Yang E, Liu F, Lu S.** 2011. A *Salmonella* small non-coding RNA facilitates bacterial invasion and intracellular replication by modulating the expression of virulence factors. PLoS Pathog **7:**e1002120. http://dx.doi.org/10.1371/journal.ppat.1002120.
58. **Espinosa E, Casadesus J.** 2014. Regulation of *Salmonella enterica* pathogenicity island 1 (SPI-1) by the LysR-type regulator LeuO. Mol Microbiol **91:**1057–1069. http://dx.doi.org/10.1111/mmi.12500.
59. **Chubiz JE, Golubeva YA, Lin D, Miller LD, Slauch JM.** 2010. FliZ regulates expression of the *Salmonella* pathogenicity island 1 invasion locus by controlling HilD protein activity in *Salmonella enterica* serovar Typhimurium. J Bacteriol **192:**6261–6270. http://dx.doi.org/10.1128/JB.00635-10.
60. **Kage H, Takaya A, Ohya M, Yamamoto T.** 2008. Coordinated regulation of expression of *Salmonella* pathogenicity island 1 and flagellar type III secretion systems by ATP-dependent ClpXP protease. J Bacteriol **190:**2470–2478. http://dx.doi.org/10.1128/JB.01385-07.
61. **Saini S, Slauch JM, Aldridge PD, Rao CV.** 2010. Role of cross talk in regulating the dynamic expression of the flagellar *Salmonella* pathogenicity island 1 and type 1 fimbrial genes. J Bacteriol **192:**5767–5777. http://dx.doi.org/10.1128/JB.00624-10.
62. **Mouslim C, Hughes KT.** 2014. The effect of cell growth phase on the regulatory cross-talk between flagellar and Spi1 virulence gene expression. PLoS Pathog **10:**e1003987. http://dx.doi.org/10.1371/journal.ppat.1003987.
63. **Cardenal-Munoz E, Ramos-Morales F.** 2013. DsbA and MgrB regulate *steA* expression through the two-component system PhoQ/PhoP in *Salmonella enterica*. J Bacteriol **195:**2368–2378. http://dx.doi.org/10.1128/JB.00110-13.
64. **Adams P, Fowler R, Kinsella N, Howell G, Farris M, Coote P, O'Connor CD.** 2001. Proteomic detection of PhoPQ- and acid-mediated repression of *Salmonella* motility. Proteomics **1:**597–607. http://dx.doi.org/10.1002/1615-9861(200104)1:4>597::AID-PROT597<3.0.CO;2-P.
65. **Golubeva YA, Sadik AY, Ellermeier JR, Slauch JM.** 2012. Integrating global regulatory input into the *Salmonella* pathogenicity island 1 type III secretion system. Genetics **190:**79–90. http://dx.doi.org/10.1534/genetics.111.132779.
66. **Zwir I, Shin D, Kato A, Nishino K, Latifi T, Solomon F, Hare JM, Huang H, Groisman EA.** 2005. Dissecting the PhoP regulatory network of *Escherichia coli* and *Salmonella enterica*. Proc Natl Acad Sci U S A. **102:**2862–2867. http://dx.doi.org/10.1073/pnas.0408238102.
67. **Mouslim C, Groisman EA.** 2003. Control of the *Salmonella ugd* gene by three two-component regulatory systems. Mol Microbiol **47:**335–344. http://dx.doi.org/10.1046/j.1365-2958.2003.03318.x.
68. **Sun G, Birkey SM, Hulett FM.** 1996. Three two-component signal-transduction systems interact for Pho regulation in *Bacillus subtilis*. Mol Microbiol **19:**941–948. http://dx.doi.org/10.1046/j.1365-2958.1996.422952.x.
69. **Oshima T, Aiba H, Masuda Y, Kanaya S, Sugiura M, Wanner BL, Mori H, Mizuno T.** 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. Mol Microbiol **46:**281–291. http://dx.doi.org/10.1046/j.1365-2958.2002.03170.x.
70. **Miller SI, Kukral AM, Mekalanos JJ.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella* Typhimurium virulence. Proc Natl Acad Sci U S A **86:**5054–5058. http://dx.doi.org/10.1073/pnas.86.13.5054.
71. **Wray C, Sojka WJ.** 1978. Experimental *Salmonella* Typhimurium infection in calves. Res Vet Sci **25:**139–143.
72. **Miller SI, Mekalanos JJ.** 1990. Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. J Bacteriol **172:**2485–2490.
73. **Jiang W, Metcalf WW, Lee KS, Wanner BL.** 1995. Molecular cloning, mapping, and regulation of Pho regulon genes for phosphonate breakdown by the phosphonatase pathway of *Salmonella* Typhimurium LT2. J Bacteriol **177:**6411–6421.