The Flagellar Regulator fliT Represses Salmonella Pathogenicity Island 1 through flhDC and fliZ

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Abstract

Salmonella pathogenicity island 1 (SPI1), comprising a type III section system that translocates effector proteins into host cells, is essential for the enteric pathogen Salmonella to penetrate the intestinal epithelium and subsequently to cause disease. Using random transposon mutagenesis, we found that a Tn10 disruption in the flagellar fliDST operon induced SPI1 expression when the strain was grown under conditions designed to repress SPI1, by mimicking the environment of the large intestine through the use of the intestinal fatty acid butyrate. Our genetic studies showed that only fliT within this operon was required for this effect, and that exogenous over-expression of fliT alone significantly reduced the expression of SPI1 genes, including the invasion regulator hilA and the sipBCDA operon, encoding type III section system effector proteins, and Salmonella invasion of cultured epithelial cells. fliT has been known to inhibit the flagellar machinery through repression of the flagellar master regulator flhDC. We found that the repressive effect of fliT on invasion genes was completely abolished in the absence of flhDC or fliZ, the latter previously shown to induce SPI1, indicating that this regulatory pathway is required for invasion control by fliT. Although this flhDC-fliZ pathway was necessary for fliT to negatively control invasion genes, fliZ was not essential for the repressive effect of fliT on motility, placing fliT high in the regulatory cascade for both invasion and motility.

Introduction

Salmonella is an important bacterial pathogen that is a leading source of food-borne illness, causing diseases ranging from transient enteritis to life-threatening septicemia. To infect its animal hosts, Salmonella first must penetrate the intestinal epithelium, a process termed invasion. Most of the genes required for invasion lie within a 40 kb gene cluster at centisome 63 termed Salmonella Pathogenicity Island 1 (SPI1), which is used by Salmonella to construct a type III secretion apparatus, the needle complex, to deliver secreted effector proteins into the host cell cytoplasm [1,2,3,4,5,6]. Once these proteins are translocated into a targeted epithelial cell, they induce cytoskeleton rearrangement and membrane ruffling, resulting in internalization of Salmonella by the host cell [7,8,9,10].

SPI1 genes are known to be controlled by several transcriptional regulators encoded within and outside SPI1 through a complex network. Four transcriptional regulators, hilD, hilC, hilA and invF are present within SPI1 [4,11,12,13,14]. Among these, hilD is at the top of the regulatory cascade and controls hilC as well as a regulator located outside SPI1, rtsA [15,16]. HilD, HilC, and RtsA are able to regulate their own gene expression and can activate expression of hilD, hilC and rtsA independent of each other to constitute a regulatory circuit for the control of the SPI1 central regulator hilA [17]. HilA controls the sic/sip operon, encoding effector proteins, and the psg/ins operon that encode proteins composing the type III secretion apparatus [13,14]. HilA also induces the expression of the transcriptional regulator invF, encoding a member of the AraC family that activates the expression of genes encoding effector proteins within and outside SPI1 [13,14]. In addition, invF has been shown to be directly regulated by HilD and HilC through a HilA-independent pathway [18]. Several genetic regulators outside SPI1 have also been shown to transcriptionally or post-transcriptionally control invasion gene expression. Regulators affecting SPI1 at the level of transcription include the two-component regulators PhoP/PhoQ, EnvZ/OmpR, PhoB/PhoR and BarA/SirA [1,19,20]. In addition, the DNA binding proteins H-NS and Hha have been demonstrated to bind to multiple A-T rich sequences in SPI1, occupying the binding sites of positive regulators, and consequently preventing transcription [21,22,23]. Among the post-transcriptional regulators of SPI1, the Csr system, PNPase, Lon protease and HilE have been shown to control invasion genes by affecting protein production or by manipulating the level or activity of HilD [24,25,26,27,28].

In addition to the mechanisms of control described above, two regulators of the flagellar regulon, flhDC and fliZ, have been described as inducers of SPI1 [19,29]. In the Salmonella flagellar regulatory cascade, the FlhDCC2 complex, encoded by flhDC, functions as a master regulator that binds to the class 2 flagellar promoters and to its own promoter to induce downstream flagellar gene expression [30,31]. However, the function of FlhDCC2 is antagonized by another flagellar protein, FliT, which associates with FlhDC and neutralizes its activity [32,33].
characterized as a class 2 flagellar gene [34]. Previous studies have shown that mutation of fliZ significantly reduces hilD expression and Salmonella intestinal colonization in mice. In addition, overexpression of fliZ increases hilA expression only when hilD is present, indicating that fliZ controls invasion gene expression through hilD [29]. Although fliZ has been demonstrated to negatively control hilD, the mechanism by which this is accomplished remains uncertain [29,35].

Expression of invasion genes can also be induced using various laboratory conditions that mimic the host intestinal environment, such as low oxygen, high osmolarity, and a near neutral pH [36,37,38]. In addition, short-chain fatty acids, produced by the intestinal microflora through fermentative metabolic pathways, have been shown to play important roles in controlling Salmonella invasion [39,40]. Among these, butyrate, which exists in high concentration in the large intestine where salmonellosis rarely occurs, represses SPI1 gene expression [40,41].

To identify additional genetic elements involved in Salmonella invasion control, here we applied a transposon mutagenesis approach and identified a mutation in the flagellar gene fliT that affects the expression of SPI1 genes. As fliT was known to be a negative regulator of the flagellar regulon, we used genetic approaches to study the role of fliT and associated regulatory elements in the repression of invasion. Here, we demonstrate that fliT controls Salmonella invasion genes through flhDC and the flhDC-regulated gene, fliZ.

Results
Identifying fliT, a novel negative regulator of Salmonella invasion, using random transposon mutagenesis
screening
Salmonella Pathogenicity Island 1 (SPI1) gene expression is controlled by various regulatory elements inside and outside the island, and is also affected by environmental cues [42]. To identify novel regulators that negatively control Salmonella invasion, we used random Tn10 transposon mutagenesis in a strain carrying a gfp reporter fusion to the SPI1 gene sipC, with the strain grown in the presence of butyrate, a short-chain fatty acid found in abundance within the mammalian intestine. As butyrate has been shown to repress SPI1 genes [40,41], the bacterial colonies carrying the sipC::gfp reporter showed little fluorescence on LB agar containing 10 mM butyric acid. We surmised that transposon insertions in negative regulators of invasion would increase sipC::gfp expression, producing fluorescent colonies. The strain used for this screen also carried a deletion of ackA, encoding acetyl kinase, as our studies showed that the ackA mutation partially restored sipC expression in media containing butyric acid (Fig. 1 and data not shown). This strain allowed the screen to be performed without the repeated isolation of ackA mutants, and thus provided the possibility of identifying novel regulators of invasion.

In total, we screened approximately 40,000 colonies, representing an 8-fold screening of the genome, with 31 fluorescent colonies being found. We next sought to determine the transposon insertion sites in candidate mutants. Previously, it had been reported that Tn10 insertions near the promoter region of the SPI1 regulator hilD could cause increased expression of the downstream regulator hilA, which is essential to induce sipC [43]. To rule out these and other potential mutations within SPI1, we examined the linkage of Tn10 insertions to sipC using P22 bacteriophage-mediated transductional mapping. The results showed that 22 candidates possessed a Tn10 insertion linked to sipC; these mutants were not further characterized.

For the remaining nine candidate colonies, the Tn10 insertions were moved by transduction into an ackA mutant carrying a MudJ insertion encoding a lacZY fusion to the sipBCDA operon to quantify the increase in invasion gene expression using β-galactosidase assays. Based upon the increased level of sipBCDA::
lacZ expression, candidates were categorized into two classes; those with increased expression only when butyrate was present (six mutants), and those with increased expression under both repressing and inducing conditions (three mutants). As individual mutants in each group possessed a similar effect on sipBCDA expression, their phenotypes suggested that they might carry Tn10 disruptions in the same gene or operon. To identify the sites of transposon insertion, we amplified the region flanking the Tn10 for one candidate from each of the two groups by arbitrary PCR [44]. We found that the mutant affected only under repressing conditions, in the presence of butyrate, carried a Tn10 insertion in fliDST, the first gene of the fliDST operon. A representative of the second class, showing increased sipC expression under both repressing and inducing conditions, carried a Tn10 insertion in pnp. Further, we determined the genetic linkage of Tn10 in all of the remaining candidates of both groups to fliD and pnp by transcriptional mapping, finding that all insertions within a group were 100% linked to these respective genes. These results, taken together, demonstrate that all of the mutations residing outside SPI1 that induced the expression of sipC under our tested conditions resulted from disruptions in or near either fliD or pnp.

**fliT is a negative regulator of Salmonella invasion**

*pnp*, encoding a 3′- to 5′-phosphorolytic exonuclease, a subunit of RNA degradosome, has been shown to affect SPI1 genes expression by interfering with RNA half-life [28]. However, genes in the fliDST operon have not been reported to control invasion by *Salmonella*. For this reason, we focused our study on the role of the fliDST operon in the control of SPI1. To quantify the effects of the Tn10 disruption of fliD in invasion, we compared sipC::*lacZ* expression in various mutants grown with or without butyric acid by β-galactosidase assays. In the wild type, *fliC* expression decreased 3.5-fold when the strain was grown in media containing 10 mM butyric acid compared to media with no additive (with all media stably buffered to pH 6.7), and an ackA mutant, as expected, demonstrated a lesser, 1.5-fold repression due to the presence of butyric acid (Fig. 1). Importantly, *sipC* expression was unaffected by butyric acid in the ackA, fliD::Tn10 double mutant (Fig. 1). As fliD is the first gene in the fliDST operon, the increase of *sipC* expression caused by the disruption of fliD compared to the wild type grown under the same repressive conditions may have resulted from polar effects on any of the downstream genes in the operon. Thus, we next determined which genes played important roles in control of *sipC* expression by testing the effects of mutations of operon genes, singly and in combination. The results showed that ackA strains with an additional deletion of fliDST, fliST, or *fliT* restored *sipC* expression in the presence of butyric acid compared to the same strains without additive (Fig. 1). There remained, however, a significant decrease in *sipC* expression by butyrate in strains with disruptions of fliD (data not shown) or *fliT* (Fig. 1), the first two genes of the fliDST operon. From these results, we concluded that the last gene of the fliDST operon, *fliT*, is required for the negative control of SPI1 gene expression. The increased *sipC* expression caused by deletion of *fliT* was, however, seen only in the ackA mutant and with the repression of SPI1 genes provided by butyric acid (Fig. 1). In addition to the repressive effects that required *fliT*, we also found that the loss of some members of the operon in the ackA null strain reduced *sipC* expression irrespective of the media conditions employed (Fig. 1; compare white bars to each other). This suggests that components of this operon under some conditions may exhibit a positive effect on invasion gene expression, but in this work we further examined only the genesis of invasion gene repression caused by these genes.

To confirm the negative effect of *fliT* on invasion, we cloned the *fliT* ORF onto a low-copy number plasmid, on which *fliT* was constitutively expressed under the control of an exogenous promoter. Again using a sipC::*lacZ* fusion, we found a significant 3.1-fold decrease in *sipC* expression in the wild type strain with the *fliT* plasmid compared to the isogenic strain carrying the control plasmid, pACYC177 (Fig. 2). This repressive effect of *fliT* on invasion gene expression, however, was limited to conditions of over-expression as a *fliT* mutant carrying a sipC::*lacZ* fusion in an otherwise wild type background and grown under SPI1-inducing conditions demonstrated no significant change in *sipC* expression (data not shown), identical to the phenotype of the fliD::Tn10 insertion shown in Figure 1. To verify that the repressive effect of *fliT* on gene expression manifested itself as a significant virulence phenotype, we next characterized changes in the levels of effector proteins of SPI1 produced and secreted by this strain. SPI1 invasion proteins encoded by the sipBCDA operon have been shown to be secreted into the culture medium when *Salmonella* is grown in laboratory media [45]. We extracted the secreted proteins from overnight bacterial cultures and examined the SPI1 effector protein profile using SDS-PAGE with Coomassie blue staining (Fig. 3). Four bands had molecular weights equivalent to the invasion proteins SipA (89 kDa), SipB (67 kDa), SipC (43 kDa) and SipD/InvJ (38 kDa) (protein sequences of these bands were determined by mass spectrometry, with the band for SipD overlapping that of another invasion protein, InvJ, due to their similar molecular weights). These bands were significantly diminished in the wild type strain carrying the *fliT* plasmid compared to the strain with the control plasmid (Fig. 3, lanes 1 and 2). In addition, we examined the invasion of the wild type strain carrying the *fliT* plasmid using a gentamicin protection assay
with the HEp-2 epithelial cell line. We found that over-expression of flIT significantly reduced the ability of Salmonella to penetrate these cells, reducing invasion by 23-fold (Fig. 4). Based upon the results of β-galactosidase assay, the secreted protein profile assays, and this invasion assay, we thus demonstrated that flIT acts as a repressor of SPI1 gene expression and Salmonella invasion when it is over-expressed.

The fact that the loss of flIT in the ackA deletion mutant could relieve the butyrate-induced repressive effect on the SPI1 gene sipC (Fig. 1) led us to speculate that butyrate might function through the induction of flIT itself. To test this hypothesis, we used a flIT-lacZ transcriptional fusion in the wild type and the ackA mutant, and examined whether flIT expression was increased by butyrate. We found that there was no significant difference in flIT expression in either strain background with the addition of butyric acid (data not shown), indicating that flIT expression is not affected by butyrate at the transcriptional level. To further investigate whether the negative effects of flIT and butyrate on invasion genes were independent, we determined whether the addition of butyrate promoted the repressive effect on SPI1 when flIT was over-produced. We over-expressed flIT in the wild type and the ackA mutant strains carrying the flIT plasmid and compared sipC expression with or without the addition of butyric acid. As expected, we found that there was a significant further reduction of sipC expression by butyrate in these strains, 3.6-fold for the wild type and 3.3-fold for the ackA mutant (Fig. 2). These results therefore demonstrate that flIT is not involved in the negative control of butyrate on SPI1 gene expression.

**flIT negatively controls invasion genes through the flagellar regulators flhDC and fltZ**

Having shown that over-expression of flIT from an exogenous promoter repressed invasion, we further asked how this member of the flagellar regulon exhibited this control. FlIT has been shown to function as a chaperone to facilitate export of the flagella capping protein, FliD, in the assembly of flagella [46,47]. More importantly, FlIT has also been demonstrated to negatively control flagellar gene expression by binding to the class 1 flagellar regulator, the FlhD4C2 complex, and preventing this transcriptional activator from binding to class 2 flagellar promoters, consequently reducing downstream flagellar gene expression [32,33]. Since FlIT can function as a negative regulator of the flagellar regulon, it is possible that the repressive effect of FlIT on invasion may result from its negative effects on other flagellar genes that can positively control invasion gene expression. In Salmonella, two flagellar genes, flhDC and the flhDC-controlled downstream regulator fliZ, have been shown to positively regulate
SPI1 [19,29]. In addition, $\beta iZ$ has been shown to regulate invasion genes through the control of the SPI1 regulator, HfID [29,35]. To test whether $\beta iT$ affected Salmonella invasion through the negative control of this $\beta hDC$-$\beta iZ$ pathway, we first examined the abilities of the $\beta hDC$ and $\beta iZ$ mutants, each carrying the $\beta iT$ plasmid or the control plasmid pACYC177, to invade cultured HEp-2 epithelial cells using a gentamicin protection assay (Fig. 4). We found that there was a significant reduction in Salmonella invasion, 26-fold for the $\beta hDC$ mutant and 22-fold for the $\beta iZ$ mutant (Fig. 4). These results demonstrate that these two flagellar genes are positive regulators of invasion, and are consistent with results published by other groups [19,29]. We also found that there was no significant difference in invasion between the $\beta hDC$ mutant carrying the $\beta iT$ plasmid or the control plasmid. However, a 1.4-fold decrease in invasion was observed in the $\beta iZ$ mutant carrying the $\beta iT$ plasmid compared to the same strain carrying the control plasmid (Fig. 4). These results suggested that $\beta hDC$ is required for $\beta iT$ to control invasion, but $\beta iZ$ may be dispensable. As flagella have been shown to affect Salmonella invasion [48], mutation of the master flagellar regulator $\beta hDC$ might cause greater effects on flagella production than mutation of $\beta iZ$, and consequently affect the results of invasion assays. Therefore, to more precisely test whether $\beta iT$ affected invasion genes through the negative control of this $\beta hDC$-$\beta iZ$ pathway, we next examined $\beta iC$ expression in the wild type, the $\beta hDC$ mutant, and the $\beta iZ$ mutant, each carrying the $\beta iT$ plasmid or the control plasmid. Using the $\beta iC$-lacZ$\beta$ fusion, there was a significant reduction of $\beta iC$ expression, in the $\beta hDC$ mutant (4.6-fold) and the $\beta iZ$ mutant (6.8-fold) (Fig. 5A). Additionally, over-expression of $\beta iT$ did not further reduce $\beta iC$ expression in the $\beta hDC$ or the $\beta iZ$ mutant (Fig. 5A). These results suggest that $\beta iT$ negatively controls $\beta iC$ through this recognized pathway of regulation. To confirm the negative effect of $\beta iT$ on SPI1 genes through $\beta hDC$ and $\beta iZ$, we further examined the secreted invasion protein profiles using culture conditions identical to those employed for the $\beta$-galactosidase assays. The result showed that the secreted invasion effector proteins SipA, SipB, SipC and SipD were significantly diminished in the $\beta hDC$ and the $\beta iZ$ mutants compared to the wild type (Fig. 3, lanes 1, 3 and 5). Additionally, there was no further reduction in these proteins in the $\beta hDC$ or $\beta iZ$ mutant carrying the $\beta iT$ plasmid (Fig. 3, lanes 4 and 6). As we had shown that downstream SPI1 effector proteins were affected by over-expression of $\beta iT$, in parallel we also determined whether their upstream regulator, Hila, was affected. As for the previous $\beta$-galactosidase results using $\beta iC$, Hila expression was significantly reduced in the $\beta hDC$ and $\beta iZ$ mutants carrying the control plasmid compared to the wild type with the same plasmid. A 2.8-fold decreased in Hila expression was also observed due to the expression of $\beta iT$, and there was no additional decrease in Hila expression in the $\beta hDC$ and $\beta iZ$ mutants carrying the $\beta iT$ plasmid compared to the same strains with the control plasmid (Fig. 3B). Based upon these results, we conclude that $\beta iT$ negatively affects Salmonella invasion gene expression through $\beta hDC$ and $\beta iZ$.

The $\beta hDC$-$\beta iZ$ pathway is required for the repressive effects of $\beta iT$ on invasion gene expression, but not for its effects on flagellar regulation

Our results demonstrate that $\beta iT$ acts as a negative regulator of invasion, and previous studies have shown that $\beta iT$ affects flagellar control in Salmonella [32,33,49]. Additionally, our data suggest that the repressive effect of $\beta iT$ on invasion genes is accomplished through the $\beta hDC$-$\beta iZ$ pathway. Since $\beta hDC$ and $\beta iZ$ have been implicated as regulators in the flagellar regulon [50], we further asked whether this $\beta hDC$-$\beta iZ$ route is used by $\beta iT$ in its control of flagella. To test this, we used the wild type, the $\beta hDC$ mutant, and the $\beta iZ$ mutant carrying either the control plasmid or the $\beta iT$ plasmid, and examined their swimming ability on 0.35% LB agar plates (Fig. 6). We found that in the wild type strain over-expression of $\beta iT$ completely eliminated Salmonella motility. The same phenotype was also observed in the $\beta hDC$ mutant whether it carried the control plasmid, pACYC177, or the $\beta iT$ plasmid. As previously described, $\beta iT$ is able to negatively control flagellar gene expression by post-translational regulation of FlhD$_C$$_C_2$ activity. Our results thus suggest that $\beta iT$ controls Salmonella motility.

Figure 5. $\beta iT$ affects SPI1 gene expression through the $\beta hDC$-$\beta iZ$ pathway. The $\beta iT$ expression plasmid pFliT (white bars) and the control plasmid pACYC177 (black bars) were tested in the wild type, the $\beta hDC$ mutant and the $\beta iZ$ mutant carrying A) the sipC::lacZ$\beta$ fusion, and B) the hila::lacZ$\beta$ fusion. Strains were cultured in LB broth with 100 mM MOPS, pH6.7, and 100 $\mu$g/ml ampicillin overnight without aeration and lacZ$\beta$ expression was measured using $\beta$-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to the $\beta iT$ expression plasmid pFliT as compared to the same strain with the control plasmid at p<0.05. A plus (+) indicates a statistically significant difference due to deletion of a gene as compared to the wild type at p<0.05.
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through fliDC, and are consistent with other studies. However, unlike fliDC, the fliZ mutant showed only a slight reduction in swimming ability, and over-expression of fliT in this strain fully inhibited its motility, suggesting that the repression of motility by fliT was not mediated through fliZ. Therefore, our results, taken together, suggested that the fliDC–fliZ pathway is specific for repression of Salmonella invasion gene expression by fliT, but this pathway is not required for fliT repression of the flagellar regulon.

Discussion

For serovars of Salmonella, the genes of SPI1 are key elements that dictate the ability of the pathogen to penetrate the intestinal epithelium and cause further systemic infection. The control of SPI1 gene expression has been shown to be evoked by complex interrelated regulatory networks. In this work, using a random transposon mutagenesis strategy, we discovered that the flagellar regulator fliT, encoded within the fliDST operon, can negatively control SPI1 gene expression (Fig. 1). In addition, we showed that fliT over-expression reduced invasion gene expression (Figs. 2, 3, and 5) and Salmonella invasion (Fig. 4), and that this repressive effect on SPI1 functioned through the negative control of the fliDC–fliZ pathway (Figs. 3 and 5). fliZ has been shown to positively control invasion genes by regulating the SPI1 regulator HilD, which exists high in the regulatory hierarchy of this pathogenicity island [16,29]. It has been suggested that FliZ post-transcriptionally controls HilD through an unidentified mechanism, rather than affecting hilD expression at the level of transcription [29]. Kage and coworkers showed that HilD protein level, when expressed from a constitutive promoter, was significantly decreased by the deletion of fliZ. However, the half-life of HilD was not changed when fliZ was missing. Their studies thus suggest that fliZ controls HilD at the translational level [35]. In contrast, Chubiz and colleagues showed that HilD, when constitutively expressed from a single-copy chromosomal tetracycline-inducible promoter, was only slightly reduced in the fliZ null strain compared to that in the wild type. Additionally, they measured the stability of HilD in the fliZ mutant and in the wild type and showed that the stability of HilD was not significantly altered in this mutant. Therefore, they suggested that the mechanism by which fliZ regulates HilD is by post-transcriptionally affecting HilD activity [29]. As we have shown that fliT negatively controls Salmonella invasion by changing the amount or activity of HilD, and subsequently affects expression of downstream invasion genes.

fliT has been shown to possess two functions in Salmonella, acting both as a regulator and a chaperone [46,49,51]. In its chaperone function, FliT directly interacts with several flagellar proteins, including FlhD, FliH, and FliJ, preventing their premature aggregation and facilitating flagellar assembly [46,47]. As a regulator of flagellar expression, FliT binds to FlhC as part of the FlhD–C2 complex and inhibits FlhDC2 from binding to its target promoters, consequently repressing downstream flagellar gene expression [32]. In our work, we showed that fliT, when deleted, was the only gene of the fliDST operon able to restore invasion gene expression under our test conditions. We would expect that if FliT had affected invasion genes through its role as a chaperone, by interacting with FlhD, the deletion of fliT would similarly restore sipC expression, as did the fliT mutant (Fig. 1). However, restoration of sipC expression was not observed in the fliD mutant under the conditions used (data not shown), suggesting that FliT does not affect SPI1 gene expression through its function as a chaperone. Instead, our results indicate that fliT acts on invasion genes in its role as a regulator, as we have demonstrated that the fliDC–fliZ pathway with which FliT is known to interact is required for its negative control of invasion genes. In addition to the repressive effects that we identified for fliT, we also found that the loss of some members of the fliDST operon caused a reduction in sipC expression when tested in the ackA null mutant, a phenotype that was independent of the media conditions used (Fig. 1). This may suggest that different components of this operon can have opposing actions. Specifically, it is possible that fliT functions as a repressor, but that other products of the operon, alone or in combination with fliT, might act as inducers. Work in this area will require further efforts.

In agreement with previous studies [32,33], our work suggests that fliDC is also required for fliT to control Salmonella motility (Fig. 6). However, we found that fliZ was required for the effects of fliT only on SPI1 gene expression, and not on motility, as only a slight reduction in swimming was observed in the fliZ mutant (Fig. 6). These results, taken together, thus demonstrate that fliT is able to coordinately regulate invasion and flagellar gene expression through the single flagellar master regulator fliDC, but that the control of these two regulators diverges at subsequent steps in their regulatory cascades.

In Salmonella, flagella and invasion have been shown to be coordinately regulated by regulators in addition to fliT through the fliDC–fliZ pathway. ClpXP is an ATP-dependent protease and has been demonstrated to repress both flagellar and SPI1 gene expression [35]. ClpXP negatively controls the flagellar regulon by facilitating the degradation of the master flagellar regulators FlhD and FliC and subsequently repressing downstream flagellar genes [52]. Kage and coworkers have demonstrated that the repressive effect of ClpXP on the fliDC–fliZ cascade is required for this protease to negatively control invasion genes [35]. TviA is another regulator that negatively co-regulates invasion and flagellar genes through this pathway. TviA is a regulator within Salmonella Pathogenicity Island 7 (SPI7) unique to Salmonella serovar Typhi that does not exist in S. Typhimurium. This regulator has been shown to respond to stimulation by low osmolarity and also negatively controls both flagellar and invasion gene expression. TviA affects flagellar genes by repressing the transcription of fliDC. This inhibitory effect on fliDC has been suggested to consequently cause the reduction of invasion gene expression through the fliDC–fliZ pathway [53]. The above two regulators and FliT have thus been demonstrated to either transcriptionally
or post-translationally affect the flagellar master regulator *flhDC*, while previous studies and the results we present here demonstrate that the *flhDC-fltZ* pathway is essential for these regulators to control invasion genes. Based on this evidence, we suggest that the *flhDC-fltZ* pathway is an important common route used by *Salmonella* to allow the flagellar regulon to coordinately control invasion gene expression.

As previously described, FltT is produced, binds existing FlhD4C2, dissociating the FlhD4C2 complex and resulting in a quick down-regulation of control FlhD4C2 activity by its interaction with FlhC and invasion gene expression. *Salmonella* flhDC–fliZ is able to interact with FlhD4C2 that has not bound to its target DNA when FliT is produced, it binds existing FlhD4C2, dissociating the flagellar regulon in response to rapidly changing environments. This protein-DNA complex was insensitive to FliT [33]. Their studies suggest a means by which *Salmonella* can efficiently control the flagellar regulon in response to rapidly changing environments. When FltT is produced, it binds existing FlhD4C2, dissociating the FlhD4C2 complex and resulting in a quick down-regulation of flagellar gene expression. When the level of FltT is low, however, the FlhD4C2 complex associates with its target DNA, leading to the dissociation of the FlhD4C2 complex in vivo [33]. However, when FlhD4C2 was pre-associated with its target DNA, this protein-DNA complex was insensitive to FltT [33]. Their studies suggest a means by which *Salmonella* can efficiently control the flagellar regulon in response to rapidly changing environments.

Materials and Methods

Construction of mutant strains

*Salmonella enterica* serovar Typhimurium strain ATCC 14028S and isogenic mutants were used throughout this study, and are shown in Table 1. Gene deletions were made using the previously reported one-step inactivation method [55]. In brief, PCR reactions were performed to amplify the fragments containing the FRT sequences flanking the antibiotic resistance markers from plasmids pKD3 or pKD4 using primers carrying 40 bases of homologous sequence flanking the coding region of the target gene. The resulting PCR product was purified and transformed into a *Salmonella* strain carrying the plasmid pKD46, which expresses the Red λ recombinase, allowing allelic exchange. The resulting deletion mutants were cultured at 42°C to remove the temperature-sensitive pKD46 plasmid, and the loss of the target gene was determined by PCR. The chromosomal sipC::gfp translational fusion was created using the above one-step gene exchange method. A promoterless gfp linked to a chloramphenicol resistance marker was amplified from the plasmid pZEPO7 [56] with primers TGAGACGTTGATCGCAATCGTGAGAAGGTTTC-CAACCTTTACCGTGAGGCTGTGAGCTTTGCG and TT-ATAATGACCATCATGATGGCAGTATAGTGACCTTTACGACATATGAATATCCTCCTTAG, which encode DNA homologous to the regions immediately adjacent to the sipC open reading frame. The resulting PCR product was purified and treated with DpnI to remove the pZEP07 template and transformed into the *Salmonella* strain carrying pKD46 with selection on 25 μg/ml chloramphenicol to allow recombination of gfp, creating a translational fusion to *gfp* with an adjacent chloramphenicol cassette. To create the *fltT* expression plasmid (pFltT), a PCR product was produced that included a synthetic ribosome binding site, based upon that of lacZ and the *fltT* ORF with an additional 175 bp 3’ of the *fltT* sequence to include the predicted transcriptional termination site. This product was amplified using primers CCCATCGATGTTTGGACGAGAGCTAGTA-TGAGCCTCAACCTGGAGGTATATACG and TCCCGCCGG-GGATATCTTATTAGCCGCGAG. The PCR product was then cloned into the unique *ClaI* and *Smal* sites of pACYC177 to place *fltT* under the control of the kanamycin resistance gene (*aph*) promoter on this vector.

*Tn10* random transposon mutagenesis screening

To create a random transposon *Tn10* library, a wild type strain carrying the IPTG-inducible *Tn10* plasmid pNK2883 [57] and an additional plasmid pMS421 [58] that expresses lacI was used. The strain was grown overnight in LB broth with 100 μg/ml of ampicillin, 100 μg/ml of spectinomycin, and 20 μg/ml streptomycin at 37°C with shaking, and sub-cultured in the same medium to mid-log phase. To induce transposon insertion, IPTG was added at a final concentration of 0.1 mM to the mid-log culture, which was grown for another 16 hours. The resulting random *Tn10* insertion library was moved into the *aekI* mutant strain carrying the chromosomal sipC::gfp translational fusion by P22 phage transduction [59]. Transductants were plated on LB agar with 25 μg/ml tetracycline, 100 mM 3- (N-morpholinopropanesulfonic acid (MOPS) pH 6.7, 10 mM ethylene glycol tetraacetic acid (EGTA), and 10 mM butyric acid and incubated at 37°C overnight. The green florescence of individual colonies was determined using an OQ100 Observation Intravital System (Olympus Corp., Tokyo, Japan).

Determining the DNA sequence flanking the *Tn10* element

The sequences flanking the *Tn10* insertions were identified using a method previously reported [44]. In brief, *Tn10* insertion strains and a control strain (the isogenic strain without a *Tn10* insertion) were grown overnight in LB broth with aeration. The overnight culture was diluted 100-fold with nuclease-free water, and bacteria were frozen and thawed three times to expose the genomic DNA. Five μl of the sample was used as a template to perform an initial PCR using primer AATTGGTGTCTTATAACGAGGCACTG in combination with arbitrary primers GGCCAGCGAGCTAAGACGACNNNGTTTCG, GGCCAG- CGAGCTAAACCGAGACNNNGATAT, and GGCCAGCGA-GCTAACCAGACNNNGATCG with a cycle of 3 min at 95°C, followed by 30 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 38°C, 90 sec extension at 72°C and an additional cycle of 3 min final extension at 72°C. Five μl of this PCR reaction was next used as template to perform a second PCR using the primer set GGCCAGCGAGCTAAGACGAC and ACCTTTGGTTCACAAAGGTTT, beginning with a cycle of 5 min denaturation at 95°C following by 30 cycles of 15 sec denaturation at 95°C, 30 sec annealing at 56°C, 90 sec extension at 72°C and a final cycle of 3 min extension at 72°C. PCR products were separated by electrophoresis on a 2% agarose gel. The DNA fragments produced from the *Tn10* insertion mutants but not from the control strain were harvested from the gel. Purified DNA fragments were sequenced using the primer ACCTTTGGTTCACAAAGGTTT.
**Table 1.** Strains and plasmids used in this study.

| Strain or Plasmid | Genotype | Source or reference |
|-------------------|----------|---------------------|
| **Strains**       |          |                     |
| *Salmonella enterica* serovar Typhimurium 14028S | wild type | American Type Culture Collection |
| CA412             | sipC::lacZY | [36] |
| CA2312            | ΔackA sipBCDA::MudI | This study |
| CA2311            | ΔackA sipC::gfp | This study |
| CA1274            | ΔackA sipC::lacZY | This study |
| CA2064            | ΔackA flhD::Tn10 sipC::lacZY | This study |
| CA2123            | ΔackA flhD::Tn10 sipC::lacZY | This study |
| CA2124            | ΔackA flhST::kan sipC::lacZY | This study |
| CA2125            | ΔackA flhS::kan sipC::lacZY | This study |
| CA2126            | ΔackA fliT::kan sipC::lacZY | This study |
| CA2047            | flhD::Tn10 sipC::lacZY | This study |
| CA2060            | fliD::cam | This study |
| CA1854            | fliZ::kan | This study |
| CA2121            | fliD::cam sipC::lacZY | This study |
| CA2122            | fliZ::cam sipC::lacZY | This study |
| **Plasmids**      |          |                     |
| pNK2883           | Plasmid carrying IPTG-inducible Tn10 transposon | [57] |
| pMS421            | Plasmid carrying lacIq | [58] |
| pZEPO7            | Plasmid carrying gfp | [56] |
| pACYC177          | Cloning vector | [61] |
| pFliT (pCA173)    | fliT ORF on pACYC177 | This study |

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**Bacteriophage-mediated transductional mapping**

The bacteriophage P22 was used to map the location of genes by transduction [59]. The donor strain carrying the antibiotic marker inserted in the bacterial chromosome was grown in LB overnight at 37°C with aeration. Four hundred μl of culture was added to 2 ml of P22 phage broth containing 5 × 10^6 pfu/ml of the phage. The mixture was grown overnight at 37°C with aeration, and 6 drops of chloroform was then added to make the phage lysate. The recipient strain, harboring a different antibiotic marker in the chromosome, was grown in LB to mid-log phase, and 10 μl of phage lysate prepared from the donor strain was added to 500 μl of mid-log culture and incubated for 30 minutes at 37°C. Then, 500 μl of 20 mM EGTA was added to the above mixture and incubated at 37°C for 1 hour. Transductants were selected on LB agar with 10 mM EGTA and the antibiotic to which the donor strain was resistant. Fifty resulting colonies were patched onto LB agar with or without the antibiotic to which the recipient strain was resistant to assess the genetic linkage between the two markers.

**β-galactosidase assays**

Triplicate cultures of tested bacterial strains were grown standing overnight at 37°C in LB broth buffered to pH 6.7 with 100 mM MOPS and with 10 mM butyric acid and 100 μg/ml ampicillin if needed. β-galactosidase activity was measured as described previously [60].

**Secreted protein isolation and analysis**

Strains were grown in LB with 100 mM MOPS, pH 6.7, and 100 μg/ml ampicillin at 37°C with shaking at 60 rpm for 16 hours. Proteins secreted into the culture supernatant were prepared and analyzed as previously described [20].

**HEp-2 cell invasion assays**

HEp-2 cells were grown in 24 well plates to confluence (approximately 5 × 10^5 cells) in RPMI-1640 with 10% fetal bovine serum. Bacteria were grown overnight as static cultures in LB with 100 mM MOPS, pH 6.7, and 100 μg/ml ampicillin at 37°C. Approximately 5 × 10^6 bacteria were added to each well. Plates were centrifuged for 10 min at 800 × g and incubated for 1 hour at 37°C. Medium was discarded, and the cells were washed three times with 0.5 ml PBS. One ml of cell culture media supplemented with 20 μg/ml gentamicin was added to each well, and the cells were incubated for 1 hour at 37°C to kill the extracellular bacteria. Medium was removed, and the cells were washed three times with 0.5 ml PBS. Then, 200 μl of 1% Triton X-100 in PBS was added to each well for 5 minutes to lyse the cells, and 800 μl of PBS was added to individual wells to produce a final volume to 1 ml. The bacterial titers of the lysate were determined by colony counts. Each bacterial culture was tested in quadruplicate wells.

**Bacterial swimming activity**

Strains were grown overnight in LB with 100 μg/ml ampicillin at 37°C with shaking at 200 rpm. Ten μl of overnight culture of each strain was dotted onto the LB swimming agar plates (containing 0.35% agar) with 100 μg/ml ampicillin, and incubated at 37°C for 7 hours in a humidified incubator.

**Statistical analysis**

Results from β-galactosidase assays and invasion assays were analyzed using a one-way analysis of variance to determine if the mean of at least one strain or condition differed from any of the others. The Tukey-Kramer HSD multiple comparison test was
then used to determine which means were statistically different. A p-value<0.05 was considered significant. Statistical analysis was performed using Jmp 9.0 software (SAS, Cary, NC).

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Author Contributions

Conceived and designed the experiments: CH CA. Performed the experiments: CH LH. Analyzed the data: CH CA. Contributed reagents/materials/analysis tools: CH. Wrote the paper: CH CA.

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