The transcriptional regulator SsrB is involved in a molecular switch controlling virulence lifestyles of *Salmonella*

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Abstract

The evolution of bacterial pathogenicity, heavily influenced by horizontal gene transfer, provides new virulence factors and regulatory connections that alter bacterial phenotypes. *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) are chromosomal regions that were acquired at different evolutionary times and are essential for *Salmonella* virulence. In the intestine of mammalian hosts, *Salmonella* expresses the SPI-1 genes that mediate its invasion to the gut epithelium. Once inside the cells, *Salmonella* down-regulates the SPI-1 genes and induces the expression of the SPI-2 genes, which favor its intracellular replication. The mechanism by which the invasion machinery is deactivated following successful invasion of host cells is not known. Here, we show that the SPI-2 encoded transcriptional regulator SsrB, which positively controls SPI-2, acts as a dual regulator that represses expression of SPI-1 during intracellular stages of infection. The mechanism of this SPI-1 repression by SsrB was direct and acts upon the *hilD* and *hilA* regulatory genes. The phenotypic effect of this molecular switch activity was a significant reduction in invasion ability of *S. enterica* serovar Typhimurium while promoting the expression of genes required for intracellular survival. During mouse infections, *Salmonella* mutants lacking SsrB had high levels of *hilA* (SPI-1) transcriptional activity whereas introducing a constitutively active SsrB led to significant *hilA* repression. Thus, our results reveal a novel SsrB-mediated mechanism of transcriptional crosstalk between SPI-1 and SPI-2 that helps *Salmonella* transition to the intracellular lifestyle.

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Author summary

*Salmonella* infect humans and a wide range of mammalian hosts. Successful infection requires the bacteria to sense their surroundings and regulate gene expression in a way that maximizes fitness in that particular environment. The two major lifestyles of *Salmonella* include extracellular stages and intracellular stages of host cell infection; however, the molecular mechanisms of how *Salmonella* transitions between these two lifestyles are not completely understood. Here we show that the transcriptional regulator SsrB functions in a dual capacity, activating genes required for intracellular survival while simultaneously repressing genes needed for extracellular stages of infection. Our data highlight how regulatory crosstalk is selective during infection, presumably because it helps facilitate rapid transitions in bacterial lifestyles that ultimately promote bacterial survival and replication.

Introduction

All organisms carefully regulate gene expression to ensure correct spatiotemporal deployment of gene products. For bacterial pathogens that reside in multiple niches, a mechanism to coordinate gene expression with environmental sensing is crucial for their ability to cause disease. This is achieved largely by two-component regulatory systems that sense external surroundings using a membrane sensor kinase that signals to a cytosolic response regulator that directs a transcriptional response [1].

In *Salmonella*, many of virulence genes required for infection are found in horizontally acquired pathogenicity islands [2]. *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) were acquired at different evolutionary times and have key roles in *Salmonella* virulence [3, 4]. Both SPI-1 and SPI-2 encode a type III secretion system (T3SS), effector proteins, chaperones, and transcriptional regulators that control the expression of the genes within each of the SPIs [3, 5]. The SPI-1-encoded T3SS (T3SS-1) and effector proteins mediate *Salmonella* invasion of host cells leading to gastroenteritis [3, 4]. Following invasion, the genes within SPI-2 are required for *Salmonella* survival and replication within its intracellular niche, the *Salmonella*-containing vacuole (SCV). The ability of *Salmonella* to replicate inside macrophages allows for dissemination, leading to systemic disease in susceptible hosts [3, 4].

Consistent with their function, the SPI-1 genes are expressed when *Salmonella* is in the intestinal lumen or associated with the epithelium [6]. SPI-1 is also expressed in a subpopulation of bacteria that replicates in the cytosol of cultured epithelial cells [7]. The SPI-2 genes are mainly expressed when *Salmonella* is inside the SCV of epithelial cells and macrophages [7–11]. *In vitro*, SPI-1 genes are expressed when *Salmonella* is grown to early stationary phase in nutrient-rich lysogeny broth (LB), whereas SPI-2 genes are expressed when *Salmonella* is grown to late stationary phase in LB or in acidic minimal media containing micromolar concentrations of phosphate and magnesium ions [12–14].

A transcriptional regulatory cascade comprised of HilD, HilA and InvF, positively controls the expression of the SPI-1 genes as well as several other genes outside this island that are required for *Salmonella* invasion of host cells [3, 15–17]. When *Salmonella* is grown to late stationary phase in LB, HilD mediates transition of the gene expression program from SPI-1 to SPI-2 through activation of the SsrA-SsrB two-component system, a master regulator of SPI-2 genes [14]. In response to chemical cues detected inside host cells, the SsrA sensor kinase (also called SpiR) phosphorylates the SsrB response regulator leading to the activation of the genes found within SPI-2 and in other regions of the genome [3, 18, 19]. SsrB binds to a degenerate
A+T-rich 18-bp palindrome sequence [20], probably making few base contacts; however, the exact mechanism by which SsrB interacts with DNA may vary from gene to gene [21].

The mechanism by which the invasion machinery is repressed following invasion of host cells is not known. Here, we report that SsrB represses the expression of SPI-1 genes directly by acting on the hilD and hilA regulatory genes. Following invasion of macrophage cells SsrB represses expression of the invasion machinery encoded in the SPI-1 genes, while activating expression of the SPI-2 genes needed for intracellular survival. Consistent with this model, Salmonella mutants lacking SsrB had high levels of hilA transcriptional activity during mouse infections, whereas introducing a constitutively active SsrB led to significant hilA repression in vivo. Thus, our results reveal a regulatory switch activity for SsrB that helps Salmonella transition to the intracellular environment.

Results

SsrB represses the expression of SPI-1 genes

In a previous study we showed that SPI-1 and SPI-2 genes are expressed during early and late stationary phase, respectively, when S. Typhimurium is grown in LB [14]. Interestingly, the expression of SsrB during late stationary phase coincided with down-regulation of the SPI-1 regulator HilA [14]. To investigate the mechanisms controlling this regulation, we examined the chromosomal expression of InvF-FLAG by Western blot in a wild-type (WT) S. Typhimurium strain that constitutively expresses SsrB from the pK3-SsrB plasmid, or a strain containing the vector control pMPM-K3. InvF is a SPI-1 regulator whose expression is dependent on HilA [3]. The chromosomal expression of SsrB-FLAG was also assessed as a control in the strain containing pMPM-K3. As expected, in the presence of the vector pMPM-K3 the protein level of InvF-FLAG was maximal in early stationary phase and decreased during late stationary phase, whereas expression of SsrB-FLAG was induced only during late stationary phase (Fig 1). In contrast, in the presence of the pK3-SsrB plasmid InvF-FLAG was not detected at any of the time points tested (Fig 1), indicating that SsrB expression leads to InvF repression. To examine the broader impact of SsrB on SPI-1, we determined the effect of SsrB on the effector secretion profile in WT S. Typhimurium grown in LB. Consistent with the results with InvF, in cells constitutively expressing SsrB there was reduced secretion of the SPI-1-encoded effectors SipA, SipB, SipC and SipD, as well as the flagellar protein FliC, in the culture supernatants (Fig 2A). Similar results were obtained using a S. Typhimurium ΔSPI-2 mutant (Fig 2A), indicating that the repressing effect of SsrB on the secretion of SipA-D and FliC proteins does not require any other SPI-2-encoded factor. Together, these results show that SsrB represses the expression of the SPI-1 and flagellar genes.

Expression of SsrB decreases S. Typhimurium invasion of HeLa cells

Invasion of Salmonella into host cells requires the cellular functions encoded in both the SPI-1 and flagellar genes [3, 22, 23]. Thus, we used gentamicin protection assays to determine whether SsrB-mediated repression of the SPI-1 and flagellar genes had a phenotypic consequence on bacterial invasion. HeLa cells were infected with WT S. Typhimurium containing the pK3-SsrB plasmid or the pMPM-K3 vector and the number of intracellular bacteria was determined 1 h post-infection. S. Typhimurium ΔhilD and ΔflhDC mutants, lacking master positive regulators for the SPI-1 and flagellar genes, respectively, were used as controls. The constitutive expression of SsrB from pK3-SsrB resulted in a 500-fold reduction in invasion (Fig 2B). As expected, the ΔhilD and ΔflhDCmutants also showed a very strong reduction in invasion (Fig 2B). These results show that constitutive expression of SsrB negatively affects Salmonella invasion of HeLa cells, consistent with its ability to repress SPI-1 and flagellar genes.
SsrB represses the SPI-1 regulatory cascade

The SPI-1-encoded regulators HilD, HilA and InvF positively control the expression of the genes within this island in a cascade fashion, where HilD induces the expression of HilA and it, in turn, activates the expression of InvF [3, 24]. To investigate how SsrB represses the SPI-1 genes, we analyzed the effect of constitutive SsrB expression on the transcription of *hilD*, *hilA* and *invF*, using *cat* transcriptional fusions. As controls for these assays the expression of *sirA* and *csrA*, which are found outside SPI-1 and encode known regulators of the SPI-1 genes, and *ssaG*, a SPI-2 gene whose expression is dependent on SsrB [3, 24], was also tested using *cat* transcriptional fusions. Constitutive expression of SsrB from pK3-SsrB nearly abolished the expression of the *hilD-cat* and *hilA-cat* fusions, whereas in the presence of the pMPM-K3 vector its expression was only induced during late stationary phase (Fig 3D).

SsrB represses the SPI-1 encoded regulator InvF

Expression of InvF-FLAG and SsrB-FLAG in the WT *S. Typhimurium* strain containing the plasmid pK3-SsrB expressing SsrB from a constitutive promoter, or the vector pMPM-K3, was analyzed by Western blot using monoclonal anti-FLAG antibodies. Whole cell lysates were prepared from samples of bacterial cultures grown in LB at 37˚C, at the OD$_{600}$ or the time indicated, representing exponential, early stationary or late stationary phases of growth. As a loading control, the expression of DnaK was also determined using monoclonal anti-DnaK antibodies.

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**Fig 1.** SsrB represses the expression of the SPI-1-encoded regulator InvF. Expression of InvF-FLAG and SsrB-FLAG in the WT *S. Typhimurium* strain containing the plasmid pK3-SsrB expressing SsrB from a constitutive promoter, or the vector pMPM-K3, was analyzed by Western blot using monoclonal anti-FLAG antibodies. Whole cell lysates were prepared from samples of bacterial cultures grown in LB at 37˚C, at the OD$_{600}$ or the time indicated, representing exponential, early stationary or late stationary phases of growth. As a loading control, the expression of DnaK was also determined using monoclonal anti-DnaK antibodies.
Together, these results demonstrate that SsrB represses the transcription of the SPI-1 regulatory genes *hilD*, *hilA* and *invF*.

**SsrB directly represses *hilD* and *hilA***

To determine whether SsrB directly or indirectly represses the expression of *hilD*, *hilA*, and *invF*, we analyzed the interaction of SsrB with the regulatory regions of these genes by electrophoretic mobility shift assays (EMSAs). Full-length SsrB is unstable in solution, but the C-terminal DNA binding domain (6H-SsrBc) is stable and can specifically bind to promoter regions of SsrB-regulated genes [18, 25]. Therefore, purified 6H-SsrBc and the DNA fragments of each gene contained in the *hilD-* and *hilA-*cat fusions were used in these assays. 6H-SsrBc bound to the DNA fragments of *hilD* and *hilA* (Fig 3E and 3F) but did not bind to the DNA fragment of *ssaG*, which was used as a positive control (Fig 3H) but it did not shift those of the *sirA* or *csrA* negative controls (S1C and S1D Fig). These results show that SsrB specifically binds to the regulatory regions of *hilD* and *hilA*.

Previous work has identified a conserved yet flexible 18 bp palindrome sequence that defines the SsrB binding sequence based on a position-specific scoring matrix [20]. Scanning with this sequence (Fig 4A) identified two putative SsrB-binding sites in the regulatory region of *hilD* and nine within the *hilA* regulatory region. Interestingly, the two putative SsrB-binding sites near *hilD* are located in the promoter, whereas in *hilA* one putative SsrB-binding site is located upstream of the promoter, overlapping a HilD-binding site, and the others are located far upstream or downstream of the promoter (Fig 4B).
To determine whether SsrB represses hilD through these two putative SsrB-binding sites, three different cat transcriptional fusions were constructed, each with distinct 5’ and 3’ deletions of the hilD-cat-364+88 fusion that showed repression by SsrB (Fig 4B). The fusions (named according to the 5’ and 3’ positions of the hilD DNA fragment with respect to its transcriptional start site) hilD-cat-108+88, hilD-cat-48+88 and hilD-cat-37+6 were tested for CAT-specific activity in the presence of pK3-SsrB or the vector pMPM-K3. Positive autoregulation of hilD is not essential for its expression [26], therefore, the hilD-cat-48+88 and hilD-cat-37+6 fusions that lack the HilD-binding site upstream of hilD, were expected to be expressed. In the presence of pMPM-K3, hilD-cat-108+88 reported expression levels similar to those from hilD-cat-364+88 (compare Figs 5A and 3A), indicating that the cis-acting elements required for maximal expression of hilD are located between positions -108 to +88. In contrast, the expression of hilD-cat-48+88 decreased by 50% relative to hilD-cat-108+88 (Fig 5A and 5B), which is consistent with the reduction in hilD expression seen in the absence of autoregulation [26]. Interestingly, the hilD-cat-37+6 fusion that contains only the promoter of hilD was activated to similar levels as the hilD-cat-108+88 fusion (Fig 5A and 5C), demonstrating that in the absence of autoregulation, SsrB can repress hilD through these sites.
of negative regulatory sequences between positions +6 to +88, the autoregulation is not required for maximal expression of \textit{hilD}. Notably, the presence of pK3-SsrB significantly reduced the expression of \textit{hilD-cat-108+88}, \textit{hilD-cat-48+88} and \textit{hilD-cat-37+6} (Fig 5A, 5B and 5C), indicating that SsrB negatively acts on the \textit{hilD} promoter. EMSAs were performed to confirm that SsrB directly regulates the promoter of \textit{hilD}. The \textit{hilD} DNA fragments contained in \textit{hilD-cat-108+88}, \textit{hilD-cat-48+88} and \textit{hilD-cat-37+6}, shifted in the presence of increasing concentrations of 6H-SsrBc (Fig 5D, 5E and 5F), indicating that SsrB binds to the promoter located between position -37 to +6 relative to the transcriptional start site of \textit{hilD}, which is consistent with our bioinformatics analysis revealing two putative SsrB-binding sites on this region (Fig 4B). These results show that SsrB binds to the promoter of \textit{hilD} and thus would repress its transcription.

To determine whether SsrB mediates repression of \textit{hilA} at any of the SsrB-binding sites we predicted bioinformatically, four different \textit{hilA-cat} transcriptional fusions were constructed.
that have 5' or 3' deletions (or both) with respect to the *hilA-cat-410+446* fusion that showed repression by SsrB (Fig 4B). The fusions (named according to the 5' and 3' positions of the *hilA* DNA fragment with respect to its transcriptional start site) *hilA-cat-410+66*, *hilA-cat-100+6*, *hilA-cat-35+6* and *hilA-cat-35+446* were tested for CAT-specific activity in the presence of pK3-SsrB or the pMPM-K3 vector. Previously, it was shown that sequences flanking the promoter repress *hilA* and in the absence of the sequence upstream or downstream of the promoter, *hilA* was expressed independently of HilD [27–29]. Therefore, *hilA-cat-410+66*, *hilA-cat-100+6*, *hilA-cat-35+6* and *hilA-cat-35+446*, which lack the repressing sequences, were expected to be expressed at high levels, regardless of whether they contain the HilD binding sites or not. As expected, in the presence of the pMPM-K3 vector, *hilA-cat-410+66*, *hilA-cat-100+6* and *hilA-cat-35+6* were expressed at higher levels than *hilA-cat-410+446* (Fig 6A, 6B and 6C and Fig 3B). In contrast, the *hilA-cat-35+446* fusion, which lacks the sequence upstream of the promoter, up to position +446, required HilD. Notably, the presence of pK3-SsrB reduced the expression of *hilA-cat-410+66* and *hilA-cat-100+6*, but it did not affect the activity of *hilA-cat-35+6* and *hilA-cat-35+446* (Fig 6A, 6B, 6C and 6D), suggesting that SsrB mediates repression of *hilA* by acting on the region between -100 to -35. Expression analysis of *hilA-lux-740+*
The transcriptional fusions further indicated that this -100 to -35 region is needed for the SsrB-mediated repression of *hilA* (S2A and S2B Fig).

To determine whether SsrB physically interacts with this region of *hilA* we used EMSAs with purified 6H-SsrBc. 6H-SsrBc shifted the *hilA* DNA fragments contained in *hilA-cat-410+66* and *hilA-cat-100+6*, but not those contained in *hilA-cat-35+6* and *hilA-cat-35+446* (Fig 6E, 6F, 6G and 6H), indicating that SsrB binds between positions -100 to -35. These results are consistent with our bioinformatics analysis that predicted a SsrB-binding site in this region, centered at position -70, overlapping a HilD-binding site (Fig 4B). To determine whether SsrB mediates direct repression of *hilA* at this site, we mutated this site in the *hilA-cat-100+6* fusion by substituting five nucleotides within the predicted SsrB-binding site (Fig 7). The expression of the WT *hilA-cat-100+6* and mutated *hilA-cat-100+6* fusions was tested in WT *S. Typhimurium* containing pK3-SsrB or the vector control pMPM-K3. Constitutive expression of SsrB from pK3-SsrB drastically reduced the expression of the WT *hilA-cat-100+6* reporter but only slightly affected the activity of the mutated *hilA-cat-100+6* fusion (Fig 7A and 7C). Moreover, EMSAs showed that 6H-SsrBc binds to the *hilA* DNA fragment contained in WT *hilA-cat-100+6*, but does not bind to the *hilA-cat-100+6* fragment containing the mutated SsrB-binding site.
site (Fig 7E and 7F). Interestingly, the mutations we created within the hilA-cat-100+6 fusion also affected the regulation and binding of HilD on hilA (S3A, S3B, S3C and S3D Fig). These results show that SsrB represses hilA by binding to the site centered at position -70 that overlaps a HilD-binding site, which suggested that SsrB inhibits the HilD-mediated expression of hilA. To test this, the expression of the WT hilA-cat-100+6 and mutated hilA-cat-100+6 fusions was tested in a S. Typhimurium ΔSPI-1 ΔrtsA ΔCthns triple mutant containing pK3-SsrB or the vector pMPM-K3. This mutant lacks HilD, HilC, RtsA and the other transcriptional regulators encoded in SPI-1, as well as H-NS. The CAT-specific activity was determined from bacterial cultures grown for 9 h in LB at 37°C. Data represents the mean with standard deviation of three independent experiments. *Statistically different values relative to the WT strain containing the pMPM-K3 vector, P < 0.0005. The WT and mutated SsrB-binding sequence are indicated; the nucleotides that were changed in the mutated sequence are underlined.

EMSAs were performed to analyze the interaction of SsrB with the hilA DNA fragments carried by the hilA-cat-100+6WT (E) and hilA-cat-100+6 Mut (F) fusions. The DNA fragments were incubated with increasing concentrations of purified 6H-SsrBc (0, 0.5, 1, 1.5 and 2 μM). DNA-protein complexes are indicated by an asterisk.

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100+6 fusions in the ΔSPI-1 ΔrtsA ΔCthns mutant (Fig 7B and 7D), which further indicates that SsrB inhibits the HilD-mediated expression of hilA. Taken together, these results strongly support that SsrB represses the expression of hilA by preventing HilD from binding. SsrB can also repress hilA through an indirect mechanism by negatively regulating the expression of hilD.

Notably, the hilD and hilA promoter sequences contained in the hilD-cat-37+6 (directly repressed by SsrB) and hilA-cat-35+6 (not repressed by SsrB) fusions, respectively, are 65% identical (S4 Fig); thus, only 15 different positions between these sequences determine binding and thus negative regulation of SsrB on the hilD promoter, but not on the hilA promoter.

SsrB simultaneously represses SPI-1 and activates SPI-2 inside RAW264.7 mouse macrophages

Our results described above indicate that SsrB represses the expression of SPI-1 genes while activating expression of SPI-2 genes. In different in vitro SPI-2-inducing growth conditions that we have tested, invF was not de-repressed in the absence of SsrB (S5A and S5B Fig), consistent with the results from a previous study [17]. Thus, detection of specific environmental cues could be required for the repression of SPI-1 by SsrB in physiological conditions, which could occur during Salmonella infection of hosts. SPI-1 and SPI-2 are known to be inversely regulated when Salmonella is within macrophages [9–11, 31], an environment where SsrB is active [3]. To explore whether SsrB is involved in this inverse regulation during intracellular stages of infection, we analyzed the expression of invF (SPI-1) and ssaG (SPI-2) in WT bacteria and in bacteria lacking SsrB following macrophage infection. For this, transcriptional fusions of invF (SPI-1) and ssaG (SPI-2) to the luciferase operon (lux) were constructed in the pCS26-Pac vector. A lux transcriptional fusion of hns, a gene constitutively expressed, was also constructed as a control.

RAW264.7 macrophages were infected with WT S. Typhimurium or its isogenic ΔSPI-2 mutant carrying the invF-lux, ssaG-lux or hns-lux fusions. At specific time points after infection the macrophages were lysed and luminescence was measured and normalized to the number of viable intracellular bacteria. As expected, the intracellular replication of the WT strain increased over time whereas the ΔSPI-2 mutant decreased (S6 Fig). The intracellular expression of invF-lux and ssaG-lux also changed as expected in the WT strain, where invF expression decreased fifteen-fold by the last time point and ssaG expression increased the same magnitude over the course of the infection (Fig 8A and 8B). When comparing the expression levels of invF-lux between the WT strain and the ΔSPI-2 mutant, two distinct stages were identified. At 1 and 4 h post-infection, the invF-lux fusion showed similar expression levels in the WT strain and the ΔSPI-2 mutant, including a decrease in expression at 4 h (Fig 8A). However, at later time points in the infection, invF-lux expression levels continued to decrease in the WT strain, by two to nine-fold, but not in the ΔSPI-2 mutant (Fig 8A and 8B). This revealed SsrB-dependent repression of invF during intracellular infection. Furthermore, the hns-lux transcriptional fusion showed similar levels of intracellular expression in the WT and ΔSPI-2 strains at all time points of the infection (S7 Fig). Thus, the differences in the intracellular expression levels shown by the invF-lux fusion in the WT strain and its derivative ΔSPI-2 mutant were not due to the different levels of intracellular bacteria at these time points. On the other hand, only background activity was detected for the ssaG-lux fusion in the ΔSPI-2 mutant (Fig 8B), consistent with its expression being dependent on SsrB [3]. Interestingly, de-repression of the invF-lux intracellular expression in the ΔSPI-2 mutant coincided with the timing of induction of the ssaG-lux intracellular expression in the WT strain (Fig 8A and 8B). As expected, the de-repression of the invF-lux intracellular expression was also evident in a ΔssrA and a ΔssrB mutant, whereas the
expression of the hns-lux control fusion was similar in the WT strain and these two mutants (S8 Fig), which indicates that both the SsrA sensor kinase and SsrB response regulator are required for intracellular repression of invF and that no other SPI-2-encoded factors are required.

Together, these results show that SsrB simultaneously represses and induces the expression of invF and ssaG, respectively, inside macrophages (Fig 8D). Therefore, our data support that SsrB is involved in a regulatory switch that helps to coordinate the intracellular reprogramming of Salmonella genes, by activating the genetic program required for intracellular survival while de-activating the genes involved in the now-completed invasion step of infection.

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SsrB negatively regulates SPI-1 during mouse infections

To determine whether SsrB represses expression of SPI-1 during mouse infections, we tested the hilA-lux-740+350 transcriptional fusion in the WT S. Typhimurium strain, its isogenic ΔssrB mutant, and in the ΔssrB mutant complemented with a constitutive active SsrB variant in which aspartic acid 56 was replaced with glutamic acid. This SsrB D56E variant was expressed from the native ssrA promoter (PssrA-ssrB D56E). C57BL/6 mice were orally gavaged with these strains and luminescence was quantified by in vivo imaging every h for 6 h post-infection. Expression of the hilA-lux fusion was greater in the ΔssrB mutant than in the WT strain at the different times tested, which was evident by quantification of total abdominal luminescence (Fig 9). The presence of SsrB D56E reduced the expression of the hilA-lux fusion in the ΔssrB mutant (Fig 9). These results show that SsrB negatively regulates SPI-1 during the course of the intestinal infection of S. Typhimurium in a mouse model.

Discussion

Salmonella has developed a complex regulatory network to express virulence genes in a highly coordinated manner within particular host niches. For example, when Salmonella is inside macrophages, it down-regulates the SPI-1 invasion machinery and flagellar-based motility genes that are required for host-cell invasion, whereas the expression of the SPI-2 genes required for intracellular survival and replication is activated [9–11, 31]. Previously, the mechanism responsible for repressing the genes involved in invasion following successful invasion events was not known. Here, we show that this mechanism involves the SsrB response regulator, which had previously known roles in activating genes required for intracellular survival. Our data support a model in which SsrB acts as a key component of the molecular switch that helps Salmonella transition from an extracellular to an intracellular lifestyle (Fig 10). Interestingly, in a previous study it was demonstrated that SsrB, in its unphosphorylated form, drives a Salmonella lifestyle switch by relieving biofilm silencing [58].

Recent transcriptomics and proteomics data support that SsrB represses the expression of the SPI-1 and flagellar associated genes in in vitro SPI-2-inducing growth conditions [17, 32], and that it represses the flagellar genes when S. Typhimurium is inside macrophages [17]. In S. Typhi, a human-restricted serovar that causes systemic infections, the transcriptional regulator TviA represses the expression of the SPI-1 and flagellar genes and reduces macrophage pyroptosis [33–36]; pyroptosis and apoptosis are programmed cell death pathways stimulated by SPI-1 and flagellar gene products [37–39]. Interestingly, S. Typhimurium lacks the TviA regulator, which implied the existence of a different pathway in non-typhoidal serovars of Salmonella. The SsrB-mediated repression of the SPI-1 and flagellar genes in S. Typhimurium might be important in order to limit pyroptosis and apoptosis following infection by this serotype. Although we have not yet examined the impact of SsrB-mediated repression of invasion genes on these host cell pathways, the mechanism uncovered here may serve to limit damage to host cells as Salmonella establishes a stable intracellular niche.

Our data strongly support a mechanism whereby SsrB represses the SPI-1 genes by directly acting on the hilD and hilA regulatory genes. The direct binding of SsrB to the promoter of hilD may be preventing RNA polymerase from binding to this region. In addition to reducing the levels of HilD, SsrB-binding to the sequence centered at position -70 of hilA, overlapping a HilD-binding site, inhibits the HilD-mediated expression of hilA. These findings provide further insight on the SsrB regulon, and demonstrate how SsrB can act as a negative transcriptional regulator, in addition to its well-known role as a transcriptional activator. Moreover, previous studies indicate that the regulation of the SPI-1 genes mostly involves the control of hilD at the post-transcriptional and post-translational level [3, 24, 40]. Our results reveal
Fig 9. SsrB represses hilA in vivo. Mice were orally gavaged with the indicated strains and luciferase activity expressed from the hilA-lux-740+350 fusion was measured by live animal imaging. Images are
another pathway for the regulation of SPI-1 that involves repression of hilD and hilA at the transcriptional level.

In *Escherichia coli*, the EnvZ-OmpR two-component system responds to osmotic stress signals [41]. The inverse regulation of the SPI-1 and SPI-2 genes by SsrB resembles the reciprocal control of *ompC* and *ompF* transcription by OmpR. OmpR is known to directly activate expression of *ssrA*-ssrB, and repress the expression of *hilD* [42, 43]. In addition to OmpR, other regulators, such as SlyA and PhoP, also positively and negatively control the expression of SPI-1.

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Fig 10. SsrB is involved in a molecular regulatory switch that aids in *Salmonella* transition to an intracellular lifestyle. (A) HilD directly or indirectly activates the expression of the SPI-1 genes and several other genes located outside SPI-1, including the flagellar regulatory operon *flhDC* required for the invasion of host cells. (B) Following its uptake into macrophages, *Salmonella* resides inside vacuoles, where SsrB induces the expression of the SPI-2 genes and other genes located outside SPI-2, which are required for survival and replication, while simultaneously repressing the expression of the *hilD* and *hilA* SPI-1 regulatory genes, and the flagellar-based motility genes. Green arrows and red blunt-end lines indicate positive and negative control, respectively, whereas gray dashed arrows denote expression of the respective genes.

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2 and SPI-1 genes, respectively [3, 11, 32, 43, 44]. Notably, OmpR, SlyA and PhoP positively control the expression of SsrB [3]. Therefore, these regulators may provide additional input into the SsrB-dependent or independent mechanisms that inversely regulates the expression of the SPI-1 and SPI-2 genes within macrophages.

In a previous study, we found that HilD mediates transcriptional crosstalk between SPI-1 and SPI-2 when *S. Typhimurium* is grown in LB, through growth-phase dependent activation of HilA and SsrB [14]. Here, we demonstrate that SsrB represses the expression of HilD and HilA, and thus the SPI-1 genes, revealing that the transcriptional communication between SPI-1 and SPI-2 is bi-directional. The degenerate palindromic sequence motif that SsrB recognizes on DNA [20] may make this response regulator particularly suited to dual-level control of gene expression. For example, the flexibility in the SsrB binding site may allow the bacterium to sample a wide array of new regulatory connections that can then be further optimized and selected by *cis*-regulatory evolution.

**Materials and methods**

**Ethics statement**

Animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care, using protocols approved by the Animal Review Ethics Board at McMaster University under Animal Use Protocol #13-07-20.

**Media and culture conditions**

Bacterial cultures were grown at 37°C in LB containing 1% tryptone, 0.5% yeast agar and 1% NaCl, pH 7.5; in N-minimal medium (N-MM) containing 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1mM KH₂PO₄, 100 mM Tris-HCl (pH 7.5), 10 μM MgCl₂ and 0.1% casamino acids; or in phosphate-carbon-nitrogen (PCN) minimal medium containing 80 mM MES (pH 5.8), 4 mM Tricine, 100 μM FeCl₃, 376 μM K₂SO₄, 50 mM NaCl, 0.4 mM K₂HPO₄/KH₂PO₄ (pH 5.8), 0.4% glucose, 15 mM NH₄Cl, 1 mM MgSO₄, 10 μM CaCl₂ and micronutrients (10 nM Na₂MoO₄, 10 nM Na₂SeO₃, 4 mM H₃BO₃, 300 nM CoCl₂, 100 nM CuSO₄, 800 nM MnCl₂, 1 mM ZnSO₄). When necessary, media were supplemented with ampicillin (200 μg ml⁻¹), kanamycin (30 μg ml⁻¹) or streptomycin (100 μg ml⁻¹). Cultures in LB, N-MM or PCN media for chloramphenicol acetyltransferase (CAT) or Western blot assays were performed as described previously [12, 14, 45]. Briefly, overnight cultures of the *Salmonella* strains were sub-cultured (1:50) into 50 ml of fresh medium contained in 250 ml flaks, which were incubated at 37°C with shaking (200 r.p.m.) in an Orbital shaker bath (GYROMAX 902, Amerex Instruments), during the indicated times.

**Construction of mutant strains and strains expressing FLAG-tagged proteins**

Bacterial strains used in this work are listed in Table 1. Deletion of *rtsA* in *S. Typhimurium* SL1344 was performed by the λ Red recombinase system, as described previously [46], using the primers shown in Table 2, generating the strain DTM91. P22 transduction was used to transfer the *invF::3XFLAG-kan* allele from strain DTM76 into *S. Typhimurium* SL1344, generating the strain DTM85, to transfer the ΔssrB::kan allele from the strain MJW112 into the strain DTM86, generating the strain DTM87, to transfer the ΔSPI-1::kan allele from the strain ΔSPI-1 into DTM92, generating the strain DTM93, to transfer the ΔCthns::kan allele from the strain DTM84 into the strain DTM94, generating the strain DTM95, to transfer the ΔssrB::kan allele from the strain 4/74 ΔssrB into *S. Typhimurium* SL1344, generating the strain DTM97,
Table 1. Bacterial strains and plasmids.

| Name                  | Genotype                                    | Reference          |
|-----------------------|---------------------------------------------|--------------------|
| **Bacterial strains** |                                             |                    |
| S. Typhimurium        | Wild type; xyl, hisG, rpsL; Sm<sup>R</sup>   | [53]               |
| SL1344                |                                             |                    |
| MJW112                | ΔssrB::kan                                   | M. Worley and F. Heffron |
| ΔSPI-2                | ΔSPI-2::kan                                   | [54]               |
| JPTM25                | ΔhilD                                        | [45]               |
| JPTM30                | ssrB::3XFLAG-kan                              | [45]               |
| DTM76                 | 14028s invF::3XFLAG-kan                      | [26]               |
| DTM84                 | 14028s ΔhilD ΔCthns::kan                     | [26]               |
| DTM85                 | invF::3XFLAG-kan                              | This study         |
| DTM86                 | invF::3XFLAG-kan                              | This study         |
| DTM87                 | ΔssrB::kan invF::3XFLAG                       | This study         |
| DTM88                 | ΔhilDC::kan                                   | [55]               |
| DTM89                 | ΔSPI-2                                       | This study         |
| DTM90                 | ssrB::3XFLAG-kan                              | This study         |
| ΔSPI-1                | ΔSPI-1::kan                                   | [31]               |
| DTM91                 | ΔrtsA::kan                                    | This study         |
| DTM92                 | ΔrtsA                                        | This study         |
| DTM93                 | ΔrtsAΔSPI-1::kan                              | This study         |
| DTM94                 | ΔrtsAΔSPI-1                                   | This study         |
| DTM95                 | ΔrtsAΔSPI-1 ΔCthns::kan                       | This study         |
| DTM96                 | ΔrtsAΔSPI-1 ΔCthns                           | This study         |
| 4/74 ΔssrB            | ΔssrB::kan                                   | [32]               |
| 4/74 ΔssrA            | ΔssrA::kan                                    | [32]               |
| DTM97                 | ΔssrB::kan                                    | This study         |
| DTM98                 | ΔssrA::kan                                    | This study         |
| DTM99                 | ΔssrB                                        | This study         |
| DTM100                | ΔssrA                                        | This study         |
| **E. coli**           |                                             |                    |
| BL21/DE3              | Strain for expression of recombinant proteins| Invitrogen         |
| DH10β                 | Laboratory strain                            | Invitrogen         |
| **Plasmids**          |                                             |                    |
| pKK232-8              | pBR322 derivative containing a promotorless chloramphenicol acetyltransferase (cat) gene, Ap<sup>R</sup> | [56]               |
| phiD-cat-364+88       | pKK232-8 derivative containing a hilD-cat transcriptional fusion from nucleotides -364 to +88 | [14]               |
| phiD-cat-108+88       | pKK232-8 derivative containing a hilD-cat transcriptional fusion from nucleotides -108 to +88 | This study         |
| phiD-cat-48+88        | pKK232-8 derivative containing a hilD-cat transcriptional fusion from nucleotides -48 to +88 | This study         |
| phiD-cat-37+6         | pKK232-8 derivative containing a hilD-cat transcriptional fusion from nucleotides -37 to +6 | This study         |
| phiA-cat-410+446      | pKK232-8 derivative containing a hilA-cat transcriptional fusion from nucleotides -410 to +446 | [14]               |
| phiA-cat-410+66       | pKK232-8 derivative containing a hilA-cat transcriptional fusion from nucleotides -410 to +66 | This study         |
| phiA-cat-100+6        | pKK232-8 derivative containing a hilA-cat transcriptional fusion from nucleotides -100 to +6 | This study         |
| phiA-cat-100+6 Mut    | hilA-cat-100+6 transcriptional fusion carrying mutations in the SsrB binding site | This study         |
| phiA-cat-35+6         | pKK232-8 derivative containing a hilA-cat transcriptional fusion from nucleotides -35 to +6 | This study         |
| phiA-cat-35+446       | pKK232-8 derivative containing a hilA-cat transcriptional fusion from nucleotides -35 to +446 | This study         |
| pinvF-cat             | pKK232-8 derivative containing a invF-cat transcriptional fusion from nucleotides -306 to +231 | [14]               |
| pssaG-cat             | pKK232-8 derivative containing a ssaG-cat transcriptional fusion from nucleotides -303 to +361 | [14]               |
| psrA-cat              | pKK232-8 derivative containing a srrA-cat transcriptional fusion from nucleotides -563 to +98 | [45]               |
| pcsrA-cat             | pKK232-8 derivative containing a csrA-cat transcriptional fusion from nucleotides -327 to +61 | [45]               |

(Continued)
and to transfer the ΔssrA::kan allele from the strain 4/74 ΔssrA into S. Typhimurium SL1344, generating the strain DTM98. The kanamycin resistance cassette was excised from the strains DTM85, ΔSPI-2::kan, JPTM30, DTM91, DTM93, DTM95, DTM97 and DTM98, by using helper plasmid pCP20 expressing the FLP recombinase, as described previously [46], generating the strains DTM86, DTM89, DTM90, DTM92, DTM94, DTM96, DTM99 and DTM100, respectively. All mutant strains were verified by PCR amplification and sequencing.

Construction of plasmids

Plasmids and primers used in this work are listed in Tables 1 and 2, respectively. To construct the plasmids containing the transcriptional fusions hilD-cat-108+88, hilD-cat-48+88, hilA-cat-410+66, hilA-cat-100+6, hilA-cat-35+446 and fliC-cat, the respective segment of the regulatory region of hilD, hilA or fliC were amplified by PCR with the primer pairs hilD-108FW/hilDRHindIII(rv), hilD-48FW/hilDRHindIII(rv), hilA1FBam(fw)/hilA+6Hind(rv), hilA-100Ba(fw)/hilA+6Hind(rv), hilA-100MutBamH(fw)/hilA+6Hind(rv), hilA+66FB(fw)/hilA2RHind(rv) or fliC-RVI-BH/fliC-FWI-Hd. The PCR products were digested with BamHI and HindIII restriction enzymes and then cloned into the BamHI and HindIII sites of the vector pKK232-8, which carries a promotorless cat gene (Amersham Pharmacia Biotechnology), generating plasmids philD-cat-108+88, philD-cat-48+88, philA-cat-410+66, philA-cat-100+6, philA-cat-35+446 and philC-cat. To construct the plasmids containing the transcriptional fusions hilD-cat-37+6 and hilA-cat-35+6, the complementary primers hilDPF-Bam(fw) and hilDPRHind(rv) or hilAPFBam(fw) and hilAPRHind(rv), each at a final concentration of 50 μM, were annealed by heating them together at 94°C for 10 min and then slowly cooling to room temperature. The obtained double-strand products carried cohesive ends for their cloning into the BamHI and HindIII sites of the vector pKK232-8, generating plasmids philD-cat-37+6 and philA-cat-35+6. To construct the plasmids containing the transcriptional fusions invF-lux-306+231, ssaG-lux-303+361 and hns-lux-967+73, the respective segment of Table 1. (Continued)

| Name           | Genotype                                      | Reference       |
|----------------|-----------------------------------------------|-----------------|
| pfliC-cat      | pKK232-8 derivative containing a fliC-cat transcriptional fusion from nucleotides -220 to +160 | This study      |
| pCS26-Pac      | pZS derivative containing a promoterless luxCDABE operon, KanR | [47]            |
| pinvF-lux      | pCS26-Pac derivative containing an invF-lux transcriptional fusion from nucleotides -306 to +231 | This study      |
| pssaG-lux      | pCS26-Pac derivative containing a ssaG-lux transcriptional fusion from nucleotides -303 to +361 | This study      |
| phns-lux       | pCS26-Pac derivative containing a hns-lux transcriptional fusion from nucleotides -967 to +73 | This study      |
| pGEN-luxCDABE  | p15A derivative low-copy-number plasmid carrying the luxCDABE operon downstream the constitutive em7 synthetic promoter, ApR | [48]            |
| philA-lux-740+350 | pGEN-luxCDABE derivative containing a hilA-lux transcriptional fusion from nucleotides -740 to +350 | This study      |
| philA-lux-36+446 | pGEN-luxCDABE derivative containing a hilA-lux transcriptional fusion from nucleotides -36 to +446 | This study      |
| pCP20         | Plasmid expressing FLP recombinase from a temperature-inducible promoter, ApR | [46]            |
| pMPM-K3       | Low-copy-number cloning vector, p15A ori, lac promoter, KanR | [49]            |
| pK3-SsrB      | pMPM-K3 derivative expressing SsrB from the lac promoter | This study      |
| pWSK129       | Low-copy-number cloning vector, pSC101 ori, KanR | [57]            |
| pPssrA-ssrB(D56E) | pWSK129 derivative expressing SsrB with the D56E mutation from the native ssrA promoter | This study      |
| pk6-HSsrBc    | pMPM-K6Ω derivative expressing 6H-SsrBc from an arabinose-inducible promoter, KanR | M.A. De la Cruz |
| pMAL-HilD1    | pMAL-c2X derivative expressing MBP-HilD from a lac promoter, ApR | [14]            |

The coordinates for the cat and lux fusions are indicated with respect to the transcriptional start site for each gene. ApR, ampicillin resistance; KanR, kanamycin resistance; SmR, streptomycin resistance.

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and to transfer the ΔssrA::kan allele from the strain 4/74 ΔssrA into S. Typhimurium SL1344, generating the strain DTM98. The kanamycin resistance cassette was excised from the strains DTM85, ΔSPI-2::kan, JPTM30, DTM91, DTM93, DTM95, DTM97 and DTM98, by using helper plasmid pCP20 expressing the FLP recombinase, as described previously [46], generating the strains DTM86, DTM89, DTM90, DTM92, DTM94, DTM96, DTM99 and DTM100, respectively. All mutant strains were verified by PCR amplification and sequencing.
the regulatory region of invF, ssaG or hns were amplified by PCR with the primer pairs invF-luxR1/invF-luxF2, ssaG-luxR1/ssaG-luxF2 or hns-luxR1/hns-luxF2, respectively. The PCR

Table 2. Oligonucleotides.

| Primer | Sequence (5'-3') | Target gene | *RE |
|--------|-----------------|-------------|-----|
| For cat transcriptional fusions and EMSAs | | | |
| hilD-108FW | AACGGATCCGACGAGGCTCGAG | hilD | BamHI |
| hilD-48FW | CAAGGATCCGACGAGGCTCGAG | hilD | BamHI |
| hilD RHindI(-rv) | CTGATATTGCGGATCGGCAATG | hilD | HindIII |
| hilD PRBam(-fw) | GATGGATCCGACGAGGCTCGAG | hilD | BamHI |
| hilAp+66F(2) | CATGACGAGGCTCGAG | hilA | BamHI |
| hilA-100Bas(2) | TAGGGATCCGACGAGGCTCGAG | hilA | BamHI |
| hilA+6Hind(2) | AGAAGCATCCGACGAGGCTCGAG | hilA | HindIII |
| hilA PRBam(2) | GATGGATCCGACGAGGCTCGAG | hilA | BamHI |
| hilA-100MutFw | TAGGGATCCGACGAGGCTCGAG | hilA | BamHI |
| fliC-RV-2 | GTGTGACATGGATGACAGGCTCGAG | fliC | BamHI |
| fliC-FW-1 | AATGGATCCGACGAGGCTCGAG | fliC | HindIII |
| For lux transcriptional fusions | | | |
| invF-luxR1 | GATGGATCCGACGAGGCTCGAG | invF | BamHI |
| invF-luxF2 | CATGACGAGGCTCGAG | invF | XhoI |
| ssaG-luxR1 | ATCCGATCCGACGAGGCTCGAG | ssaG | BamHI |
| ssaG-luxF2 | GGTTCGCACTCGAG | ssaG | XhoI |
| hns-luxR1 | CTGATATTGCGGATCGGCAATG | hns | BamHI |
| hns-luxF2 | GCCGTCGACGAGGCTCGAG | hns | XhoI |
| EC30F | GCGGGATCCGACGAGGCTCGAG | EC30F | KpnI |
| EC30R | CGGCGGATCCGACGAGGCTCGAG | EC30R | SacI |
| DTMI7F | ACGCGTCAACGGAGTCTGGGTGCTGG | DTMI7F | Sall |
| DTMI7.2R | TGTAGGGAGTCTGGGTGCTGG | DTMI7.2R | Xbal |
| DTM299F | CATGACGAGGCTCGAG | DTM299F | D56E |
| DTM299R | TGTAGGGAGTCTGGGTGCTGG | DTM299R | D56E |
| For gene cloning | | | |
| SRBF19-KpnI | GCGCGGATCCGACGAGGCTCGAG | ssrB | KpnI |
| ABR15-SacI | GCGCGGATCCGACGAGGCTCGAG | ssrB | SacI |
| DTM17F-Sall | ACGCGTCAACGGAGTCTGGGTGCTGG | PssrA | Sall |
| DTM17.2R | TGTAGGGAGTCTGGGTGCTGG | PssrA | Xbal |
| For gene deletions | | | |
| rtsA H1P1 | TATTATTTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT |
products were digested with BamHI and XhoI enzymes and then cloned into the same restriction sites of the pCS26–Pac vector, which carries a promotorless lux operon [47], generating the pirvF-lux, pssaG-lux and phns-lux plasmids. The hilA-lux-740+350 and hilA-lux-36+446 transcriptional fusions were constructed by replacing the em7 promoter in the pGEN-lux-CDABE plasmid [48] (Addgene plasmid # 44918) with the respective regulatory region of hilA. The regulatory region of hilA was amplified by PCR with the primer pairs EC30F/EC30R or EC76F/EC77R. The PCR products were digested with BamHI and SnaBI enzymes and then cloned into the same restriction sites of the pGEN-luxCDABE, generating the pinvF-lux, pssaG-lux and phns-lux plasmids. The hilA-lux-740+350 and hilA-lux-36+446 transcriptional fusions were constructed by replacing the em7 promoter in the pGEN-luxCDABE, generating the pinvF-lux, pssaG-lux and phns-lux plasmids. The hilA-lux-740+350 and hilA-lux-36+446 transcriptional fusions were constructed by replacing the em7 promoter in the pGEN-luxCDABE, generating the pinvF-lux, pssaG-lux and phns-lux plasmids. The hilA-lux-740+350 and hilA-lux-36+446 transcriptional fusions were constructed by replacing the em7 promoter in the pGEN-luxCDABE, generating the pinvF-lux, pssaG-lux and phns-lux plasmids.

Protein secretion analysis and Western blotting

Protein secretion and Western blot assays were performed as we described previously [45]. Immunoblots were performed with anti-FLAG M2 (Sigma) or anti-DnaK (StressGen) monoclonal antibodies at 1:4,000 and 1:20,000 dilutions, respectively. Horseradish peroxidase-conjugated anti-mouse (Pierce) at a dilution of 1:10,000 was used as the secondary antibody.

CAT assays

The CAT assays and protein quantification to calculate CAT specific activities were performed as previously described [50].

Expression and purification of 6H-SsrBc

E. coli BL21/DE3 containing pK6-HSsrBc was grown in 200 ml of LB at 37˚C with shaking. At an optical density (OD600) of 0.6, expression of 6H-SsrBc was induced by adding 0.1% L-arabinose and cultures were incubated for an additional 4 h. Bacterial cells were harvested by centrifugation at 4˚C and the 6H-SsrBc protein was purified from pellet as previously described [25].

Expression and purification of MBP-HilD

Maltose binding protein (MBP)-HilD was expressed in E. coli BL21/DE3 containing pMAL--HilD1 and purified by using an amylose column, as described previously [14].

Electrophoretic mobility shift assays (EMSA)

Fragments of the regulatory regions of hilD, hilA, invF, ssaG, sirA and csrA were obtained by PCR amplification with the same primer pairs used to construct the respective transcriptional fusion to the cat reporter gene. PCR products were purified using the QIAquick PCR purification kit (Qiagen). Each PCR product (≈100 ng) was mixed with increasing concentrations of
purified 6H-SsrBc in a binding buffer containing 10 mM Tris (pH 7.5), 50 mM KCl, 2.5% glycerol, 5 mM MgCl₂ and 0.05% Nonidet P-40, in a final volume of 20 μl. Protein-DNA binding reactions were incubated at room temperature for 20 min; then separated by electrophoresis in 6% non-denaturing acrylamide gels in 0.5 X Tris-borate-EDTA buffer, at room temperature. The DNA fragments were stained with ethidium bromide and visualized with an Alpha-Imager UV transilluminator (Alpha Innotech Corp.).

Invasion assays
Gentamicin protection assays were performed as previously described [22]. HeLa (human cervical adenocarcinoma epithelial) cells (ATCC) were grown in high-glucose Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO 12100–046) supplemented with 10 mM sodium pyruvate solution (SIGMA S8636), 20 mM L-glutamine (GIBCO 25030–081) and 10% (v/v) heat-inactivated fetal bovine serum (ByProductos 13001), at 37˚C in a humidified atmosphere with 5% CO₂. HeLa cells were seeded 20 h prior to infection in 24-well tissue culture plates at 1 x 10⁷ cells per well. Overnight Salmonella cultures were sub-cultured 1:33 in 20 ml of fresh LB and incubated at 37˚C with shaking for 4 h. The sub-cultures were diluted (1:5) in LB to OD₆₀₀ of 0.6. At this point, 1 ml of each sub-culture was spun and resuspended in 1 ml of 1X PBS. Then, 10 μl of these bacterial suspensions were used to infect the HeLa cells at a multiplicity of infection (MOI) of 30:1 (bacteria to eukaryotic cell) for 10 min. Cells were then washed twice with pre-warmed 1X PBS and incubated for an additional 20 min with DMEM at 37˚C. Following this incubation time, monolayers were incubated with DMEM containing 50 μg/ml gentamicin for 1 h to eliminate any extracellular bacteria. The media was then removed and the cells were lysed in 1 ml of 0.2% (w/v) sodium deoxycholate in 1 X PBS. The cell lysates and the initial starting inoculums were serial diluted and plated onto LB agar supplemented with streptomycin at 100 μg ml⁻¹.

Bioluminescent reporter assays
Overnight cultures of the Salmonella strains containing the hilA-lux transcriptional fusions were sub-cultured (1:50) into LB broth at 37˚C until the cultures reached mid-exponential phase (OD₆₀₀ = 0.5). The cultures were sub-cultured (1:50) again into LB in black 96-well polystyrene plates. Plates were incubated at 37˚C with shaking, and luminescence and OD₆₀₀ were measured every 30 min using the PerkinElmer Plate Reader. Luminescence was normalized to OD₆₀₀.

To determine intracellular gene expression using the lux bioluminescent reporter, we performed infection assays using RAW264.7 murine macrophage-like cells (ATCC), as described for the invasion assays with HeLa cells. The RAW264.7 cells were seeded at a density of 1.5 x 10⁶ cells/plate in 100 mm x 20 mm culture dishes (Corning 430167) and infected with the Salmonella strains carrying the lux-transcriptional fusions at an MOI of 10:1 (bacteria to eukaryotic cell). Following gentamicin treatment, the cells were lysed at 1, 4, 8, 12, and 16 h post-infection in 600 μl of 0.2% (w/v) sodium deoxycholate in 1 X PBS. A 200 μl sample of the cell lysates was loaded in duplicate into a white 96 well assay plate with a clear flat bottom (Corning 3610) and luminescence was measured using the GloMax-Multi Detection System (Promega). Cell lysates were also plated and luminescence was normalized to bacterial CFUs. Replication was determined by enumerating the recovered CFUs at 4, 8, 12, and 16 h post-infection. Fold-replication represents the CFUs recovered at 4, 8, 12 or 16 h relative to the CFUs at 1 h post-infection.

In vivo bioluminescent imaging
One day prior to infection, C57BL/6 mice were orally gavaged with 20 mg of streptomycin and abdominal fur was removed using clippers and depilatory cream. The WT S. Typhimurium
SL1344 strain and its isogenic ΔssrB and ΔssrB complemented with the pPsrrA-ssrB (D56E) plasmid, each containing the hilA-lux-740+350 fusion, were grown overnight with shaking at 37°C in LB supplemented with 100 mg ml⁻¹ ampicillin and 50 mg ml⁻¹ kanamycin. Bacteria were washed twice in 0.1 M HEPES (pH 8) + 0.9% NaCl and mice were orally gavaged with 1x10⁸ CFUs. Following infection, mice were anaesthetized with 2% isoflurane carried in 2% oxygen and imaged dorsally in an IVIS Spectrum (PerkinElmer). Grey scale and luminescent images were captured every hour for six hours. Total abdominal luminescence was quantified at each time point.

Bioinformatics analysis

Computational analyses were performed with the regulatory sequence analysis tools (RSAT) [51, 52]. The position-specific scoring matrix (PSSM) for the DNA-binding consensus sequence of SsrB was generated using the conserved 18 bp palindrome sequence that SsrB is known to recognize [20]. The prediction of SsrB-binding sites in the regulatory regions of hilD and hilA was performed with the matrix-scan program and the PSSM we created, using a P-value of 1e-3. Default parameters were used in these computational programs unless otherwise indicated.

Statistical analysis

Data were analyzed with GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA) using unpaired Student’s t-test. For in vivo bioluminescence analyses, data outliers were identified using the Grubbs test. One data point was identified as an outlier and was omitted from the analysis in the WT (1 h) group.

Supporting information

S1 Fig. Negative controls for cat reporter assays and EMSAs. Expression of the transcriptional fusions sirA-cat (A) and csrA-cat (B) was determined in the WT S. Typhimurium strain containing the vector pMPM-K3, or the plasmid pK3-SsrB, which expresses SsrB from a constitutive promoter. The CAT-specific activity was determined from bacterial cultures grown for 4 and 9 h in LB at 37°C. Data represents the mean with standard deviation of three independent experiments. EMSAs were performed to examine whether SsrB binds to the DNA fragments in the sirA-cat (C) and csrA-cat (D) fusions. The DNA fragments were incubated with increasing concentrations of purified 6H-SsrBc (0, 0.5, 1, 1.5 and 2 σM). DNA-protein complexes are indicated by an asterisk. (TIFF)

S2 Fig. Repression of hilA by SsrB requires the DNA sequence located upstream of the hilA promoter. Expression of the hilA-lux-740+350 (full length) (A) and hilA-lux-36+446 (truncated) (B) transcriptional fusions was determined in the WT S. Typhimurium strain and its isogenic ΔssrB mutant containing or not the pWSK129 vector, or the pPssrA-ssrB (D56E) plasmid expressing SsrB with the D56E mutation, from its native promoter that is located upstream of ssrA. Luminescence (RLU) was quantified from bacterial cultures grown in LB at 37°C. RLUs were normalized to OD₆₀₀ at each time point. Data represents the mean with standard deviation of three and two independent experiments for (A) and (B), respectively. (TIFF)

S3 Fig. Mutations in hilA affecting repression by SsrB also affect activation by HilD. Expression of the hilA-cat-100+6 WT (WT SsrB binding site) (A) and hilA-cat-100+6 Mut (mutated SsrB binding site) (B) fusions was determined in the WT S. Typhimurium strain and its isogenic ΔhilD mutant. The CAT-specific activity was determined from bacterial cultures
grown for 9 h in LB at 37˚C. Data represents the mean with standard deviation of three independent experiments. *Statistically different values relative to the WT strain, P < 0.0005.

EMSAs were performed to analyze the interaction of HilD with the hilA DNA fragments carried by the hilA-cat-100+6 WT (C) and hilA-cat-100+6 Mut (D) fusions. The DNA fragments were incubated with increasing concentrations of purified MBP-HilD (0, 0.1, 0.5 and 1 μM). DNA-protein complexes are indicated by an asterisk.

(TIFF)

S4 Fig. Comparison of the promoter sequences contained in the hilD-cat-37+6 and hilA-cat-35+6 transcriptional fusions. Common nucleotides are indicated by shading. The two predicted SsrB binding sites in hilD are shown by red letters. The transcriptional start site (+1) and the -35 and -10 promoter sequences are underlined.

(TIFF)

S5 Fig. invF is not de-repressed in the absence of SsrB during in vitro growth conditions. (A) The expression of InvF-FLAG was analyzed by Western blot in the WT S. Typhimurium strain and in a ΔssrB mutant, using monoclonal anti-FLAG antibodies. As a loading control, the expression of DnaK was also determined using monoclonal anti-DnaK antibodies. Expression of the invF-cat (B) and ssaG-cat (C) transcriptional fusions was measured in the WT and ΔssrA strains with chromosomally FLAG-tagged invF. Data represents the mean with standard deviation of three independent experiments. *Statistically different values with respect to the WT strain are indicated, P < 0.0005. Expression of InvF-FLAG, and the invF-cat and ssaG-cat fusions was determined from bacterial cultures grown for 4 and 9 h in LB or at OD600 of 0.3 in PCN, at 37˚C.

(TIFF)

S6 Fig. Growth rates of the WT S. Typhimurium strain and its isogenic ΔSPI-2 mutant inside macrophages. Fold-replication represents the CFUs recovered at the different post-infection times relative to the CFUs at 1 h post-infection for each strain. The dashed line is used to distinguish between increased and decreased replication levels. Data represents the mean with standard deviation of three independent experiments.

(TIFF)

S7 Fig. Expression of hns in macrophages is not affected by the absence of SsrB. The intracellular expression of the hns-lux transcriptional fusion was examined in the WT S. Typhimurium strain and its derivative ΔSPI-2 mutant (lacking SsrB) in RAW264.7 murine macrophage-like cells. Luminescence was quantified and normalized to CFU counts at 1, 4, 8, 12, and 16 h post-infection. The dashed line represents the relative luminescence per CFU of the WT S. Typhimurium strain with the promoterless pCS26-Pac vector in RAW264.7 cells. Data represents the mean with standard deviation of three independent experiments.

(TIFF)

S8 Fig. SsrA and SsrB repress the expression of invF inside macrophages. Expression of the invF-lux (A) and hns-lux (B) transcriptional fusion was analyzed in the WT S. Typhimurium strain and its isogenic ΔssrA and ΔssrB mutants inside RAW264.7 murine macrophage-like cells. Monolayers of macrophages were infected with an equal number of bacteria of the respective Salmonella strain. At 16 h post-infection the cells were lysed and luminescence and CFU counts were determined as described in Materials and Methods. Data represents the mean with standard deviation of three independent experiments. *Statistically different values with respect to the WT strain, P < 0.005.

(TIFF)
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