The *Salmonella enterica* PhoP Directly Activates the Horizontally Acquired SPI-2 Gene *sseL* and Is Functionally Different from a *S. bongori* Ortholog

Ohad Gal-Mor¹²³*, Dana Elhadad¹³*, Wanyin Deng², Galia Rahav¹³, Brett B. Finlay²

¹ Infectious Diseases Research Laboratory, Sheba Medical Center Tel-Hashomer, Tel-Hashomer, Israel, ² Michael Smith Laboratories, University of British Columbia, Vancouver, British Columbia, Canada, ³ Sackler Faculty of Medicine, Tel Aviv University, Tel-Aviv, Israel

Abstract

To establish a successful infection within the host, a pathogen must closely regulate multiple virulence traits to ensure their accurate temporal and spatial expression. As a highly adapted intracellular pathogen, *Salmonella enterica* has acquired during its evolution various virulence genes via numerous lateral transfer events, including the acquisition of the *Salmonella* Pathogenicity Island 2 (SPI-2) and its associated effectors. Beneficial use of horizontally acquired genes requires that their expression is effectively coordinated with the already existing virulence programs and the regulatory set-up in the bacterium. As an example for such a mechanism, we show here that the ancestral PhoPQ system of *Salmonella enterica* is able to regulate directly the SPI-2 effector gene *sseL* (encoding a secreted deubiquitinase) in an SsrB-independent manner and that PhoP plays a part in a feed-forward regulatory loop, which fine-tunes the cellular level of SseL. Additionally, we demonstrate the presence of conserved cis regulatory elements in the promoter region of *sseL* and show direct binding of purified PhoP to this region. Interestingly, in contrast to the *S. enterica* PhoP, an ortholog regulator from a *S. bongori* SARC 12 strain was found to be impaired in promoting transcription of *sseL* and other genes from the PhoP regulon. These findings have led to the identification of a previously uncharacterized residue in the DNA-binding domain of PhoP, which is required for the transcriptional activation of PhoP regulated genes in *Salmonella* spp. Collectively our data demonstrate an interesting interface between the acquired SsrB regulon and the ancestral PhoPQ regulatory circuit, provide novel insights into the function of PhoP, and highlight a mechanism of regulatory integration of horizontally acquired genes into the virulence network of *Salmonella enterica*.

Introduction

*Salmonella* spp. infects various animal and human hosts and is a major cause of diverse diseases including enteric fever, gastroenteritis, bacteremia, and systemic infections worldwide. Current taxonomy of the genus *Salmonella* includes the species *S. enterica* with a large number of clinically important serovars that infect animal and human hosts and *S. bongori*, which represents a phylogenetically older lineage of the *Salmonella* genus and is mainly associated with cold-blooded vertebrates [1].

As with other pathogens, lateral gene transfer plays a key role in the evolution of *Salmonella* in adaptation to changes in the environment and exploitation of new niches [2]. Horizontal acquisition of mobile genetic elements has been shown to provide a wealth of genetic diversity and a source of various virulence factors required during the infection. In some cases, the acquired elements consist of large virulence gene clusters, designated *Salmonella* pathogenicity islands (SPIs) such as SPI-1 and SPI-2. The acquisition of these SPIs into the bacterial genome is considered to be a ‘quantum leap’ in *Salmonella* evolution [3]. SPI-1 is present in both *Salmonella* species and required for *Salmonella* invasion into the intestinal epithelium and non-phagocytic cells [4]. SPI-2 is found in *S. enterica* species, but not in *S. bongori*, and represents a second, more recent event in *Salmonella* evolution [5]. SPI-2 is essential for intracellular survival and replication and has a crucial role in systemic infections in mammals [6,7]. Both SPI-1 and SPI-2 encode a separate type three secretion system (T3SS) used to deliver effector proteins directly into the cellular environment of the eukaryotic host, manipulating various host pathways (reviewed in [8]).

*SseL* is a SPI-2 translocated effector encoded outside of the SPI-2 locus and, like other SPI-2 effectors, is absent from the *S. bongori* genome [9]. A *S. enterica* serovar Typhimurium (*S. Typhimurium*) *sseL* mutant strain is attenuated for virulence in mice [9,10] and SseL functions as a deubiquitinase [10] that modulates host inflammatory responses by suppressing NF-κB activation and IkBα ubiquitination and degradation [11].
Bacterial pathogenicity is a multifactorial trait that requires appropriate expression of numerous virulence genes. Fine-tuning of bacterial virulence factor expression is achieved by a synchronized operation of regulatory pathways in response to environmental cues. Among others, *S. enterica* utilizes two pivotal two-component regulatory systems known as PhoPQ and SsrAB to control the expression of genes required for its intracellular life-style.

PhoQ is a sensor for extracellular Mg\(^{2+}\) that modifies the phosphorylation state of the DNA-binding protein PhoP [12,13]. PhoP belongs to the OmpR family of winged-helix transcription factors (reviewed in [14]) and controls the expression of a large number of genes that mediate adaptation to low Mg\(^{2+}\) environments and/or virulence in several Gram-negative species including *Salmonella enterica* and *Escherichia coli* [15]. It is believed that by monitoring extracellular Mg\(^{2+}\), PhoPQ allows *Salmonella* to sense the transition from an extracellular environment to a subcellular location and to activate a set of virulence factors, which are required for intracellular infection [16]. Indeed, *S. Typhimurium* strains lacking the PhoPQ system are highly attenuated for virulence in mice and unable to proliferate within macrophages [16,17].

While the PhoPQ pathway is conserved among *Salmonella* and related species (PhoP and PhoQ of *E. coli* and *S. enterica* are 93% and 86% identical, respectively [18]) and considered as an ancestral regulatory system, the SPI-2 encoded SsrAB system is unique to *S. enterica*. SsrAB is composed of the histidine kinase sensor, SsrA, and the response regulator, SsrB, a member of the NarL/FixJ subfamily. The expression of SPI-2 genes and SPI-2 T3SS-associated effectors located outside of SPI-2 is induced within host cells and is strictly dependent on the SsrAB system (reviewed in [19]). In addition, expression of the SsrAB regulon members was shown to be positively affected by the two-component system EnvZ-OmpR [20,21], the transcriptional regulator SlyA [22,23], and several nucleoid-associated proteins such as the integration host factor (IHF) and the factor for inversion stimulation (Fix; reviewed in [24]). Interestingly, an epistatic interaction between PhoP and the SsrAB system has been demonstrated by the ability of PhoP to bind the *sseB* promoter and regulate SsrA post-transcriptionally [25].

Here we characterized the role of PhoP in governing the expression of the SPI-2 effector gene, *sseL*. We demonstrate a regulatory integration of a horizontally acquired virulence gene into the ancestral PhoPQ networks and show that PhoP directly controls *sseL* expression in a feed-forward regulatory loop. Moreover, we demonstrate that a *S. bongori* SARC 12 strain is impaired in activation of the PhoP regulon due to a single amino acid substitution in the C-terminal DNA binding domain of PhoP.

**Results**

PhoP facilitates *sseL* transcription in an SsrB-independent manner

A differential fluorescence induction (DFI) screen of a *gfp* reporter-gene library was performed to identify *S. Typhimurium* genes, which are induced under a defined set of conditions (low Mg\(^{2+}\), low phosphate, acidic pH) assumed to mimic the intracellular milieu (our unpublished results). In the final pool of 93 sequenced clones we found a significant representation of various known PhoP regulated genes including *pagO* (3 times) [26]; *mig-5* (also known as *PSL1046*; 2 times) [27]; *pmrD* (2 times) [28]; and *ybjX* [29], together with a dominant presence of *sseL*, which was hit 8 times. A previous study showed that SsrB is absolutely required for *sseL* expression and that it is induced inside macrophages [9]. Nonetheless, co-isolating PhoP regulon members with *sseL* under the same enrichment protocol prompted us to look more closely on the potential role of PhoP as a regulator that controls *sseL* expression in conjunction with SsrB.

To elucidate the involvement of PhoP in the regulation of *sseL*, a reporter-gene fusion between *sseL* and a promoterless β-galactosidase was constructed using the vector pMC1403 [30]. Comparison of *sseL*:lac\(_Z\) expression under several growth conditions showed the contribution of minimal medium, acidic pH, and phosphate/magnesium starvation cues to the induction of *sseL* and indicated that maximal expression was reached during the stationary phase in defined low phosphate, low magnesium minimal (LPM) medium (Fig. 1). These results closely matched the previously described high protein levels of SseL-HA [9] and induction of other SsrB regulated genes [31,32,33] under similar conditions, and also demonstrated the capability of our experimental system to correctly report different levels of *sseL* expression.

Subsequently, we were interested in assessing the relative contribution of PhoP and SsrB to the integrated regulation of *sseL*. Examining the expression levels of *sseL*:lac\(_Z\) in the ssrB and *phoP* *S. Typhimurium* mutant strains showed a significant (P<0.0001) decrease by ~20, and 4-fold, respectively, in comparison to its expression in the wild-type (Fig. 2A). These data highlighted the fundamental role of SsrB and indicated that PhoP is also involved in the *sseL* regulatory network, directly or indirectly.

To better understand the mechanism by which PhoP facilitates *sseL* expression, we explored the expression of *sseL*:lac\(_Z\) in a *phoP* *ssrB* (OG2011) double mutant strain. The rationale behind this experiment was the assumption that if PhoP contributes to the expression in an SsrB-dependant manner, i.e. functioning

![Figure 1. *sseL*:lac\(_Z\) is induced in response to low phosphate, low magnesium and acidic pH environmental cues. *S. Typhimurium* strains carrying *sseL*:lac\(_Z\) were grown for 16 h at 37 °C in LB, LPM (pH 7.4) LPM (pH 5.8) supplemented with 10 mM MgCl\(_2\), and LPM (pH 5.8), and were assayed for β-galactosidase activity presented in Miller units (M.U.). Basal lac\(_Z\) expression of *S. Typhimurium* harboring pMC1403 (vector) that was grown in LPM (pH 5.8) is also shown. doi:10.1371/journal.pone.0020024.g001](image-url)
upstream of SsrB in the same regulatory cascade, the expression in the double mutant strain is likely to be similar to the expression in the ssrB background; however, if the contribution by PhoP is independent of SsrB, then an accumulative effect is expected, leading to further reduced expression in the double mutant background. sseL::lacZ expression in the phoP ssrB double mutant showed ~2-fold lower expression compared to that in the ssrB strain (P<0.0001) supporting the possibility that PhoP and SsrB have accumulative effects on the expression of sseL, and that PhoP contributes to the expression of sseL by an SsrB-independent manner, in addition to its epistatic regulation of sseL. To further test this hypothesis, we conducted quantitative reverse transcription PCR (qRT-PCR) determining directly the mRNA levels of sseL transcripts in the wild-type, ssrB, phoP, and phoP ssrB backgrounds. RNA harvested from Salmonella cultures that were grown in LPM medium showed a prominent reduction of ~5600 and 500-fold in the abundance of sseL transcripts in the ssrB and phoP backgrounds, respectively, relative to the wild-type strain. In the phoP ssrB double mutant strain, the transcription of sseL was further reduced to levels that were below the detection threshold of the RT-PCR [Fig. 2B]. These data provided further evidence that PhoP can contribute to sseL transcription in an SsrB-independent mechanism.

PhoP is required for sseL expression during intracellular infection

sseL was shown to be readily expressed inside macrophages [9,10] and therefore, we were interested in characterizing the role of PhoP in sseL induction during macrophage infection. J774.1 macrophages were infected with wild-type, phoP and ssrB S. Typhimurium strains harboring sseL::lacZ or the vector only. At six hours post-infection, the cells were harvested, and the intracellular expression of the reporter strains, as well as their intracellular survival, was determined. As shown in Fig. 2C, a remarkable reduction was observed in the normalized intracellular expression of sseL::lacZ when macrophages were infected with phoP or ssrB mutant strains. sseL::lacZ expression results in the intracellular bacteria were correlated with the in-vitro analyses and showed that in addition to SsrB, PhoP is required for maximal sseL expression in the intramacrophage environment.

sseL promoter is conserved among S. enterica subspecies I and harbors two putative PhoP boxes

As opposed to some SPI-2 effector genes with only limited serovar distribution, sseL is present in many different serotypes of S. enterica subspecies I. Comparison of the regulatory region of sseL in 13 S. enterica serovars (Fig. 3A) showed a very high degree of promoter conservation, including in serovars that are either host-restricted (Typhi and Paratyphi to human and Gallinarum to poultry) or host-adaptive (Dublin to cattle and Choleraesuis to swine). These highly conserved promoters might reflect the outcome of a selective pressure against genetic changes in this locus and suggest that sseL is similarly regulated by different serovars and within different hosts.

To identify putative cis regulatory elements in the context of a functional promoter, we determined the transcription start site of sseL in S. Typhimurium by an RNA ligase mediated (RLM) rapid amplification of cDNA ends (RACE). This analysis identified a transcriptional initiation site corresponding to a T residue located 97-bp upstream to the predicted start codon of SseL (GI:267994379; or 28-bp upstream from a differently predicted start codon according to GI:308065958). In the sequences upstream of the transcription initiation site, probable ρT35 (TTFACA) and ρT−35 (CATATT) elements separated by 18

Figure 2. PhoP activates the expression of sseL in an SsrB-independent manner. (A) β-galactosidase expression of S. Typhimurium wild-type (WT), phoP, ssrB and phoP ssrB double mutant strains harboring sseL::lacZ grown in LPM medium (pH 5.8). (B) Expression of sseL transcripts in ssrB, phoP, and phoP ssrB double mutant backgrounds vs. the wild-type strain as determined by q-RT-PCR. RNA was harvested from Salmonella strains grown in LPM medium to late logarithmic phase. The purified RNA was reverse-transcribed and the expression of sseL was examined by quantitative real-time PCR. The fold change in the abundance of sseL transcripts in the different mutants relative to their expression in the wild-type strain is presented. Expression was normalized using the housekeeping rpoD gene as a control. The results represent the mean of 8 RT-PCR reactions from two independent RNA preparations with a standard error shown by the error bars. (C) Expression of sseL::lacZ during macrophages infection. S. Typhimurium wild-type (WT) harboring sseL::lacZ or the vector (pMC1403) only and phoP, ssrB isogenic mutant strains carrying sseL::lacZ were used to infect J774.1 macrophages. Six hours post-infection, the cells were harvested; β-galactosidase activity was measured and normalized by the intracellular CFU count. The values are presented in normalized Miller units and are the mean of four independent infected cultures.

doi:10.1371/journal.pone.0020024.g002

PhoP Directly Activates SPI-2 Effector Gene sseL
PhoP Directly Activates SPI-2 Effector Gene sseL

**A**

| Typhimurium_SL1344 | Agona | Schwarzengrund | Paratyphi_A | Heidelberg | Gallinarum | Paratyphi_C | Newport | Dublin | Enteritidis | Choleraesuis | Typhi |
|-------------------|-------|----------------|-------------|------------|-----------|-------------|---------|-------|-------------|-------------|-------|
| TTATTTTTTACAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT | ATATTTTTTTCCAAATTTGATATTAATGATTTATCAGTACATTACGTTTACCAGAAAACAGAGAAAAT |

**B**

\[(T/G)TTT\text{-}5\text{ bp spacing-} (T/G)TTTA\]

**C**

| Relative \(\beta\)-galactosidase activity (in %) |
|-----------------------------------------------|
| WT | phoP | S1 | S2 | S1+S2 |
| 110 | 90 | 70 | 50 | 30 |

**D**

| Relative \(\beta\)-galactosidase activity (in %) |
|-----------------------------------------------|
| phoP sseB | phoP sseB + phoP | phoP sseB + pSad |
| 0 | 0 | 0 |
nucleotides were also identified (Fig. 3A). These elements are in agreement with the consensus sequences TTTGACA (−35) and TATAAT (−10) [34] and fit the preferred spacing range of 15–19bp between the −35 and −10 elements [35]. The results suggesting that sseL is activated by PhoP have led us to search for the presence of potential PhoP binding sites. A conserved repeat of the hexanucleotide (T/G)GTTTA separated by 5 nucleotides, known as PhoP box in Salmonella and in E. coli [29,36]. A conserved thymine in the first hexanucleotide (at position 3) and two conserved thymines together with one conserved adenine in the second hexanucleotide (at positions 3, 4, and 6, respectively) were necessary and sufficient for DNA binding of PhoP in in-vitro DNA footprinting assays [37,38,39,40]. The sseL promoter region was found to harbor two putative sites resembling the characterized PhoP binding sequences as shown in Fig. 3A. Interestingly, one putative PhoP box was found downstream from the transcription start site (+1) of sseL, while the other overlapped with the transcription start site and the −10 element.

In order to examine whether these sites contribute to the expression of sseL, the two conserved thymines in the second hexanucleotide of each site were replaced with guanine residues. As illustrated in Fig. 3C, site directed mutagenesis of either site 1 (S1) or site 2 (S2) resulted in a moderate but significant (P<0.0001) decrease in the expression of sseL::lacZ. Additionally, an sseL manipulated promoter containing both mutations (S1+S2) demonstrated accumulative reduction in the expression of sseL::lacZ by more than 2-fold (P<0.0001), indicating that the S1 and S2 sites are required for optimal expression of sseL, possibly due to their role as PhoP binding sites. To further examine this idea and to rule out the possibility that these positions are used as SsrB binding sites, we analyzed the expression of an altered sseL promoter harboring both mutations in a phoP:ssrB background complemented with PhoP or SsrB, provided in trans. In the presence of SsrB, this mutated promoter was readily able to induce sseL::lacZ expression, suggesting that SsrB does not bind to these sites. In contrast, providing PhoP in trans did not induce sseL::lacZ expression from the modified promoter (Fig. 3D), supporting the function of S1 and S2 loci as potential PhoP binding sites.

**PhoP binds directly to the promoter region of sseL**

The above results implied that PhoP might interact directly with sseL to activate its expression. To investigate the possibility of direct binding of PhoP to the promoter region of sseL, we conducted an electrophoretic mobility shift assay (EMSA) using a S. Typhimurium N-terminally His-tagged PhoP protein (His-PhoP). In this analysis, purified His-PhoP protein (Fig. 4A) was incubated for 30 min at 37°C with a 128-bp dUTP-digoxigenin labeled DNA fragment corresponding to the predicted regulatory sequence of sseL. Subsequently, the DNA-protein complex was resolved on a 6% native polyacrylamide gel and imaged. EMSA analysis showed that His-PhoP alone could gel shift the promoter region of sseL in a dose dependent manner and in the presence of a 100-fold excess of a non specific DNA (dl-dC), indicating the ability of PhoP to bind in-vitro to the sseL promoter (Fig. 4B).

The *S. enterica* PhoP, but not an impaired ortholog from a *S. bongori* strain, activates *sseL* expression

To further characterize the relative contribution of PhoP and SsrB to the transcriptional output of *sseL*, we sought to analyze sseL::lacZ expression in two SsrB-free heterologous hosts, *E. coli* and *S. bongori*, representing closely related species that lack the entire SPI-2 locus and its related effectors. Expression of sseL::lacZ in *E. coli* and *S. bongori* was found to be ~18-fold lower relative to the expression in *S. Typhimurium*. We hypothesized that these differences resulted from the lack of the primary SPI-2 regulator, SsrB. Interestingly, providing SsrAB in trans to these backgrounds caused a dramatic induction (~50-fold) of *sseL* in *S. bongori* (to a much higher level than in *S. Typhimurium*), but only limited expression increase in *E. coli* (Fig. 5A), suggesting possible involvement of other sseL regulator(s), (see Discussion).

We further characterized sseL expression in *S. bongori* as an SsrB-negative host. A *S. bongori* phoP in-frame deletion mutant (DE.1.10.3) was constructed and the expression of sseL::lacZ was examined in the presence and absence of its native PhoP. In contrast to the reduced expression in the absence of PhoP in *S. Typhimurium* (SL1344 sseB vs. SL1344 phoP sseB; Fig. 2), no significant change in the expression of sseL::lacZ was found in the *S. bongori* phoP strain relative to the parental strain, and both expressed low levels of *sseL*. Nevertheless, providing in trans the *S. Typhimurium* PhoP (pPhoPST), but not the *S. bongori* PhoP (pPhoPSB) resulted in elevated (>5-fold; P<0.0001) expression of sseL in the *S. bongori* phoP strain background (Fig. 5B). To confirm these results, using a *S. Typhimurium* host, we expressed sseL::lacZ in an SL1344 phoP strain complemented with either pPhoPSX or pPhoPSB. Although the expression level following complementation was much lower in this background relative to *S. bongori* (further supporting the notion that an sseL-repressor might play a role in *S. Typhimurium*), higher levels of sseL::lacZ were constantly detected upon complementation with pPhoPSX in comparison to pPhoPSB (P<0.0001; Fig. 5C).

In agreement with the previously presented data, these results provided a further line of evidence that the *S. Typhimurium* PhoP can induce *sseL* expression independently of SsrB. However, unlike
S. Typhimurium PhoP, the S. bongori ortholog did not seem to activate *sseL* expression.

Possible differences between the PhoP of *S. bongori* and *S. enterica* were intriguing. Comparing the sequence of PhoP from the *S. bongori* strain to its ortholog in *S. Typhimurium* (Fig. 4D) revealed a single amino acid difference at the C-terminal domain, harboring phenylalanine (F) instead of valine (V) at position 169. The C-terminal domain of the OmpR family members is thought to be involved in DNA-binding and protein-protein interactions with the RNA-polymerase (reviewed in [14]). The structure of the C-terminal domain of *E. coli* OmpR (OmpRc) has been determined (PDB ID: 1ODD) and was shown to compile 3 α-helices and 7 β-sheets [41]. Secondary structure prediction of the *Salmonella* PhoP using the SOPMA program [42] predicted very similar organization of the secondary elements and positioned residue 169V within the β5 strand (Figure 5D). To test whether V169F amino acid substitution in PhoP is responsible for the disability of the *S. bongori* strain to activate *sseL* expression, we engineered a construct harboring the *S. bongori* PhoP sequence with a GTC (valine) codon instead of TTC (phenylalanine) at position 169 (pPhoPSB169V). Introducing pPhoPSB169V, but not the original pPhoPSB, into a *S. bongori* phoP strain elevated *sseL*::lacZ expression to a similar level that was found in the presence of the *S. Typhimurium* PhoP (pPhoPST, Fig. 5B). We concluded from this analysis that the PhoP of the *S. bongori* strain was unable to activate *sseL* expression due to a single amino acid change at the C-terminal domain of this regulator.

The PhoP of the *S. bongori* strain is impaired in activating the PhoPQ regulon

Next we asked whether the identified disparity in *S. bongori* PhoP affects its ability to activate other genes from the PhoP regulon, besides *sseL*. To answer that we investigated the expression of an ancestral [ybjX] and two *S. Typhimurium*-specific [pagO and *mg-5*] genes known to be under PhoP control, in *S. Typhimurium* and *S. bongori* hosts. As expected, this analysis showed significantly higher expression levels of *ybjX::lacZ*, *mg-5::lacZ* and *pagO::lacZ* in *S. Typhimurium* compared to a *S. Typhimurium phoP* strain. Up-regulation was observed when a *S. bongori* phoP strain was complemented with either *S. Typhimurium* PhoPQ (pPhoPST) or with a *S. bongori* PhoPQ harboring V169 residue (pPhoPST169), but to a significantly lesser extent when it was complemented with the native *S. bongori* PhoPQ system (pPhoPSB; Figure 6). Taken together, these results suggested that the PhoP of the *S. bongori* strain is attenuated in activating expression of not only *sseL*, but also other members of the PhoP regulon, and that the valine residue at position 169 is required for the regulatory activity of PhoP.

Discussion

Understanding the regulatory circuits governing the expression of virulence traits is important for a more complete understanding of bacterial pathogenesis and host-pathogen interactions. The virulence potential of many pathogens is greatly facilitated by the acquisition of new traits via lateral gene transfer. The ability of the recipient organism to benefit from these “imported goods” is largely dependent on their successful incorporation into a functional preexisting regulatory network.

Previous studies have shown a role for PhoP in controlling SPI-2 associated genes by indirect mechanisms. PhoP has been shown to bind the *ssrB* promoter and to affect SsrA levels post-transcriptionally [25]. In addition, PhoP affects the expression of SlyA [23]. Since SsrB and SlyA are both positive regulators of the SPI-2 regulon [22,43], indirect effect by PhoP on SPI-2 gene expression occurs. Nonetheless, it seems that the functional integration of the PhoPQ pathway into the regulatory network governing SsrB-activated genes is even more convoluted. Here we elucidate...
another level of regulation and establish that the SPI-2 effector gene, sseL, is directly regulated by this ancestral regulator. Several lines of evidence indicated the ability of PhoP to promote expression of sseL in an SsrB-independent fashion: (i) reduced expression of sseL::lacZ was demonstrated in a phoP ssrB double mutant strain compared to the ssrB background in S. Typhimurium (Fig. 2A); (ii) diminution in the abundance of sseL transcripts in the phoP ssrB background in relation to the ssrB strain (Fig. 2B); (iii) a PhoP-mediated induction of sseL::lacZ in a S. bongori SsrB-free heterologous host (Fig. 5); (iv) the presence of two putative Pho boxes in the promoter region of sseL (Fig. 3); and (v) direct in-vitro binding of His-PhoP to the promoter region of sseL in a gel mobility shift assay (Fig. 4). Collectively, our data suggest that PhoP directly activates sseL by a feed-forward regulatory mechanism. The feed-forward loop is a very efficient regulatory circuit [44], in which a transcription factor (PhoP) regulates a second transcription factor (SsrB) and both of them jointly control the expression of a third gene (sseL). This mode of regulation allows tuning up gene regulation and effective integration of different environmental signals sensed by PhoQ and SsrA. Controlling sseL expression by PhoP could be mediated either by transcription activation per se or by counteracting nucleoid-like proteins, such as H-NS, YdgT, and Hha that were shown to bind A+T rich sequences and repress transcription of SPI-2 genes (reviewed in [24]) including sseL specifically [9]. Such an activity of PhoP has been shown for pagC, in which PhoP counteracts H-NS silencing [45,46].

Promoter analysis followed by site directed mutagenesis identified two putative PhoP binding sites in the promoter region of sseL, whereas one of them overlaps with the −10 element of sseL. Although this arrangement is not typical of PhoP dependent promoters, similar promoter architecture has been previously described. The PhoP-activated promoters of ugtL [47] and invL [48] harbor two PhoP binding sites, and one of the sites in each promoter overlaps with the −10 element. Furthermore, it is now apparent that some PhoP-regulated promoters do not possess a
canonical PhoP box in their PhoP binding sites. One of the PhoP-binding sites of \( ugL \) and the \( ssrB \) promoters does not contain a PhoP box at all [25,47]. Another interesting finding is that the second putative PhoP-binding site is downstream from the \( sseL \) site of transcriptional initiation. Although it is not very common among transcriptional regulators, accumulating evidences show that binding sites for transcriptional activators can be located downstream of the transcriptional initiation sites. For example, both the response regulator Rns from \( E. coli \) and SlyA from \( Salmonella \) have been shown to have binding sites downstream of the transcription start sites of target genes [47,49]. Multiple binding sites for OmpR, which belongs to the same subfamily of response regulators as PhoP [50], were observed downstream of the transcriptional start sites of \( ssrA \) and \( ssrB \) in \( Salmonella \) [51].

Noteworthy, many of the PhoP-governed genes that do not have an orthodox PhoP promoter organization also require additional regulators to activate their transcription [29], including \( ugL \) that requires SlyA in addition to PhoP [47]; and the \( ssrB \) promoter, which is autoregulated by SsrB [25]. These observations support the notion that the expression of some PhoP-regulated genes involves the function of PhoP in conjunction with other transcription regulators and that the promoter architectures of regulons such as PhoP or SsrB can be modular and evolvable over time [52].

The possibility that \( sseL \) expression requires the coordinated activity of several regulators is also supported by comparing its expression in \( E. coli \) and \( S. bongori \) in the presence of SsrB. Introducing SsrAB into these hosts led to a profound expression of \( ybjX::lacZ \) in \( S. bongori \), but to only moderate levels in \( E. coli \) (Fig. 5A). These results suggest that certain \( Salmonella \) spp. factors, which are not present (or at least different enough) in \( E. coli \) (Fig. 5A), are required for maximal \( sseL \) expression, in conjunction with SsrB. Similarly, introducing SsrB or PhoP into \( S. bongori \) resulted in a much higher induction of \( sseL \) expression than in \( S. Typhimurium \) background (Fig. 5), implying the possibility that a particular \( sseL \)-negative regulator, not present in \( S. bongori \), may play a role in \( S. Typhimurium \).

Surprisingly, as opposed to the \( S. Typhimurium \) PhoP, an impaired ortholog from a \( S. bongori \) SARC 12 strain was not able to activate \( sseL \) transcription. We showed that this distinction resulted from a single amino acid variation at position 169, containing phenylalanine in place of valine (Fig. 5D), that was important for the expression of \( sseL \), as well as for other PhoP-regulated genes (Fig. 5 and Fig. 6). A secondary structure prediction of \( Salmonella \) PhoP and comparison with the crystal structure of OmpRc positioned V169 within the \( \beta5 \) domain of the PhoP C-terminus region. A mutation at the equivalent position (Leu 175) was identified in the VirG regulator (also belonging to the OmpR family) and demonstrated to be part of a conserved internal hydrophobic core, important for stabilizing protein conformation [14,53]. Hydrophobic core residues are highly conserved across the OmpR family members, which presumed to share a common fold [14]. Based on that, we believe that the V to F substitution at this position changes protein conformation and therefore interferes

Figure 6. The PhoP from a \( S. bongori \) SARC 12 strain is impaired in regulating the \( phoPQ \) regulon. \( S. Typhimurium \) (ST), \( S. Typhimurium \) \( phoP \) (ST \( phoP \)), \( S. bongori \) \( phoP \) (SB \( phoP \)) and \( S. bongori \) \( phoP \) complemented with a \( S. bongori \) \( phoP \) (p\( PhoP_{SB} \)), or the \( S. bongori \) \( phoP \) harboring V169 (p\( PhoP_{SB}^{169V} \)) expressing \( ybjX::lacZ \) (A), \( mig-5::lacZ \) (B) or \( pagO::lacZ \) (C) were examined for their \( \beta \)-galactosidase activity following growth under inducing conditions. The means with a standard error shown by the error bars are presented.

doi:10.1371/journal.pone.0020024.g006
with the regulatory activity of PhoP in S. bongori. It is worth pointing out that PhoP from E. coli K-12 (accession number BAA33952) and other S. bongori strains (ABG34164, ABG34169) harbor a V169 residue, suggesting that this missense mutation in the SARC 12 strain probably arose relatively recently.

Collectively, the experimental setup described here demonstrates an interesting interface between the SsrB and the PhoP pathways and shows the evolution of a feed-forward regulatory loop to control newly acquired virulence genes. The interaction between the conserved housekeeping regulator PhoP and a horizontally acquired SsrB-regulated gene not only provides an interesting example for functional integration of incoming genes into the core regulatory network of a pathogen, but also reveals dynamics and plasticity in the evolution of regulatory circuits.

It is tempting to hypothesize that PhoP contributes directly to the expression of other SPI-2 effector genes as well. Previously we showed that expression of the effectors SseK1 and SseK2 was significantly reduced under SPI-2 induction conditions in the S. bongori phoP-Δ169 strain as compared with wt. This observation, together with the presence of PhoP-box resembling sequences in the promoter regions of sseK1 and sseK2 (data not shown), is in agreement with this idea.

The acquisition of new traits and their regulatory assimilation are central to the ability of prokaryotes to evolve novel phenotypes, adapting to and occupying new niches. Coordination of laterally acquired virulence genes by several systems is expected to contribute to fine-tuning of a Salmonella intracellular virulence program and provide more flexibility and sensitivity in its response to the host milieu.

Materials and Methods

Bacterial strains and in vitro growth conditions

Bacterial strains and plasmids utilized in this study are listed in Table 1. S. Typhimurium SL1344 and S. bongori SARC 12 (a generous gift from Prof. M. Hensel) were used as the wild-type strains. S. Typhimurium phoP:ssrB (OG2011) double mutant strain was generated by P22 transduction of phoP::kan into SL1344 ssrB background. A spontaneous streptomycin-resistant S. bongori SARC 12 strain was isolated on LB agar plates containing 100 µg ml⁻¹ streptomycin and was used as the parental strain for a S. bongori phoP mutant (DE1.10.3). DE1.10.3 carries an in-frame deletion (amino acids 8–217) of phoP and was generated by allelic exchange using the counter-selectable suicide vector pRE112 [53] as previously described [56].

Bacterial cultures were routinely maintained in Luria-Bertani (LB) liquid medium or on LB agar plates supplemented with the appropriate antibiotic(s) at the following concentrations: chloramphenicol, 25 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; ampicillin, 100 µg ml⁻¹; and streptomycin, 100 µg ml⁻¹. To examine expression under SPI-2 induction conditions, Salmonella strains were grown in low phosphate low magnesium minimal (LPM) medium containing 90 mM MES (pH 5.8), 5 mM KC1, 7.5 mM (NH4)2SO4, 0.5 mM K2SO4, 337 µM KH2PO4/KHPO4 (pH 7.4), 20 mM MgCl2, 30 mM glycerol, and 0.1% Casamino acids [57] with aeration at 37°C.

Construction of lacZ reporter fusions

PCR fragments containing ~500-bp from the upstream region and the first seven amino acid codons of sseL, mig-3, pagO, and ybjX were amplified using the primers OG-140 and OG-141; OG-132 and OG-133; OG-128 and OG-129; OG-130 and OG-131, respectively. PCR products were cloned into pCR-Blunt, and moved into pMC1403 digested with EcoRI and BamHI, resulting in pOG-sseL::lacZ, pOG-mig-3::lacZ, pOG-pagO::lacZ, and pOG-ybjX::lacZ.

To generate an sseL promoter harboring nucleotides substitution in the putative PhoP box 1, 325-bp and 593-bp PCR products were amplified using pOG-sseL::lacZ as template and the primer pairs OG-171 and OG-185, and OG-172 and OG-173, respectively. The resulting PCR products were used as template for a secondary PCR reaction using the primers OG-167 and OG-185. The obtained 895-bp PCR product was cloned using EcoRI and BamHI into pMC1403, generating pOG-sseL::lacZ-S1. Similarly, the primers pairs OG-169, OG-185 and OG-170, OG-167, were used to amplify 360-bp and 556-bp PCR products, respectively, that were used to create a parallel mutation in PhoP box 2. The obtained PCR fragment was cloned as described above into pMC1403, generating pOG-sseL::lacZ-S2. To generate an sseL::lacZ promoter harboring both S1 and S2 mutations, we used pOG-sseL::lacZ-S1 as a template in PCR reactions with primers OG-167, OG-170 and OG-169, OG-185. The resulting PCR products were used as a template for a subsequent PCR amplification with primer OG-167 and OG-185, and the obtained 895-bp PCR product was cloned into pMC1403, generating pOG-sseL::lacZ-S1+2. All the final plasmids described above were verified by DNA sequencing.

Construction of PhoPQ and SsrAB complementation constructs

A 4802-bp PCR fragment corresponding to the S. Typhimurium or S. bongori phoPQ was amplified using oligonucleotides DE-12 and DE-13 containing XhoI and SphI sites, respectively. The digested fragment was cloned into pACYC184, generating pDE-PhoPQST or pDE-PhoPQSB. Similarly, the primers DE-23 and DE-24 were used to amplify a 3610-bp DNA fragment containing the ssrB operon. The resulting PCR product was cloned into pACYC184, resulting in the plasmid pDE-SsrAB. To generate a construct harboring a S. bongori PhoP with V residue at position 169, 999-bp and 2108-bp PCR products were amplified using pDE-PhoPQSB as template and the primer pairs DE-11, DE-101, and DE-12, DE-102, respectively. The resulting PCR products were used as a template for a secondary PCR reaction using the primers DE-11 and DE-12. The obtained 3067-bp PCR product was cloned using BglII and SphI into pDE-PhoPQSB, generating pDE-PhoPQ-SB169V.

β-galactosidase assays in vitro

β-galactosidase assays were performed according to [61]. Salmonella strains were grown in LB or in LPM medium. The assays were performed with 100 µl of culture, and the substrate for β-galactosidase hydrolysis was o-nitrophenyI-β-D-galactopyranoside (ONPG, Sigma).

β-galactosidase assays in macrophages

1 ml of an overnight culture grown in LB+Bamp was harvested and resuspended in 1 ml of DMEM. 100 µl of the suspended bacteria were used to inoculate each well of a six-well plate containing J774.1 mouse macrophages at a multiplicity of infection of 100. Plates were centrifuged at 300 g for 5 min and incubated at 37°C/5% CO2 for 20 min to allow adherence. Infected cells were washed three times with PBS, 2 ml DMEM containing 50 µg/ml gentamicin was added to each well, and the plates were incubated at 37°C/5% CO2. After 6 h, macrophages were washed with PBS, scraped from the plates, centrifuged, and resuspended in 115 µl of PBS. 15 µl of the suspended cells was added to 135 µl of PBS buffer containing 1% Triton X-100 and 0.1% SDS. Serial
Table 1. Bacterial strains and plasmids used in the study.

| Strain or plasmid | Genotype and description* | Reference or source |
|-------------------|---------------------------|---------------------|
| **Salmonella**    |                           |                     |
| S. Typhimurium SL1344 | wild-type Sm' xyl hisG rpsL | ATCC               |
| OG2011 (phoP ssrB) | SL1344 phoP:Tn10 Tc' ssrB::kan transduced by P22 from ssrB | This study          |
| phoP              | SL1344 phoP:Tn10 Tc'       | [58]                |
| ssrB              | SL1344 ssrB::kan transduced by P22 from 14028s | [59]                |
| S. bongori SARC 12 |                           | [60]               |
| DE1.10.3 (phoP)   | S. bongori SARC12Δ phoP   | M. Hensel lab       |
| **E. coli**       |                           |                     |
| BL21(DE3)         | F- ompT hsdSdeLr.mcrA cml gal (DE3) torA | Lab collection |
| DH5α              | F- w80lacZ::M15 ΔlacZYA-argFΔU169 deoR recA1 endA1 hsdR17(rk-, mK') supE44 thi-1 gyrA96 relA1 λ- | Lab collection |
| SM10 Δpir         | thi thr leu tonA lacY supE recA::RPlpA-Tc-Mu Km λpir | Lab collection |
| TOP10             | mcrA Δ(mrr-hsdRMS-mcrBC) w80lacZ ΔM15 ΔlacX74 deoR recA1araD139 Δ(ara, leu) 7697 galU galK λ- rpsL endA1 mupG | Invitrogen        |
| **Plasmids**      |                           |                     |
| pACYC184          | Tc' Cm' cloning vector    | NEB                |
| pCR-Blunt         | Kan' cloning vector       | Invitrogen          |
| pDE-PhoPQ88       | S. bongori phoPQ cloned into pACYC184 | This study          |
| pDE-PhoPQ88Δ65    | S. bongori phoP harboring V169 cloned into pACYC184 | This study          |
| pDE-PhoPQ87ST     | S. Typhimurium phoPQ cloned into pACYC184 | This study          |
| pDE-SsrAB87       | S. Typhimurium ssrA8 cloned into pACYC184 | This study          |
| pET-2Ba(+)        | Kan' cloning vector for N-terminal His tag fusions | Novagen            |
| pRE112            | pGP704 suicide plasmid; pir dependent; sacB | [55]                |
| pMC1403           | Amp' lacZ cloning vector  | [30]                |
| pOG-His-PhoP      | N-terminus 6-His tag of PhoP in pET28-a | This study          |
| pOG-pagO-lacZ     | pagO fused to lacZ in pMC1403 | This study          |
| pOG-mig5-lacZ     | fkpA fused to lacZ in pMC1403 | This study          |
| pOG-PCR-ssel-RACE | RLM-RACE of ssel cloned into pCR-Blunt | This study          |
| pOG-ssel-lacZ     | ssel fused to lacZ in pMC1403 | This study          |
| pOG-ssel-lacZ-S1  | S1 mutation in ssel promoter fused to lacZ in pMC1403 | This study          |
| pOG-ssel-lacZ-S2  | S2 mutation in ssel promoter fused to lacZ in pMC1403 | This study          |
| pOG-ssel-lacZ-S1+2| S1+S2 mutations in ssel promoter fused to lacZ in pMC1403 | This study          |
| pOG-ybX-lacZ      | ybX fused to lacZ in pMC1403 | This study          |
| pUC19             | Amp' high-copy number cloning vector | NEB                |

*S, streptomycin; Cm, chloramphenicol; Kan, kanamycin.

dilutions were plated on LB to determine intracellular bacterial count. The remaining 100 μl of the cell suspension was directly used for β-galactosidase assay by adding 0.9 ml of Z buffer, three drops of chloroform, and two drops of 0.1% SDS followed by vortexing. An aliquot of 200 ml of ONPG (4 mg/ml) was added and reaction was stopped by the addition of 0.5 ml of 1 M Na2CO3. Following centrifugation, optical density of the supernatant was measured at 420 nm. The β-galactosidase activity was calculated according to: OD420/[l(min)xvolume (ml)], and was normalized according to the intracellular bacterial count.

**Quantitative real-time PCR analysis**

Salmonella RNA was extracted from late-exponential phase cultures that were grown in LPM medium for 6 h using the Qiagen RNAprotect Bacteria Reagent and the RNaseasy mini kit according to the manufacturer’s instructions, including an on-column DNase digest using the RNase-free DNase set (Qiagen). The quantity and quality of the extracted RNA were determined by an ND-1000 spectrophotometer [NanoDrop Technologies]. To diminish any genomic DNA contamination, RNA was secondarily treated with an RNase-free DNase I (Invitrogen). 0.5 μg of DNase I-treated RNA was subjected to a first strand cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR reactions were performed in an Applied Biosystems 7500 Fast Real-time PCR System. Each reaction was carried out in a total volume of 10 μl on a 96-well optical reaction plate (Applied Biosystems) containing 5 μl FastStart Universal SYBR Green Master (ROX) mix [Roche Applied Science], 1 μl cDNA,
and two gene-specific primers at a final concentration of 0.3 μM each. Real-time cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 s, 60°C for 1 min. No-template and no reverse-transcriptase controls were included for each primer set and template. Melting curve analysis verified that each reaction contained a single PCR product.

Relative quantification of sseL transcripts was evaluated using the comparative Ct method. The housekeeping gene, rpoD, was used as the endogenous normalization control. The ΔCt values were calculated by determining the difference in threshold values for sseL and rpoD in the wild-type vs. the mutant strains. Calculation of ΔΔCt involved the subtraction of the normalized wild-type ΔCt value from the normalized ΔCt value of the compared mutant. Fold-differences in gene expression were calculated as 2^{-ΔΔCt}.

Gene-specific primers were designed using PRIMER3 software (http:// primer3. sourceforge.net/), are listed in Table 2, and correspond to the following genes: rpoD, OG-220 and OG-221; sseL, OG-233 and OG-234.

5' RLM-RACE

RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was used to determine the transcription start site of sseL. Total RNA was extracted from S. Typhimurium SL1344 culture grown to late exponential phase in LPM medium under SPI-2 induction conditions. Rapid amplification of 5' cDNA ends was carried out using a FirstChoice RLM-RACE kit (Ambion) according to the manufacturer’s instructions, excluding a CIP treatment. Briefly, 300 ng of Salmonella extracted RNA was subjected to a Tabacco Acid Pyrophosphatase treatment followed by 5' RACE adapter ligation and reverse transcription reactions. The nested-PCR conditions for 5' outer PCR were 1 μl from the RT reaction, 10 pmol gene-specific outer primer OG-246, 10 pmol 5' RACE outer primer (Ambion), 2.5 units of PfuTurbo DNA polymerase (Stratagene), 5 μl of 10× PhiiTurbo PCR buffer, 4 μl of dNTP mix, and 35 μl of H2O. The PCR conditions were as follows: 1 cycle of 95°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s; and 1 cycle of 72°C for 10 min. The 5' RACE inner PCR reaction was carried out with 10 pmol gene-specific inner primer OG-245 and 10 pmol 5' RACE inner primer (Ambion) using the products generated with the outer primer set as a template. The same conditions as for the 5' outer PCR were used, but the annealing temperature was 53°C. The resulting PCR products were analyzed on 2% agarose gels, purified, and then cloned into pCR-Blunt (Invitrogen). Four individual clones were sequenced to determine the transcription initiation site of sseL.

Construction of S. Typhimurium His tagged PhoP

A 706-bp fragment containing the S. Typhimurium phoP gene was amplified by PCR using the primers OG-146 and OG-147. The resulting PCR fragment was cloned into pCR-Blunt, generating pOG-PCR-phoP. The latter was digested with NdeI and BamHI, and the resulting insert was cloned into the pET-28a vector (Novagen) digested with the same enzymes resulting in the plasmid pOG-His-PhoP. This plasmid expresses full-length S. Typhimurium PhoP fused to a His6 tag on the N terminus, with a predicted molecular mass of 27.8 kDa, under the control of the T7 promoter.

Over expression of His-tagged PhoP and protein purification

E. coli BL-21 (DE3) strain carrying pOG-His-PhoP was grown overnight in LB medium containing 30 μg/ml Kan at 37°C with vigorous aeration. The culture was diluted 1:200 into 300 ml of fresh LB and grown for 2.5 h. To induce expression of the recombinant protein, IPTG was added to 1 mM final concentration, and the culture was grown for an additional 3 h at 30°C and harvested by centrifugation. The pellet was resuspended in 10 ml lysis buffer [50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 1 mM MgCl2 in the presence of DNase I (5 μg/ml) and RNase A (10 μg/ml)]. Cells were disrupted using a French Press (three passages, 1000 PSI) and then centrifuged at 20,000 rpm for 30 min. His6-PhoP was purified from 1 ml of the soluble fraction by a nickel-affinity chromatography using AKTApurifier FPLC system (GE Healthcare) and a HiTrap FF 1 ml column (GE Healthcare) at 4°C. The column was washed with 5 ml of washing buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole and 1 mM MgCl2). His6-PhoP was eluted with 5 ml elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole and 1 mM MgCl2) and analyzed on an SDS-polyacrylamide gel. The selected elute fractions were then collected and dialyzed against dialysis buffer [20 mM HEPES (pH 8.0), 100 mM KCl, 20%...
glycerol, 1 mM DTT and 1 mM MgCl₂ on a 200-ml filter column. Dialyzed fractions were analyzed by UV absorbance at 280 nm and on an SDS-polyacrylamide gel. The appropriate fractions were pooled and concentrated using a 10 kDa Amicon Ultra centrifugal filter devices (Millipore). Protein samples were stored at −80°C until use.

Mobility shift assay

Gel retardation assays were carried out using the Dig Gel Shift Kit (Roche). A 129-bp fragment corresponding to the upstream regulatory sequences of sseL was generated using the primers OG-259 and OG-240 and labeled with digoxigenin (DIG)-dUTP on an SDS-polyacrylamide gel. The binding reactions were stopped by the addition of 5 µl of loading dye and were immediately resolved on a 6% nondenaturing polyacrylamide gel in 0.5 x Tris-glycine running buffer (25 mM Tris, 250 mM Glycine; pH 8.3) at 100 V. Subsequently, the gels were electroblotted onto a positively charged nylon membrane (Hybond-N+, Amersham Biosciences) in 0.5 x TBE buffer at 400 mA for 1 h and fixed by UV cross-linking. Detection of the DIG-labeled DNA probes by anti-DIG Fab fragment-alkaline phosphatase conjugate (Roche) in the presence of CSPD substrate was performed according to the manufacturer’s instructions.

Statistical analysis

Data of the β-galactosidase and the RT-PCR assays are expressed as mean ± standard error. The statistical significance between different values was calculated by the unpaired t-test with two-tailed P value. P<0.05 was considered to be statistically significant.

Acknowledgments

We would like to thank Dr. Amit Bhavsar for helpful assistance with protein purification and Prof. M. Hensel for the S. bongori SARC 12 strain.

Author Contributions

Conceived and designed the experiments: OG DE WD BF GR. Performed the experiments: OG DE WD. Analyzed the data: OG DE WD BF. Wrote the paper: OG WD BF GR.

References

1. Baumler AJ, Toleis RM, Feicht TA, Adams LG (1998) Evolution of host regulation in Salmonella enterica. Infect Immun 66: 4579-4587.
2. Ochsner H, Lawrence JG, Grossman EA (2000) Lateral gene transfer and the nature of bacterial innovation. Nature 405: 299-304.
3. Grossman EA, Ochsner H (1996) Pathogenicity islands: bacterial evolution in open genomes. Cell 87: 791-794.
4. Mills DM, Rajaj V, Lee CA (1995) A 40 kb chromosomal fragment encoding the regulatory sequences of Kit (Roche). A 128-bp fragment corresponding to the upstream
5. Kasahara M, Nakata A, Shinagawa H (1992) Molecular analysis of the invasion genes is absent from the corresponding region of Salmonella typhimurium. Infect Genet Evol 3: 1-9.
6. Ochsner H, Soncini FC, Solomon F, Grossman EA (1996) Identification of a pathogenicity island required for Salmonella survival in host cells. Proc Natl Acad Sci U S A 93: 7600-7804.
7. Shear JE, Hensel M, Gleeson C, Holden DW (1996) Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. Proc Natl Acad Sci U S A 93: 2593-2597.
8. Hensel M (2004) Evolution of pathogenicity islands of Salmonella enterica. Int J Med Microbiol 294: 169-192.
9. Coutelier EK, Lovenden MJ, Bishop JL, Wickham ME, Brown NF, et al. (2007) SwL is a Salmonella-specific translocated effector integrated into the SseL-related pathogenicity island 2 type III secretion system. Infect Immun 75: 574-580.
10. Rydmark A, Poh J, Garneny J, Boyle C, Thompson A, et al. (2007) SseL, a Salmonella drubhiquitine required for macrophage killing and virulence. Proc Natl Acad Sci U S A 104: 3502-3507.
11. Li Negrate G, Faustin B, Welsh K, Loeffler M, Krajewska M, et al. (2008) Salm...tial gene expression by PhoP. Proc Natl Acad Sci U S A 105: 1507-1512.
12. Liu H, Huang M, He C, Wang Y, Shi D, et al. (2007) Salmonella typhimurium promotes SsrA-ssrB in Salmonella pathogenicity island 2. J Bacteriol 189: 791-794.
13. Montague M, Martel A, Le Mosul H (2001) Characterization of the catalytic activities of the PhoQ histidine protein kinase of Salmonella enterica serovar Typhimurium. J Bacteriol 183: 1787-1794.
14. Martinez-Hackett E, Stock AM (1997) Structural relationships in the Ompr family of winged-helix transcription factors. J Mol Biol 269: 301-312.
15. Grossman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol 183: 1893-1893.
16. Miller SI, Kuwahara AM, Mekalanos JJ (1989) A two-component regulatory system (phoP/phoQ) controls Salmonella typhimurium virulence. Proc Natl Acad Sci U S A 86: 5054-5058.
17. Galan JE, Curtis R, 3rd (1989) Virulence and vaccine potential of phoB mutants of Salmonella typhimurium. Microb Pathog 6: 433-443.
18. Kettanna M, Nair S, Shinagawa H (1992) Molecular analysis of the Escherichia coli phoP-phoQ operon. J Bacteriol 174: 492-498.
19. Weisberger SR, Holden DW (2004) Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system. Cell Microbiol 5: 501-511.
20. Lee AK, Detwiler CS, Fallow S (2000) OmpR regulates the two-component system SsrA-SsrB in Salmonella pathogenicity island 2. J Bacteriol 182: 771-781.
21. Grossman EA, Ruiz-Arjona G, Lobo M, Feldman MD (2001) The role of SsrA-SsrB in the transcription of genes encoding the Salmonella typhimurium SPI-2 type III secretion system. Microbiology 147: 2835-2936.
22. Linehan SA, Rytkonen A, Yu XJ, Liu M, Holden DW (2005) SsrA regulates function of Salmonella pathogenicity island 2 SPI-2 and expression of SPI-2-associated genes. Infect Immun 73: 4354-4362.
23. Nakamura Y, Shinagawa H (1992) Molecular analysis of the invasion genes is absent from the corresponding region of Salmonella typhimurium. Infect Genet Evol 3: 1-9.
24. Kasahara M, Nakata A, Shinagawa H (1992) Molecular analysis of the invasion genes is absent from the corresponding region of Salmonella typhimurium. Infect Genet Evol 3: 1-9.
25. Kasahara M, Nakata A, Shinagawa H (1992) Molecular analysis of the invasion genes is absent from the corresponding region of Salmonella typhimurium. Infect Genet Evol 3: 1-9.
the regulator of two-component system sensing external magnesium availability. Mol Microbiol 45: 423–438.

38. Minagawa S, Ogasawara H, Kato A, Yamamoto K, Eguchi Y, et al. (2003) Identification and molecular characterization of the Mg2+ stimulon of Escherichia coli. J Bacteriol 185: 3906–3902.

39. Lejona S, Aguierre A, Cabeza ML, Garcia Vescovi E, Sounci FC (2003) Molecular characterization of the Mg2+ responsive PhoP-PhoQ regulon in Salmonella enterica. J Bacteriol 185: 3696–3702.

40. Monsieurs P, De Keersmaecker S, Navarre WW, Bader MW, De Smet F, et al. (2005) Comparison of the PhoPQ regulon in Escherichia coli and Salmonella typhimurium. J Mol Evol 60: 462–474.

41. Kenney LJ (2002) Structure/function relationships in OmpR and other winged-helix transcription factors. Curr Opin Microbiol 5: 135–141.

42. Feng X, Oropeza R, Kenney LJ (2005) Dual regulation by phospho-OmpR of ots/B gene expression in Salmonella pathogenicity island 2. Mol Microbiol 48: 1131–1143.

43. Perez JC, Groisman EA (2009) Evolution of transcriptional regulatory circuits in bacteria. Cell 138: 233–244.

44. Mangan S, Zaslaver A, Alon U (2003) The coherent feedforward loop serves as a sign-sensitive delay element in transcription networks. J Mol Biol 334: 197–204.

45. Kong W, Weatherspoon N, Shi Y (2008) Molecular mechanism for establishment of signal-dependent regulation in the PhoP/PhoQ system. J Biol Chem 185: 6287–6294.

46. Geourjon C, Deleage G (1995) SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. Comput Appl Biosci 11: 681–684.

47. Worley MJ, Ching KH, Heffron F (2000) Salmonella SsrB activates a global regulon of horizontally acquired genes. Mol Microbiol 36: 749–761.

48. Mangan S, Zaslaver A, Alon U (2005) The coherent feedforward loop serves as a sign-sensitive delay element in transcription networks. J Mol Biol 334: 197–204.

49. Muuson GP, Scott JR (2000) Rns, a virulence regulator within the AraC family, requires binding sites upstream and downstream of its own promoter to function as an activator. Mol Microbiol 36: 1391–1402.

50. Kenney LJ (2002) Structure/function relationships in OmpR and other winged-helix transcription factors. Curr Opin Microbiol 5: 135–141.