Coordinated Regulation of Virulence during Systemic Infection of *Salmonella enterica* Serovar Typhimurium

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Abstract

To cause a systemic infection, *Salmonella* must respond to many environmental cues during mouse infection and express specific subsets of genes in a temporal and spatial manner, but the regulatory pathways are poorly established. To unravel how micro-environmental signals are processed and integrated into coordinated action, we constructed in-frame non-polar deletions of 83 regulators inferred to play a role in *Salmonella enteriditis* Typhimurium (STM) virulence and tested them in three virulence assays (intraperitoneal (i.p.), and intragastric (i.g.) infection in BALB/c mice, and persistence in 129X1/SvJ mice). Overall, 35 regulators were identified whose absence attenuated virulence in at least one assay, and of those, 14 regulators were required for systemic mouse infection, the most stringent virulence assay. As a first step towards understanding the interplay between a pathogen and its host from a systems biology standpoint, we focused on these 14 genes. Transcriptional profiles were obtained for deletions of each of these 14 regulators grown under four different environmental conditions. These results, as well as publicly available transcriptional profiles, were analyzed using both network inference and cluster analysis algorithms. The analysis predicts a regulatory model and showed that the response regulator SsrB and the MarR type regulator, SlyA, are the terminal regulators in a cascade that integrates multiple signals. Furthermore, experiments to demonstrate epistatic relationships showed that SsrB can replace SlyA and, in some cases, SlyA can replace SsrB for expression of SPI-2 encoded virulence factors.

Introduction

Gastrointestinal infections are the second most common cause of childhood mortality in the developing world and Typhoid alone (caused by serovar Typhi) is estimated to result in 500,000 deaths per year [1]. In addition to fluid and electrolyte loss, non-typhoidal *Salmonella* often results in septicemia in children and in HIV infected adults in developing countries with a fatality rate of 25% or greater [2]. *Salmonella enteriditis* serotype Typhimurium (referred to simply as *Salmonella* or Typhimurium below) is a paradigm for understanding intracellular pathogenesis because of its established genetics and simple and inexpensive animal model - the mouse. All strains of *Salmonella enteriditis* share at least 95% sequence identity; the differences are associated with growth in a specific host or survival in an environmental niche. More than 4% of the entire genome is required for Typhimurium to infect the mouse [3]. These genes are widely distributed around the entire circular chromosome including many genes not involved in metabolic processes nor required for growth under laboratory conditions. Numerous studies have assigned a small fraction of these genes to specific steps in mouse infection but most are still a mystery. Many virulence genes are attributable to horizontally acquired DNA sequences that are not present in nonpathogenic but related bacteria. These regions include two 40 kb stretches of DNA termed *Salmonella* pathogenicity islands 1 (SPI-1) and 2 (SPI-2) [4–9]. SPI-1 and SPI-2 encode a secretion apparatus resembling a needle and related to the bacterial flagella that uses the proton motive force to secrete proteins directly into the cytoplasm of the eukaryotic cell [10]. Secretion can take place from extracellular bacteria that are juxtaposed to the surface of the cell or from intracellular bacteria located in vacuoles. The two type III secretion systems are expressed under different environmental conditions and play distinct roles in pathogenesis. SPI-2 is known to be required for systemic infection whereas SPI-1 plays an essential role during intestinal infection and in mouse persistence [11–14].

During the course of systemic infection in mice, bacteria are found within neutrophils, monocytes, dendritic cells, and B and T cells but are not found extracellularly until the last one or two days immediately before death of the mouse [15–17]. How *Salmonella* survives and replicates within the host and how it expresses virulence genes at the appropriate time during systemic infection is little understood and the subject of this work. Technological advances in the last 10 years such as microarrays, whole genome sequences, and global proteomics have provided a more complete picture of gene expression for a number of bacteria. The goal of the current work is to develop a predictive model for host-pathogen interactions that will provide insight into how *Salmonella* responds to specific conditions in the host. This approach was based on identification of regulators that were necessary for
Author Summary

We have used the intracellular pathogen Salmonella to investigate how pathogenic bacteria correctly express virulence factors during infection. After ingestion by its host, Salmonella confronts multiple challenging microenvironments such as acidic pH in the stomach, nutrient starvation, and host innate immune responses. However, Salmonella overcomes host defenses through expression of an array of virulence factors. We defined 14 Salmonella regulators out of 83 tested that are required for systemic infection. We then analyzed expression of Salmonella genes in each mutant strain grown under various conditions. Surprisingly, these regulators coordinately activate expression of a subset of virulence genes required for intracellular survival and replication. Computer-aided analysis based on our data established a regulatory network in which all 14 regulators are connected. Furthermore, the analysis identified many additional genes with no known function that are expressed under conditions identical to those that express known virulence factors. This work suggests that many environmental signals are processed through diverse regulators and integrated to induce virulence genes allowing Salmonella to survive and multiply within its host.

Salmonella to cause a systemic infection and transcriptional profiling of isogenic derivatives missing the regulator under a variety of growth conditions. The transcriptional profiles provided more than 300,000 data point, necessitating computer analysis. We have used SEBINI (Software Environment for Biological Network Inference; [18]) to directly compare multiple network algorithms. The network inference algorithm that we have used is the context likelihood of relatedness (CLR) to analyze the gene expression profiles [19]. CLR is an extension of the relevance network class of machine learning algorithms [20] and provides the highest precision of several algorithms tested [19]. At a 60% true positive rate, CLR identified 1,079 regulatory interactions in E. coli, of which 330 were in previously known networks and 741 were novel predictions (ibid). The analysis of our data provided a testable regulatory hierarchy and a list of genes with similar expression profiles as described below.

Results

Salmonella regulators required for systemic mouse infection

Typhimurium encodes a surfeit of regulators (more than 330 based on annotation cited in NCBI) presumably because it can survive and replicate in many different environments, cause infection in diverse hosts, and can use multiple carbon sources and terminal electron acceptors. We focused on 83 regulators presumed to play roles in virulence, based on published data including negative selection experiments [13,21–23]. We constructed non-polar in-frame deletions in which each regulator gene was replaced with a "scar" sequence using bacteriophage lambda-mediated recombination [24,25]. The list of 83 regulator genes is provided in Supporting Information (Table S1). For the two-component regulator ssrA/ssrB we constructed in-frame deletions missing ssrA, ssrB or both; for the other two two-component regulators, phoP/phoQ and ompR/envZ, both the signal sensor and response regulator were deleted. As an initial screen two 4–6 weeks-old BALB/c mice were infected intraperitoneally (i.p.) with 200 colony forming units (CFU) of each deleted strain (about 100× the LD50). Mutations that resulted in either no deaths, one death, or delayed death of infected mice were retested with groups of 5 mice at the same dose (Figure 1A). Based on this preliminary screen we chose the regulators spaR, fruR, himD, phoP/phoQ, ssrA/ssrB, slyA, hnr, tpoE, smpB, csrA, rpoS, crp, ompR/envZ, and hfq for further investigation (see Figure 1 and Table 1 for references and descriptions; a complete list of virulence phenotypes is provided in Table S1). It is possible that additional regulators would be identified if a larger group of mice were used in the initial screen. The LD50 for each of the mutants was computed as shown in

Figure 1. Attenuation of S. typhimurium strains encoding deletions of the indicated regulatory gene. A. Five BALB/c mice were i.p. infected with ~200 cfu (approximately 100 × LD50) of the Salmonella strain indicated and observed for 21 days. The percentages of surviving mice are shown for each strain. Salmonella strains lacking smpB, hnr, csrA, fruR, or crp caused death of some mice during the observation period and the other strains indicated with an asterisk resulted in no deaths. B. Each Salmonella strain was administered i.p. to 3 groups of 3 BALB/c mice at 1 × 10⁵, 1 × 10⁴, and 1 × 10³ cfu respectively and monitored for 1 month to estimate the approximate LD50 values.

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Figure 1B. Compared to the parental strain (ATCC14028), which has an LD50 of 1–2 cfu, all of the derivatives were attenuated for virulence. The 14 deletion strains lacking regulators reported in this study were selected from i.p. BALB/c mice infection to eliminate other regulators required only for gastrointestinal infection or persistence but most of these strains were avirulent in another strain of mice (129X1/SvJ) and by other route (intragastric) of infection (see Table S1). Therefore the regulators investigated in this study might be considered the most central regulators for virulence. In the other virulence assays, the 83 derivatives were tested for virulence by intragastric (i.g.) BALB/c infection and by a more sensitive competitive index (CI) experiment. In the competitive index experiment all mutants were co-administered to 129X1/SvJ mice and the number of each surviving mutant bacteria was determined 7 days after i.p. inoculation. BALB/c mice are missing natural resistance-associated macrophage protein (Nramp1) and thus succumb to Typhimurium infection within a week when mice are infected peritoneally with less than 10 bacteria of the strain employed here (14028). Unlike BALB/c, 129X1/SvJ mice have a functional copy of Nramp1 and the bacteria persist for several weeks without killing the mouse [13,26]. The CI test identified 30 (out of 83) regulator mutants that were attenuated in comparison to the wild type control (see Table S1). The 14 derivatives chosen for this study were among those showing the poorest survival by comparison to the parent in this competitive index experiment.

The 14 regulators identified were diverse and included alternative sigma factors (rhoS and rhoE), two-component regulators (ompR/envZ, phoP/phoQ, and ssaA/ssrB), a response regulator for which the signal sensor is unknown (hnr or mvdA), post-transcriptional regulators (csrA, hflQ and smpB), a binding protein essential for some types of recombination (hflQ) and an assortment of other DNA binding proteins (hnrR, rhoR, crp, and slyA). Transcription profiling was carried out for isogenic deletion mutants in each of these 14 genes under four different conditions and analyzed to infer a regulatory hierarchy during systemic infection. Salmonella transcription profiles for the following regulators have already been reported: rhoE [27]; phoP/phoQ [28]; ssaA/ssrB [29]; csrA [30]; slyA [31]; hflQ [32]; hflQ [33]. Our results closely match these published results.

The large degree of attenuation we observed for an spoR mutation following i.p. infection was surprising, as most studies had limited its effect to the i.g. route of infection. To validate this result we complemented the spoR mutation in trans and found complete complementation in mouse virulence (Figure S1). The differences between our results and others are most likely related to the different strains of Salmonella used in the studies [34]. For the other mutations we have mobilized the mutation into a new genetic background by P22 transduction to ensure that there is no secondary mutation that influences the results [35].

The ability of Salmonella to survive and replicate in macrophages is necessary but not sufficient for mouse virulence

Previous work demonstrated that Salmonella mutants that were unable to survive within elicited peritoneal macrophages were attenuated for virulence during systemic mouse infection [36]. In fact, fluorescence-activated cell sorting analysis of infected blood and spleen using Salmonella that expresses green fluorescent protein does not identify any extracellular bacteria [16,37,38]. Salmonella is within blood monocytes and in other WBC in the spleen including neutrophils, dendritic cells, and B and T cells in these reports (ibid). It is possible that growth in cells types other than macrophages is necessary for Salmonella to cause a systemic infection in mice following i.p. administration. Thus, some of the regulatory mutations described here may affect growth in cells types other than macrophages. We therefore wished to determine if there is a direct relationship between growth in macrophages and mouse virulence. In these studies we used primary bone marrow-derived macrophages (BMDM) from the same strain of mouse as used in the original identification of attenuated regulatory mutants (BALB/c). The identical number of input bacteria and the identical number of macrophages were used in every infection experiment. As observed by others, following phagocytosis there is some bacterial killing that varied from strain to strain followed by intracellular growth. We monitored the number of intracellular bacteria at an early time (30 min) to determine the number of bacteria internalized (Figure 2). Even at the shortest time few intracellular bacteria were recovered from macrophage infection with an spoE mutant, suggesting that this

| Gene no. | Gene symbol | Description | Reference |
|----------|-------------|-------------|-----------|
| pSLT041  | spoR        | Salmonella plasmid virulence: regulation of spo operon (LysR family) | [42]       |
| STM0118  | rhoR        | transcriptional repressor of rho operon and others (GlnR/Lac family) | [95]       |
| STM0982  | himD        | integration host factor (IHF), beta subunit; site-specific recombination | [32]       |
| STM1230/1| phoP/phoQ   | two-component regulatory system responding to Mg^2+ | [28]       |
| STM1391/2| ssaB/ssrA   | two-component regulatory system | [7,8]      |
| STM1444  | slyA        | transcriptional regulator for hemolysin (MarR family) | [31]       |
| STM1753  | hnr         | response regulator in protein turnover | [96]       |
| STM2640  | rpoE        | sigma E factor of RNA polymerase, response to periplasmic stress | [27]       |
| STM2688  | smpB        | small protein B; tmRNA-binding protein | [97]       |
| STM2826  | csrA        | carbon storage regulator | [30]       |
| STM2924  | rhoS        | sigma S factor of RNA polymerase, major sigma factor during stationary phase | [98]       |
| STM3466  | crp         | catabolite activator protein (CAP), cyclic AMP receptor protein (CRP family) | [99]       |
| STM3501/2| envZ/ompR   | two-component regulatory system responding to osmolarity and pH | [100]      |
| STM4361  | hflQ        | host factor f for bacteriophage Q beta replication, a growth-related protein | [33]       |

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Table 1. List of virulence regulators analyzed in this study, gene number, gene symbol, description and reference.
strain is very sensitive to microbicidal factors released by macrophages on contact with bacteria or doesn’t get internalized very well. At 2.0 hrs post infection there was a decrease in bacterial numbers that varied from strain to strain presumably reflecting variation in the sensitivity to bacterial killing by the oxidative burst, acidic pH, and antimicrobial peptides. Finally, at 18 hrs bacterial numbers were enumerated to monitor intracellular replication as well as the ability to withstand nitrous oxide oxidation and other late antimicrobial factors [39,40]. No effect was found at any time point for a mutant in the plasmid-encoded regulator \( \text{spvR} \) in murine macrophages in agreement with other investigators [41,42]. Small differences in intracellular growth were observed for \( \text{ssrA}/\text{ssrB} \) and \( \text{spvA} \) compared to the parent although larger differences have been observed previously [43–45] perhaps reflecting BMDM preparation techniques, bacterial strain differences, or opsonization differences [46]. Mutations of \( \text{himD} \), \( \text{rpoE} \), \( \text{crp} \), or \( \text{hfq} \) drastically reduce the number of viable bacteria that can be recovered from macrophages even at short times (Figure 2). These results also demonstrated that growth in macrophages \( \text{per se} \) does not duplicate \( \text{in vivo} \) infection and that some regulator mutants that were totally avirulent in the mouse showed no differences in growth in these primary macrophages.

Transcription profiling of avirulent strains

Because some of the Typhimurium regulator mutants survived so poorly within macrophages that preparing mRNA from intracellular bacteria was not possible we have used \( \text{in vitro} \) growth conditions that duplicate some of the intracellular conditions [low pH, minimal media; [47,48]]. The 14 virulence regulators, whose absence was identified by attenuated virulence \( \text{in vivo} \) in this study, presumably sense specific environmental signals within the host and respond by expressing the appropriate complement of virulence factors. However the specific environmental signals are not known and therefore each strain was grown in four different conditions; to log phase or stationary phase in Luria-Bertani (LB) broth and in a low pH/low magnesium, minimal medium [47,48].

Two different minimal media conditions were used depending only on pre-growth conditions as described in the Materials and Methods (called acidic minimal media (AMM)1 and AMM2 here); these conditions are identical to conditions previously reported for expression of SPI-2; [47,48]. The fate of intracellular Typhimurium is determined by pre-growth conditions prior to macrophage infection and for that reason we used both conditions AMM1 and 2 [49]. To identify global transcriptional changes for each regulator, mRNAs were isolated from each strain grown under the four conditions, converted to cDNA, and used to probe a spotted non-redundant pan-Salmonella orf microarray using hybridization of total genomic Salmonella DNA as an internal control [50]. Each microarray was probed with RNA from the parent or an isogenic deletion of one of the regulator mutants prepared from bacterial cells grown under one of the four conditions. In all there were 4 biological replicates of the parent strain but one for each mutant under each growth condition. Transcriptional profiles were significantly different from the parent for known virulence factors required during systemic infection, but only if cells were grown in acidic minimal media (AMM1 or AMM2; see Figure 3; complete microarray data available at http://www.ohsu.edu/microbiology/heffron/r01.html). Z-scores (score—mean/standard deviation) were computed for each gene in the Typhimurium genome in each mutant background under each growth condition (Table S2). Statistical analysis of the complete transcriptional data for gene expression in AMM1 identified at least 237 genes that were reduced 4-fold or more in common comparing the mutations to the parent strain (at least 3 standard deviation based on the technical replicates). Because transcription is reduced in the mutant compared to the parent, it suggests that normally the regulator activates transcription of these genes as is shown dramatically in Figure 3 for all SPI-2 encoded virulence genes. Conversely, only 45 genes showed a 4-fold or more up-regulation in the mutant backgrounds when cells were prepared under the same growth condition, suggesting that the regulator normally represses these genes. A more precise statistical
analysis of genes that are co-regulated with SPI-2 is provided below (Table 2). All in all, these results suggest that the normal function of each of the 14 regulators is to activate transcription of virulence factors necessary for systemic infection but only under specific environmental conditions. Acidic minimal media (AMM1) provides the best induction condition of those that were examined.

Validation of SPI-2 regulation

SPI-2 encodes a type III secretion system and secreted effectors required for systemic mouse infection [8,9]. To assess the effects of growth conditions on expression of the SPI-2 secretion apparatus we constructed a \( \text{lacZ} \) transcriptional fusion to \( ssaG \), a component of the secretion apparatus, and tested expression in each mutant background under each of the four growth conditions. At the same time we determined transcript levels via quantitative real-time PCR (qRT-PCR), using transcripts from \( rpoD \) and \( gyrB \) as controls [51]. We observed that the results determined by these two methods matched closely (Figure S2) and that the level of transcription of \( ssaG \) was highest when \( \text{Salmonella} \) was grown in minimal acidic media. In agreement with the microarrays, the effect of these regulators on \( ssaG \) expression was minimal if the bacteria were grown in rich media (LB broth). Because the type III secretion system and associated virulence factors encoded within SPI-2 were most highly expressed in acidic minimal media we therefore focused on growth in this media and used qRT-PCR to measure transcript levels. To validate the transcriptional profiles we prepared RNA from mutants and parent bacteria grown in acidic minimal medium and used as template for qRT-PCR. Six promoters have been identified for the type III secretion system encoded within SPI-2 (see Figure 4; [52]). We monitored transcription of seven genes within SPI-2, covering each operon at least once, and used \( gyrB \) transcript as an internal control. We observed a decrease in transcription of all 7 genes in 11 of the 14 mutants. The three exceptions were \( \text{spvR}, \text{fruR}, \text{and rpoS} \). Strains containing mutations in \( \text{phoP/} \text{phoQ}, \text{sdhA}, \text{ssaB}, \text{dfmA}, \text{and} \text{ompR/envZ} \) showed at least 100-fold decreases in transcription of all SPI-2 genes (Figure 4). Mutations of \( \text{ihf} \) (\( \text{himD} \)) and \( \text{csrA} \) showed an intermediate level of 8-16-fold decreased transcription whereas \( \text{hnr} \), \( \text{rpoE} \), \( \text{smpB} \), \( \text{crp} \) and \( \text{hfq} \) showed a modest decrease of 2-8-fold. The results of this analysis are generally concordant with the microarray results although the dynamic range was larger for qRT-PCR than for the microarrays as has been observed before [53]. Note that \( \text{rpoE} \) and \( \text{hfq} \) mutants showed a dramatic reduction in macrophage survival, but little difference in transcription of

![Figure 3. Transcriptional profiles of SPI-2 genes encoding components of the type III secretion apparatus.](https://example.com/figure3.png)

In each case we have computed the ratio of the microarray results for a given gene in a specific mutant background to the results of the parental strain grown under identical conditions. The results were determined under four different growth conditions (from left to right rich medium (LB) logarithmic phase, LB stationary phase, acidic minimal medium 1 (AMM1) and AMM2). Rows correspond to individual genes located within SPI-2 while columns represent regulator mutants. A \( crp \) deletion was excluded from transcriptional profiling for AMM1 because it does not grow under this condition. Blue represents a decrease in the mutant and yellow an increase using the computer program Genesis for display (log2 values; [94]). Thus blue indicates genes that are positively regulated (i.e. the level of expression decreases in the corresponding mutation compared to the parent). The results show that the 14 virulence regulators under study activate expression of most genes located in SPI-2 but the effect is only observed when bacteria are grown in minimal acidic media.
Cluster analysis identifies additional genes that are co-regulated with known virulence factors

The results from the microarray analysis identified many genes that appeared to be coordinately regulated including all SPI-2 encoded genes that are part of the type III secretion system (Table S2). We searched for additional previously unidentified virulence factors encoded elsewhere on the chromosome that show the same pattern of expression as those located within SPI-2. A systematic way of identifying co-regulation is with one of several computer algorithms that group genes together if their expression is similarly changed under like conditions. The diverse growth conditions used

SPI-2 genes during growth in AMM. It has recently been reported that the translational regulator Hfq, regulates translation of RpoE explaining in part why the two mutations behave similarly [54]. Furthermore, Hfq regulates translation of more than 20% of all Salmonella proteins explaining why a mutation in this gene has such a dramatic phenotype ([54]; Charles Ansong, Hyunjin Yoon, Steffen Porwollik, Heather Mottaz-Brewer, Briana Ogata-Petriis, Navdeep Jaitly, Joshua N. Adkins, Michael McClelland, Fred Heffron, and Richard D. Smith; Global systems-level analysis of small RNA-mediated translational regulation: Implications for virulence and global protein translation; Submitted).
in this study, as well as differences between isogenic strains containing mutations in virulence regulators, make our data set ideal for this type of analysis. In addition, we also performed cluster analysis using public data sets acquired from the NIH sponsored public repository of microarray data, gene expression omnibus (GEO). We chose 120 transcriptional profiles from GEO that were derived by extracting RNA from the same parent Salmonella strain grown under a wide variety of culture conditions (GSE2456; G. Yun et al., unpublished data). Expression profiles for all genes were uploaded to SEBINI (Software Environment for

Figure 4. Validation of microarray results by qRT-PCR for 7 genes located within SPI-2. The bottom part of the figure shows a map of the type III secretion system located within SPI-2 with the genes that were quantified colored the same as the corresponding bars above. The top part of the figure shows expression of 7 SPI-2 genes in each regulator mutant in comparison to the parental strain when grown in AMM1. The results are plotted on a logarithmic scale. Values were normalized using *gyrB* mRNA level and represent the average of RNA prepared from three independent biological samples. The results show that mutations in 6 regulators (*himD*, *phoPQ*, *ssrA/ssrB*, *slyA*, *csrA*, and *ompR/envZ*) strongly decrease expression of SPI-2, in 5 strains there is a significant decrease (*hnr*, *rpoE*, *smpB*, *crp*, *hfq*) while in the remaining 3 no decrease or even an increase is observed for *fruR* (the others showing no change are *spvR* and *rpoS*).

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Biological Network Inference [18]). SEBINI incorporates statistical and network algorithms into a framework for network inference [55,56]. To detect dependencies among genes over different conditions we employed the context likelihood of relatedness algorithm (CLR; [19]), which is a plugin for SEBINI. The results were visualized as a network of similarity relationships using Cytoscape [57]. A recent study with *E. coli* shows that the CLR algorithm was the most accurate in computing the correct network for experimentally verified regulatory interactions [19]. The results shown in Figure 5 use a force-directed network layout algorithm where genes (shown as small colored circles) are generally closer together when their statistical association, and thus degree of predicted co-regulation, is stronger (the cutoff for the genes shown is 5 standard deviations from the mean or greater; *p* < .0001). All SPI-2 secretion apparatus genes as well as associated effectors and chaperones were found to very tightly cluster in the network. In support of this conclusion, the cluster analyses based on our data and based on publicly available data sets are consistent. The cluster analysis identified 92 genes that are co-regulated with the SPI-2 encoded type III secretion system but not located within SPI-2. This includes known SPI-2 secreted effectors encoded elsewhere on the chromosome as well as many genes for which no function has been assigned (all total 123 genes; see Table 2). Several of these genes are A+T rich (>60% compared to 48% for the *Salmonella* chromosome) and located within sequences that are not present in close relatives of *Salmonella* suggesting that they may be unidentified secreted effectors or other virulence determinants that have been acquired from other pathogens. All in all, the implication of the cluster data is that the genes shown in Table 2 are co-regulated with SPI-2. One interesting point is that the algorithm does not distinguish between positive and negative regulation only that it is coincident. We analyzed expression of each of the 123 genes and found that only two genes (pepA and mopB) are negatively regulated; the remaining 121 are positively regulated.

### Computer aided analysis of the regulatory network

The fact that all of the 14 regulators in this study have biological functions during infection suggests they could be part of a coordinated network. Acidic minimal medium is a well-established *in vitro* condition for SPI-2 expression; accordingly, we focused on examining regulation of genes expressed under these conditions [47,48,58]. To identify coordinated regulation we compared expression of each regulator in each mutant background when grown in minimal acidic medium (Figure 6A). RNA samples were prepared from three separate cultures and used as a template in separate qRT-PCR experiments. A matrix with 14 mutants in columns and 14 regulatory genes and SPI-2 genes (6 genes except for *ssrB* as used in Figure 4) in rows was constructed based on qRT-PCR data and *z*-scores were calculated based on average and standard deviations from columns and rows. A network among regulators and SPI-2 was mapped as described in Lee *et al.* by sorting out values that changed in a specific mutant background [59]. We visualized the resulting relationships using Cytoscape.
Coordinated Regulation of *Salmonella* Virulence

Nodes indicate regulators or SPI-2 and red and blue arrows indicate activation and repression respectively (see Figure 6B). In the computed network multiple regulators act both directly and indirectly to control SPI-2 expression. However, direct or indirect regulation cannot be distinguished without additional experimental verification. So, in Figure 6B all regulatory effects on SPI-2 have been removed except for those mediated directly by *slyA* and *ssrB* based on additional genetic data as described below. The network suggests that both *slyA* and *ssrA/ssrB* could coordinate regulation of SPI-2 and other virulence factors by integrating signals from multiple regulators as was tested next. An overall network was also generated by integrating 4 data sets; two CLR algorithm data from the complete microarray results and GSE2456 public microarray database and two matrix analysis data from the transcription profiles and qRT-PCR results (Figure S3; the limitation is that no distinction is made between positive and negative regulation in CLR algorithm data). The consensus network combining all data reported in this study includes the network computed from qRT-PCR (Figure 6B) in part and suggests a predictive regulatory cascade that merits a test.

**SsrB can complement other regulators of SPI-2 transcription**

SsrB binds within SPI-2 and activates SPI-2 genes for transcription. However the location of binding sites varies suggesting that SsrB regulation is unusual [52]. Some of the 14 regulators in this study have been previously shown to directly regulate *ssrA/ssrB*; OmpR binding to P_{ssrA/ssrB} [60,61]; SlyA binding to P_{ssrA} [62]; PhoP binding to P_{ssrB} [45]. To distinguish direct from indirect effects, we carried out epistasis experiments and determined if *ssrB* expression could suppress the phenotype of the other regulators of SPI-2 expression. Each isogenic derivative was transformed with either pBAD30SsrB or the empty vector control. We investigated the same seven SPI-2 genes as in the qRT-PCR assays above, and results are shown in Figure 7A [52]. Expression of an episomal copy of *ssrB* resulted in expression of SPI-2 genes in each mutant background, suggesting that SsrB is epistatic to other regulators for SPI-2 transcription. This result is in agreement with the model shown in Figure 6 where all positive regulation takes place via either *ssrB* or *slyA* or both as tested further below. Furthermore, SsrB could complement all three deletions Δ*ssrB*, Δ*ssrA*, and Δ*ssrA/ssrB* (Figure 7B), suggesting that over-expression of *SSrB* may compensate for differences in phosphorylation that normally play a role in regulation [52].

**SlyA can complement other regulators for SPI-2 expression**

The number of inputs at each regulator shown in Figure 6B corresponds to the number of regulators that directly or indirectly control its expression. The number of regulators that act on *slyA* was surprising. Quantitative RT-PCR confirmed that *slyA* transcription was reduced in 11 of 14 mutant backgrounds (Figure 6A). *slyA* transcriptional activation by PhoP has been reported [63,64]. The complementation result suggests that many of the regulators may function on SPI-2 through SlyA activation of *ssrB* or alternatively via both regulators (*slyA* and *ssrB*) acting together. To determine epistatic relationships, we introduced a *slyA*-expressing plasmid, pBAD30SlyA, into each regulatory mutant and examined complementation in each mutant background compared to an empty vector control (Figure 7C). The construct strongly complemented *ompR*: *ompR*, *phoP*, *phoQ*, *cscA*, and *hmdD*, suggesting that the effect of these regulators on SPI-2 expression may be indirect via regulation of *slyA*. Surprisingly, the expression of SPI-2 genes depended upon *slyA* even in an *ssrA/ssrB* background.

![Figure 6. A model for regulatory interactions. A.](image-url)

**Figure 6. A model for regulatory interactions. A.** Transcription of all 14 regulatory genes in each regulator mutant background was determined by qRT-PCR using RNA isolated from bacteria grown in AMM1. Horizontal axis represents the genes under study; vertical represents an in-frame deletion of the regulator indicated. Values shown were normalized to gyrB mRNA level. Color intensity represents an average expression ratio between mutant and parent in a log2 scale from three independent RNA samples; blue and yellow blocks indicate activation and repression by corresponding regulators respectively. Results are displayed in Genesis (log2) [94].

**B.** Cytoscape image of the hierarchical order of regulation under SPI-2 inducing conditions was constructed based on qRT-PCR data as described in Lee et al. [59]. Nodes indicate regulators or SPI-2 and red and blue arrows indicate activation and repression described as "edges". Six SPI-2 genes (*ssaE*, *ssrA*, *ssaA*, *ssaG*, *ssaH*, and *ssaN*) were used in the matrix construction and depicted as one node in the network for the simplicity. The arrows or edges do not distinguish between direct and indirect effects except as determined experimentally. A similar regulatory hierarchy was predicted using the CLR algorithm data and matrix analysis data based on all of the expression data and is included as Figure S3.

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Figure 7. Determination of epistatic relationships among the regulators. A. To determine if expressing ssrB in trans can compensate for mutations in the 14 regulators we monitored transcription of 7 SPI-2 genes by qRT-PCR (triplicate biological samples, normalized results shown in each case compared to the empty vector control). Each strain containing a specific regulator deletion was transformed with pBAD30SsrB or pBAD30 and grown under AMM1 condition in the presence of 0.02% L-arabinose for 4 hours. The results are presented as expression ratio comparing the strain that over-expresses ssrB to the empty vector control displayed in a logarithmic scale. The values shown are averages from three separate RNA samples and grouped into three parts based on the magnitude of the effect. The results show that ssrB transcription is sufficient to up regulate transcription of these 7 SPI-2 genes in each mutant background. The magnitude of the effect varied from several hundred-fold to 2-fold for cpr.

B. ssrB was expressed in strains missing the response regulator (ssrB), the signal sensor (ssrA), or both and β-galactosidase expression was monitored using P:ssaG::lacZ (pFssaGTC; see Materials and Methods for details). The results show that ssrA is not necessary for ssrB to induce transcription of downstream genes (in this case ssaG) at least when ssrB is over-expressed.

C. Complementation with pBAD30SlyA was performed in the same way as in Fig. 7 A in the 15 strains being investigated. The results show that ssrA is not necessary for ssrB to induce transcription of downstream genes (in this case ssaG) at least when ssrB is over-expressed.

D. slyA expression increases transcription of some SPI-2 genes even when ssrB is deleted. SsrB-independent SlyA activation on SPI-2 was further tested by slyA expression in double deletions of himD/ssrAB, phoPQ/ssrAB, slyA/ssrAB,
deleted strain. This suggests that \textit{slyA}\ can regulate expression of SPI-2 in a way that is independent of \textit{ssB}. To test this possibility we constructed double deletions of \textit{ssAB}/\textit{ompRenvZ}, \textit{ssEB}/\textit{phoPQ}, \textit{ssAB}/\textit{csrA} and \textit{ssAB}/\textit{himD}. As shown in Figure 7D, \textit{slyA}\ is capable of activating expression of SPI-2 genes independently of \textit{ssB}, \textit{ompRenvZ}, \textit{phoPQ}, \textit{csrA} and \textit{himD} (\textit{shf}), although the effects are not as strong as \textit{ssB}. Furthermore there was a clear dichotomy in expression between the first two SPI-2 operons encoding \textit{ssaB-ssaE} and \textit{ssaA-ssaG} and the operons further downstream (\textit{ssaF-ssaQ}) suggesting that \textit{slyA}\ may act at different sites within SPI-2. However, these experiments are based on over-expression of \textit{slyA}\ that may complicate the analysis because of binding to sites that would normally not be occupied.

**Discussion**

During systemic mouse infection, \textit{Salmonella} processes multiple environmental cues via more than 14 regulators

We performed virulence assays on 83 regulators previously identified as required for \textit{Salmonella enteritidis} virulence by one or more negative selection experiments in various hosts including calves, chickens, and mice [13,21–23]. The mutant strains devoid of 83 regulators were tested in three mouse virulence assays to identify a subset of 14 that were most highly attenuated in a systemic mouse infection model. These 14 regulators are very diverse including alternative sigma factors (\textit{tpoS} \textit{tpoE}), two-component regulators (\textit{ompRenvZ}, \textit{phoPQ}, and \textit{ssAR}), post-transcriptional regulators (\textit{csrA}, \textit{hflC} and \textit{ampB}), a binding protein (\textit{hsf}) and an assortment of other DNA binding proteins (\textit{fruR}, \textit{spvR}, \textit{crp}, \textit{shy}, and \textit{hnr}; see Table 1 for references). \textit{Salmonella} follows a short course of infection after intraperitoneal infection, as we have used here, passing through the lymph nodes in the peritoneal cavity to the blood stream and then colonizing and replicating within the spleen and liver. During this entire trip the bacteria are located within cells either neutrophilcs or monocytes although at lower numbers in B and T cells and dendritic cells of every subclass [16,37,38,65,66]. Because the bacteria are exclusively intracellular we tested the hypothesis that replication in macrophages could be a surrogate for systemic mouse infection. Mutations that were attenuated for growth in primary macrophages were also attenuated within the mouse but the converse was not always true. Next, we used expression profiling to define the regulatory pathways. Transcriptional profiles from intracellular bacteria at different times after infection are likely to match most closely the environments encountered by \textit{Salmonella} during infection. However, isolation of RNA from intracellular bacteria was not used because we wished to test a spectrum of regulatory mutants several of which simply did not survive within cells long enough to allow RNA preparation. RNA was therefore prepared using four laboratory growth conditions two of which partially mimic the intracellular environment (acidic minimal media).

We compared the transcriptional profiles we observed using laboratory growth conditions that mimic intracellular conditions, to those that have been performed during intracellular replication within J774 macrophage cells. We computed the z-score for each \textit{Salmonella} gene from our data and from the data provided by Eriksson et al. [67] thus providing a value that can be compared across different experiments. To compare the two sets of data we subtracted the z-scores computed from our data for \textit{AMM1} from that computed from expression profiles of intracellular \textit{Salmonella}. As the expression pattern changes with time after infection we computed the difference for each time as well as an average for all three-time points reported (4, 8, and 12 hours after infection). There were 102 genes where the difference in z-score was 2 or greater, and 54 genes of 102 were annotated as putative, hypothetical, or conserved hypothetical. Four of the 5 most strongly differentially induced genes include magnesium transporters (\textit{mgdB} and \textit{mgdC}), an acid shock protein (\textit{STM1485}), and a high affinity phosphate transporter (\textit{pstB}) suggesting that the acidic minimal media we used may not be low enough in magnesium, phosphate, or may not be sufficiently acidic. Many genes are transcribed inside cells but may be only weakly transcribed or not at all transcribed in acidic minimal media. Examples of such genes include \textit{slyA}\ and the operon STM5117–3120. This operon encodes some of the most abundantly expressed proteins by intracellular \textit{Salmonella}. This result is striking, given that STM5117–3120 are not transcribed under a variety of \textit{in vitro} conditions including \textit{AMM1} and 2 and the many conditions corresponding to the transcriptional profiles reported for microarrays archived in GEO ([68], J. McDermott and L. Shi, Unpubl. Obs.). The nature of the inducing signal(s) that results in expression of these genes during intracellular growth is not known.

The regulatory network controlling expression of the genes necessary for systemic infection is complex. In our transcriptional network each pink node represents a regulator (Figure 6B) and lines represent positive or negative transcriptional interactions (positive in red and negative in blue). In \textit{E. coli} most regulation has been shown to follow one of three motifs: feedforward in which a regulator controls a second regulator, single input in which a regulator uniquely controls a set of downstream genes, and so called dense overlapping regulons in which there are multiple regulatory inputs to a single operon [69]. A feedforward loop can act as an electronic “AND-gate” preventing expression except when two or more signals are sensed as we see for \textit{hsf} (upstream) and \textit{ssB} (downstream); \textit{fruR} (upstream) and \textit{crp} (downstream). Many of these predicted relationships have been demonstrated already but some are new and merit further investigation. The single input motif is found in systems of genes that form a protein complex such as both of the type III secretion systems in \textit{Salmonella}. For SPI-2 \textit{ssB} plays this role and for SPI-1 \textit{bshA} is the central regulator. The multiple promoters located within SPI-2 presumably respond to differences in \textit{ssB}/\textit{slyA} activation, perhaps establishing part of the temporal order of expression following phagocytosis of \textit{Salmonella}. There are other regulators required for systemic infection in \textit{BALB/c} mice, including those whose absence reduces viability without a compensating mutation (\textit{hnr}; [70]), those that require deletion of two unlinked genes for inactivation (\textit{ppGpp}; reviewed in [71] and \textit{ydqT/hha} [48]), or those that were simply missed in the screens (STM0410; [72]; these regulators will be included in subsequent analyses.

**What are the signals being sensed during systemic infection and how are they integrated to express virulence factors appropriately?**

There have been several studies to identify environmental factors that regulate expression of the type III secretion system encoded within SPI-2. Carbon limitation, low concentrations of
Mg$^{2+}$ or Ca$^{2+}$ [73], and acidic pH [47,61] induce SPI-2 expression in vitro. Inside professional phagocytic cells, divalent cation concentrations and the presence of defense-like molecules signal through phoP/phoQ [74,73], and acidic pH and osmolarity signal through ampR [76]. AmpR has been shown to respond to acidic pH via conA [77]. The signal(s) received by SsrA, the sensor of the SsrA/SsrB system, is not yet established. It is surprising that over-expression of srrA or srrA/srrB can compensate for a deletion of ssaI or ssaJ/ssrC. Previous results have shown that a conservative replacement of the amino acid that is the essential phosphate acceptor eliminated expression of SPI-2 genes suggesting that SsrB requires phosphorylation for activity. Yet, expression of srrA without ssaI results in expression of all 7 SPI-2 genes examined (Figure 7A). It is possible that SsrB can be phosphorylated from other sources as noted by Walthers et al. [52] or that the over-expression results in dimerization and self activation similar to what has been observed for PhoP [78].

Mutations in rpoE, smpB, rpoS, and hfg showed only small decreases in SPI-2 gene transcription during growth in minimal acidic media but some of them showed large defects in survival assays performed in murine macrophages. For rpoE and rpoS it is possible that the environmental conditions that distinguish growth in minimal media from those in the SCV inside hosts are sensed via these two alternative sigma factors. Figure 6B shows that there is a close relationship between these two regulators and that they can both affect each other through the ampR [76] two-component regulator. Post-transcriptional SPI-2 regulation is a likely explanation for the large intracellular growth defects observed in the mutant smpB and hfg derivatives relative to small transcriptional effects. Strains containing mutations in rpoR and hnr showed comparable survival levels to wild-type in macrophages from BALB/c mice despite the fact that they are attenuated in the same mouse strain. Perhaps these regulators are selected during growth in other cell types or within phagocytic cells that are activated as a consequence of the inflammatory response generated by the bacteria.

**SlyA and SsrA/SsrB together activate transcription of the SPI-2 encoded secretion apparatus**

SlyA is a DNA-binding protein with high affinity for inverted repeat sequences [79] and the binding ability of SlyA to the ssaI promoter region was previously reported [62]. SlyA binds to a specific palindromic sequence, but also to other binding sites that do not fit a consensus sequence [79,80]. Similarly SsrB binds upstream of ssaI in a region of the promoter typical of response regulators but has no obvious recognition site and binds within the operons that encode structural components of the type III secretion system [52,81]. Four nucleoid-like proteins in enteric bacteria, H-NS, StpA, Hha, and YdgD have a predilection for binding to A+T rich sequences and repress transcription of SPI-2 genes (rev. in [14]). These proteins have a degenerate recognition sequence and the ability to polymerize along and bridge adjacent stretches of DNA repressing transcription apparently by occlusion of RNA polymerase. It has been shown that virulence regulator slyA, and close homologues msrA [82] and tspT [83] act to relieve the repression caused by H-NS at specific promoters [48,70,84,85]. SPI-2 regulation by SsrB and SlyA may be explained in a similar mechanism where both SlyA and SsrB counteract the silencing activity of H-NS/YdgD/Hha by competing for binding to the same target sequences or by altering the structure of the DNA to promote transcription for SPI-2. Walthers et al. [52] and Feng et al. [81] found that there was no consensus SsrB binding site or distance relative to the transcription start sites and Walthers [ibid] proposes that different promoters may be activated by distinct SsrB mechanisms. Our results support this conclusion and extend the observation to slyA at least for specific SPI-2 transcripts. A proposed mechanism for activation is that SlyA competes with a repressor for binding to the promoter and subsequently facilitates RNA polymerase access [80]. This model is supported by SlyA binding sites that are located downstream of transcriptional start sites [31,86], which is unusual for a traditional transcriptional activator. In agreement with this model, SlyA and PhoP counteract H-NS silencing at pagC [85,87]. Thus, one explanation for the transcription we observe following overexpression of srrA or slyA may be that both SlyA and SsrB counteract binding of small nucleoid-like proteins including both H-NS and YdgD/Hha in this A+T rich SPI-2 region [70]. This possibility may be reflected in the differences in expression of the five operons within SPI-2 following srrA and slyA over-expression.

**Clustering analysis and co-expression**

The complex regulatory network suggested by these studies has been partially confirmed. Complete elucidation of the virulence pathways must include proteomic studies to identify steps that require translational regulation, binding studies to distinguish direct from indirect effects and additional regulators not included in this preliminary group of 14. In this study, cluster analysis was used to identify genes that show similar expression profiles under a variety of environmental conditions and from the isogenic Salmonella regulatory mutants we describe. The average G+C content of the 92 genes outside of SPI-2 that show the same regulation as SPI-2 is 47% while the average for Salmonella is 52% (p<e$^{-25}$). Most of these genes are not found in closely related non-pathogenic bacteria (overall comparison eliminating SPI-2 genes p<e$^{-11}$). Thus application of the CLR algorithm identified a very interesting group of genes that are co-regulated with SPI-2 and were horizontally transferred to Salmonella. Analysis of microarrays and other expression data was used to construct a predictive model for how the regulators may interact and coordinate regulation within the host. A few of the predictions have been verified and more are being tested. The construction of a complete interaction network describing both the host and pathogen is a long-term goal of this and other related research efforts.

**Materials and Methods**

**Bacterial strains and growth conditions**

STM ATCC14028s was used as the parent for all of the constructions described. Eighty-three non-polar deletions of regulatory genes were constructed using derivatives of pKD13 (AY048744, GeneBank) designed to include a DNA ‘bar code’ allowing strains to be distinguished within a mixture [24,25]. Using these plasmids as template we prepared eighty-three PCR products with sequences homologous to target genes at the 5’- and 3’- ends of the linear fragment. Deletions were constructed as described [24], confirmed by PCR and P22 transduced into the parent STM 14028s. The eighty-three deletion strains are listed in Table S1. Double deletions including himD/ssrAB, phoPQ/ssrAB, slyA/ssrAB, csrA/ssrB, and ampRemI/ssrAB were generated in the same way. In the construction of the himD knockout strain, himD was cloned onto pCP20 (ibid) allowing expression of flip recombinase even in an hisB mutant, which does not support replication of pCP20. A more detailed description of these strains will be described elsewhere.

pFssaGTC was constructed by cloning lacZ and the promoter for ssaG (P$_{ssaG}$) including 450 bp of upstream sequence into pZC320 (U26464, GeneBank) via a SalI site. pZC320 was kindly
provided by B. D. Jones (University of Iowa, USA) and D. Lane (CNRS, France). The primer sets for PCR amplifications of lacZ and Fur are shown in Table S3. pKG137, a derivative of pCE37 (AY061943, GeneBank) was used as template for lacZ PCR. For pBAD30/SsrB/pBAD33/SsrB or pBAD30/SlyA, ssrB and slyA were amplified by PCR using the oligos listed in Table S3 and subsequently cloned into pBAD30 or pBAD33 [88]. srrB and slyA were induced by addition of 0.02% L-arabinose to acidic minimal medium 1 (see below). To complement a ysrB deletion, a 1.7 kb DNA fragment containing ysrB and its promoter was PCR amplified and cloned into pWSK29 (AF016889, GeneBank) via BamHI and EcoRI. All primers used in plasmids construction are amplified and cloned into pWSK29 (GeneBank) via BamHI and EcoRI. All primers used in plasmids construction are listed in Table S3. Kanamycin, chloramphenicol and carbenicillin were added at 50 μg/ml, 10 μg/ml and 100 μg/ml respectively as necessary for selection.

Bacteria were grown under four different conditions to cover expression of a large number of Salmonella genes. They were: log phase in Luria-Bertani (LB) medium, stationary phase in LB medium, acidic minimal media 1 (AM1; grown in AMM media overnight, diluted 1:100 and grown for an additional 4 hrs) and AMM2 (grown in LB to stationary phase and resuspended in the same volume of acidic minimal media [47,48]). The latter two media partially mimic the intracellular environment of the Salmonella containing vacuole and differ from each other only in the pre-growth conditions [47,48]. Because of the density of the culture and the fact that the bacteria are in stationary phase the pre-growth conditions [47,48]. Furthermore, during growth in minimal media there were substantial differences in growth rate for some regulator mutants (csp, hfd, and casf). For this reason a csp deletion strain was excluded from transcriptional profiling for AMM1 although expression of relevant virulence factors was analyzed for a csp mutant by qRT-PCR at several time points as described. A total of 55 RNA pools from 14 strains in 4 conditions were prepared. An additional 12 RNA samples were prepared from the parental strain. All were subjected to microarray analysis according to the procedures described [50]. Total RNAs were reverse transcribed with random hexamers and Superscript II (Invitrogen) and subsequent reverse transcripts were labeled by Cy3-linked dCTP (Amersham Biosciences). Genomic DNA from 14028s was isolated using GeneElute bacterial genomic DNA kit (Sigma) and labeled with Cy5-encoded dCTP (Amersham Biosciences) and the fluorescent signal intensities were quantified using ScanArray Express (Packard Bioscience, BioChip Technologies) has identical triplicate arrays as technical replicates. Slides hybridized with Cy3/Cy5-labeled probes were scanned using ScanArray Express (Packard Biosciences, BioChip Technologies) and the fluorescent signal intensities were quantified using QuantArray software (Packard Biosciences, BioChip Technologies) and exported to Excel files. The raw data was processed in WebArray [92] for statistical normalization.

Quantitative RT-PCR analysis
Total RNA was isolated using RNase protect Bacteria Reagent (Qiagen), mRNAeasy mini kit (Qiagen), and DNase to remove residual chromosomal DNA (Qiagen) according to the manufacturer’s recommendations. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). cDNA was synthesized using the iScript cDNA synthesis kit (Bio Rad) and cDNA corresponding to 10 ng of input RNA was used as template in each real-time PCR using SYBR green reagent to detect duplex DNA product (ABI 7700, Applied Biosystems). Primers used in RT-PCR are listed in Table S3. RT-PCR was carried out at 95 °C for 15 s (ABI 7700, Applied Biosystems). Primers used in RT-PCR are listed in Table S3. RT-PCR reaction was carried out at 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min for 50 cycles. The expression ratio of each mutant by qRT-PCR was normalized to the level of gyrB [51,70].

β-Galactosidase assay
β-Galactosidase activity was assayed according to the method described previously [93]. Salmonella cells were grown as described above. The activity was measured in triplicate using independently grown cultures and average enzyme activities were normalized to the number of input bacteria in the assay to compute Miller units.
Accession numbers of 14 genes investigated in this study

\(\text{spvR}, 1256197; \text{flaB}, 1251636; \text{hmu}, 1252500; \text{phoP}, 1252749/1252748; \text{ssaA}/\text{ssaB}, 1252910/1252909; \text{crp}, 1252962; \text{hnr}, 1253272; \text{ssaD}, 1254163; \text{ssrA}, 1254211; \text{csrA}, 1254549; \text{spvR}, 1254447; \text{cpp}, 1254989; \text{ompR}/\text{emc}, 1255025/1255024; \text{hfq}, 1255087.\) Accession number assigned here refers to Entrez Gene ID in NCBI.

Supporting Information

**Figure S1**

Complementation of an \(\text{spvR}\) deletion in strain 14028s restores virulence. A PCR fragment containing \(\text{spvR}\) and its 600 bp upstream region was cloned on pWSK29 (AF016889, GenBank) via BamHI and EcoRI. Three Salmonella strains of 14028s, \(\text{spvR}\), and \(\text{spvR}\) harboring pWSK29spvR were i.p. administered to 4 BALB/c mice respectively with dose of 200 cfu per mouse. Mice survival after Salmonella infection was monitored for 21 days. Found at: doi:10.1371/journal.ppat.1000306.s001 (0.14 MB TIF)

**Figure S2**

Comparison of \(\text{ssaG}\) transcript level as determined by different methods. The transcript level of \(\text{ssaG}\) was determined in each isogenic mutant under all four growth conditions used in this study by qRT-PCR in A (\(\text{gfp}\) transcript as a control). An alternative method of comparing transcription levels is shown in B in which a lac\(_Z\) reporter was used. The construct was made on a low copy F derivative plasmid as described in methods. Values reported are for \(\beta\)-galactosidase assays of each strain in each growth condition and show similar patterns to those from qRT-PCR. As a measure of transcript levels we chose to use qRT-PCR because we observed differences in \(\beta\)-galactosidase translation that did not reflect transcription. Found at: doi:10.1371/journal.ppat.1000306.s002 (0.23 MB TIF)

**Figure S3**

Regulatory consensus network integrating 4 data sets. To produce an overall transcriptional regulatory network that links the 14 essential regulators, we combined the two CLR algorithm results described in Figure 5 and two matrix analyses from our gene profiles and GSE2456 public database. The network was visualized in Cytoscape using a hierarchical layout algorithm.

Found at: doi:10.1371/journal.ppat.1000306.s003 (0.23 MB TIF)

**Table S1**

List of 83 regulators inferred to have a virulence function and their deletion phenotypes examined in this study for mouse virulence.

Found at: doi:10.1371/journal.ppat.1000306.s004 (0.13 MB DOC)

**Table S2**

List of genes highly co-regulated by virulence regulators in this study when grown in minimal acidic media. Genes were grouped into categories based on known function listed on Clusters of Orthologous Groups (COGs) in NCBI.

Found at: doi:10.1371/journal.ppat.1000306.s005 (0.06 MB DOC)

**Table S3**

Primers used in plasmids construction (A) and qRT-PCR (B).

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Author Contributions

Conceived and designed the experiments: FH. Performed the experiments: HY SP. Analyzed the data: HY JEM FH. Contributed reagents/materials/analysis tools: MM. Wrote the paper: HY FH.

References

1. Graham SM (2002) Salmonellosis in children in developing and developed countries and populations. Curr Opin Infect Dis 15: 507–512.
2. Holmaa EL (2001) Nontyphoidal salmonellosis. Clin Infect Dis 32: 263–269.
3. Bose F, Lipsi CJ, Toosi RM, Gromisz E, Heffron F, et al. (1998) At least four percent of the Salmonella typhimurium genome is required for fatal infection of mice. Infect Immun 66: 3372–3377.
4. Galan JE, Ginocchio C, Costes P (1992) Molecular and functional characterization of the Salmonella invasion gene invA: homology of InvA to members of a new protein family. J Bacteriol 174: 4338–4349.
5. Dunlap NE, Benjamin WH Jr, Briles DE (1994) The intracellular nature of Salmonella Pathogenicity Island 2 Is Expressed Prior to Penetrating the Intestine. PLoS Pathog 1: e32. doi:10.1371/journal.ppat.0010032.
6. Drecktrah D, Knodler LA, Steele-Mortimer O (2004) Modulation and utilization of host cell phosphoinositides by Salmonella spp. Infect Immun 72: 4331–4335.
7. Lawley TD, Chan K, Thompson LJ, Kim CC, Govoni GR, et al. (2006) Genome-wide screen for salmonella genes required for long-term systemic infection of the mouse. PLoS Pathog 2: e11. doi:10.1371/journal.ppat.0020011.
8. Marshall DG, Bose F, Hau C, Douglas G, Dorman CJ (2000) DNA topology and adaptation of Salmonella typhimurium to an intracellular environment. Philos Trans R Soc Lond B Biol Sci 355: 563–574.
9. Dunlap NE, Benjamin WH Jr, Beles DE (1994) The intracellular nature of Salmonella infection during the early stages of mouse typhoid. Immunol Rev 160: 303–312.
10. Geddes K, Cruz F, Heffron F (2007) Analysis of Cells Targeted by Salmonella typhimurium and Shigella flexneri. Embo J 12: 301–306.
11. Brown NF, Vallance BA, Coombes BK, Valdez Y, Coburn BA, et al. (2005) At least four SPI-2 genes although the \(\text{spa}\)I_O results described in Figure 7 demonstrate that not all of the SPI-2 encoded operons are regulated similarly. Interconnections between the regulators and SPI-2 were reduced based on the experimental data described in the manuscript. The network was visualized in Cytoscape using a hierarchical layout algorithm.

Found at: doi:10.1371/journal.ppat.1000306.s003 (0.23 MB TIF)
18. Taylor RG, Shah A, Treadman C, Blevins M (2006) SEBINJ: Software Environment for Biodigital Network Inference. Bioinformatics 22: 2769-2770.

19. Faith JJ, Hayete B, Thaden JT, Mogno I, Wierzbowski J, et al. (2007) Large-scale mapping and validation of Escherichia coli transcriptional regulation from a compendium of expression profiles. PLoS Biol 5: e103. doi:10.1371/journal.pbio.0050088.

20. Eisent MP, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95: 14863-14868.

21. Chan K, Kim CC, Falkows S (2005) Microarray-based detection of Salmonella enterica serovar Typhimurium transposon mutants that cannot survive in macrophages and mice. Infect Immun 73: 3430-3449.

22. Shah DH, Lee MJ, Park JH, Lee JH, Eo SK, et al. (2005) Identification of Salmonella gallinarum virulence genes in a chicken infection model using PCR-based signature-tagged mutagenesis. Microbiology 151: 3957-3968.

23. Morgan E, Campbell JD, Rowse SC, Bispahm J, Stevens MP, et al. (2004) Identification of host-specific colonization factors of Salmonella enterica serovar Typhimurium. Mol Microbiol 54: 994-1010.

24. Datensko KA, Wanner BL (2001) One-step inactivation of chromosomal homologs in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 98: 6640-6645.

25. Pierse A, Fung EL, Jaramillo DF, Chu AM, Davis RW, et al. (2006) A unique and universal molecular barcode array. Nat Methods 3: 601-603.

26. Vidal SM, Malo D, Vogan K, Skameene E, Gross P (1993) Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. Cell 73: 499-485.

27. Crouse ML, Becker LA, Bang IS, Tanabe H, Ouellette AJ, et al. (2005) The ssrA/B gene expression in Salmonella pathogenicity island 2. Mol Microbiol 56: 789-799.

28. Mangan MW, Lucchini S, Danino V, Croinin TO, Hinton JC, et al. (2006) The global regulation by CsrA in Salmonella typhimurium. Mol Microbiol 48: 1633-1645.

29. Pierce SE, Fung EL, Jaramillo DF, Chu AM, Davis RW, et al. (2006) A unique and universal molecular barcode array. Nat Methods 3: 601-603.

30. Navarre WW, Halsey TA, Walthers D, Frye J, McClelland M, et al. (2005) Coordinated Regulation of Salmonella enterica serovar Typhimurium, limits intracellular replication and decreases virulence. Infect Immun 73: 4358-4358.

31. Lawhon SD, Frye JG, Suyemoto M, Porwollik S, McClelland M, et al. (2003) Global regulation by CsrA in Salmonella typhimurium. Mol Microbiol 47: 1653-1664.

32. Monsieurs P, De Keersmaecker S, Navarre WW, Bader MW, De Smet F, et al. (2006) The global post-transcriptional regulator, Hfq. PLoS Genet 4: e1000163. doi:10.1371/journal.pgen.1000163.

33. Smith VA, Jarvis ED, Hartemink AJ (2002) Influence of network topology and data collection on network inference. Pac Symp Biocomput. pp 164-175.

34. Beuzon CR, Banks G, Deiwick J, Hensel M, Holden DW (1999) pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of Salmonella enterica serovar Typhimurium. Mol Microbiol 33: 806-816.

35. Coombe BK, Wickham ME, Lowden MJ, Brown NF, Finlay BB (2005) Negative regulation of Salmonella pathogenicity island 2 is required for control of virulence during typhoid. Proc Natl Acad Sci U S A 102: 17460-17465.

36. Rydstrom A, Wick MJ (2007) Monocyte recruitment, activation, and function in the gut-associated lymphoid tissue during oral Salmonella infection. J Immunol 178:5702–5709.

37. Porwollik S, Frye J, Florica LD, Blackmer F, McClelland M (2003) A non-redundant microarray of genes for two related bacteria. Nucleic Acids Res 31: 4609-4616.

38. Persson DA, Heffron F (2005) sci8, an icsm homolog in Salmonella enterica serovar Typhimurium, limits intracellular replication and decreases virulence. Infect Immun 73: 4358-4358.

39. Mastroeni P, Vazquez-Torres A, Xu Y, Khan S, et al. (2000) Secretion of a Salmonella-specific translocated effector integrated into the SPI-2 controlled salmonella pathogenicity island 2 type III secretion system. Infect Immun 75: 574-580.

40. Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC (2000) Extraintestinal dissemination of Salmonella by CD18-expressing inflammatory cells in vivo via MyD88. J Infect Immun 72: 2556-2565.

41. Libo SF, Richmond VA, Belanger SD, Messina LI, et al. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. J Exp Med 192: 237-248.

42. Mastroeni P, Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial killing by activated peritoneal macrophages in vitro. J Exp Med 192: 227-236.

43. Libby SJ, Lesnick M, Hasegawa P, Weidnhammer E, Guiney DG (2004) The Salmonella virulence plasmid svp genes are required for cytotoxicity in pathophanomy monocyt-derived macrophages. Cell Microbiol 2: 49-58.

44. Libby SJ, Adamo LG, Ficht TA, Allen C, Whitford HA, et al. (1997) The svp genes on the Salmonella dublin virulence plasmid are required for septic arthritis and systemic infection in the natural host. Infect Immun 65: 1766-1772.

45. Bijuja MJ, Grosman EA (2005) The PhoP/PhoQ system controls the intramacrophage type three secretion system of Salmonella enterica. Mol Microbiol 57: 85-96.

46. Coombe BK, Brown NF, Valdez Y, Brunnell NH, Finlay BB (2004) Expression and secretion of Salmonella pathogenicity island 2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SHL. J Biol Chem 279: 49004-49013.

47. Beuzon CR, Banks G, Dewick J, Herold M, Holden DW (1999) pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of Salmonella enterica serovar Typhimurium. Mol Microbiol 33: 806-816.

48. Coombe BK, Wickham ME, Lowden MJ, Brown NF, Finlay BB (2005) Negative regulation of Salmonella pathogenicity island 2 is required for control of virulence during typhoid. Proc Natl Acad Sci U S A 102: 17460-17465.

49. Coombe BK, Wickham ME, Lowden MJ, Brown NF, Finlay BB (2005) Negative regulation of Salmonella pathogenicity island 2 is required for control of virulence during typhoid. Proc Natl Acad Sci U S A 102: 17460-17465.
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72. Haapanen E, Galan JE (2007) Identification of a transcriptional regulator that controls intracellular gene expression in Salmonella Typhi. Mol Microbiol 64: 1549–1561.

73. Deiwick J, Nikolaus T, Erdogun S, Hensel M (1999) Environmental regulation of Salmonella pathogenicity island 2 gene expression. Mol Microbiol 31: 1759–1773.

74. Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, et al. (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122: 461–472.

75. Garcia Vescovi E, Soncini FC, Grosima EA (1996) Mg2+ as an extracellular signal; environmental regulation of Salmonella virulence. Cell 84: 165–174.

76. Slauch JM, Silhavy TJ (1989) Genetic analysis of the switch that controls porin gene expression in Escherichia coli K-12. J Mol Biol 210: 281–292.

77. Lejona S, Castelli ME, Cabeza ML, Kenney LJ, Garcia Vescovi E, et al. (2004) PhoP can activate its target genes in a PhoQ-independent manner. J Bacteriol 186: 2476–2480.

78. Stapleton MR, Norte VA, Read RC, Green J (2002) Interaction of the Salmonella typhimurium transcription and virulence factor SlyA with target DNA and identification of members of the SlyA regulon. J Biol Chem 277: 17630–17637.

79. Ellison DW, Miller VL (2006) Regulation of virulence by members of the MarR/SlyA family. Curr Opin Microbiol 9: 153–159.

80. Feng X, Wallthers D, Oropeza R, Kenney LJ (2004) The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at Salmonella pathogenicity island 2. Mol Microbiol 54: 823–835.

81. Heroven AK, Nagel G, Tran HJ, Parr S, Dersch P (2004) RovA is autoregulated and antagonizes H-NS-mediated silencing of invasin and rovA expression in Salmonella enterica serovar Typhimurium. J Bacteriol 186: 2417–2425.

82. Lejona S, Castelli ME, Cabeza ML, Kenney LJ, Garcia Vescovi E, et al. (2004) PhoP can activate its target genes in a PhoQ-independent manner. J Bacteriol 186: 2476–2480.

83. Yu RR, DiRita VJ (2002) Regulation of gene expression in Vibrio cholerae by ToxT involves both antirepression and RNA polymerase stimulation. Mol Microbiol 43: 119–134.

84. Shi Y, Latiif T, Cronie MJ, Grosima EA (2004) Transcriptional control of the antimicrobial peptide resistance ugtL gene by the Salmonella PhoP and SlyA regulatory proteins. J Biol Chem 279: 38618–38625.

85. Kong W, Weatherspoon N, Shih Y (2008) Molecular mechanism for establishment of signal-dependent regulation in the PhoP/PhoQ system. J Biol Chem 283: 16612–16621.

86. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177: 4121–4130.

87. Reed LHAM (1938) A simple method of estimating fifty per cent endpoints. Am J Hyg 27: 493–497.

88. Buchmeier NA, Helfron F (1991) Inhibition of macrophage phagosome-lysosome fusion by Salmonella typhimurium. Infect Immun 59: 2272–2278.

89. Ramsey CJ, Chatfield S, Higgins CF, Hayward C, Dougan G (1989) Identification of attenuated mutants of Salmonella choleraesuis. Infect Immun 60: 4881–4890.

90. Coombes BK, Wickham ME, Brown NF, Lemire S, Bossi L, et al. (2005) Genetic and molecular analysis of the gogB, a phage-encoded type III-secreted substrate in Salmonella enterica serovar typhimurium with autonomous expression from its associated phage. J Mol Biol 348: 817–830.

91. Xia X, McClelland M, Wang Y (2005) WebArray: an online platform for microarray data analysis. BMC Bioinformatics 6: 306.

92. Miller JH (1972) Experiments in Molecular Genetics. Plainview: Cold Spring Harbor Laboratory Press.

93. Sturn A, Quackenbush J, Trajanoski Z (2002) Genesis: cluster analysis of microarray data. Bioinformatics 18: 207–208.

94. Allen JH, Uiley M, van den Bosch H, Nuijten P, Wérdiet M, et al. (2000) A functional cya gene is required for Salmonella enterica serovar typhimurium virulence in B ALB/c mice. Infect Immun 68: 3772–3773.

95. Xia X, McClelland M, Wang Y (2005) WebArray: an online platform for microarray data analysis. BMC Bioinformatics 6: 306.

96. Xia X, McClelland M, Wang Y (2005) WebArray: an online platform for microarray data analysis. BMC Bioinformatics 6: 306.