RESEARCH ARTICLE

A Horizontally Acquired Transcription Factor Coordinates *Salmonella* Adaptations to Host Microenvironments

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ABSTRACT **The transcription factors HilA and SsrB activate expression of two type III secretion systems (T3SSs) and cognate effectors that reprogram host cell functions to benefit infecting *Salmonella* in the host. These transcription factors, the secretion systems, and the effectors are all encoded by horizontally acquired genes. Using quantitative proteomics, we quantified the abundance of 2,149 proteins from *hilA* or *ssrB Salmonella in vitro*. Our results suggest that the HilA regulon does not extend significantly beyond proteins known to be involved in direct interactions with intestinal epithelium. On the other hand, SsrB influences the expression of a diverse range of proteins, many of which are ancestral to the acquisition of *ssrB*. In addition to the known regulon of T3SS-related proteins, we show that, through SodCI and bacterioferritin, SsrB controls resistance to reactive oxygen species and that SsrB down-regulates flagella and motility. This indicates that SsrB-controlled proteins not only redirect host cell membrane traffic to establish a supportive niche within host cells but also have adapted to the chemistry and physical constraints of that niche.**

IMPORTANCE **Expression of T3SSs typically requires a transcription factor that is linked in a genomic island. Studies of the targets of HilA and SsrB have focused on almost exclusively on T3SS substrates that are either linked or encoded in distinct genomic islands. By broadening our focus, we found that the regulon of SsrB extended considerably beyond T3SS-2 and its substrates, while that of HilA did not. That at least two SsrB-regulated processes streamline existence in the intracellular niche afforded by T3SS-2 seems to be a predictable outcome of evolution and natural selection. However, and importantly, these are the first such functions to be implicated as being SsrB dependent. The concept of T3SS-associated transcription factors coordinating manipulations of host cells together with distinct bacterial processes for increased efficiency has unrealized implications for numerous host-pathogen systems.**

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During pathogenesis of enteritis or typhoid, *Salmonella* exists at numerous sites within the host that present distinct microenvironments to the infecting *Salmonella*, including those within host cells (1–3). Commensurate with this, *Salmonella* employs numerous transcriptional regulators to allow it to respond to these microenvironments appropriately (4). Typically, knockout mutants defective for these regulators show severe phenotypes in laboratory animals, and the hypothesis that these functions are appropriately repressed is also supported by experimental evidence (5).

The ability of *Salmonella* to both penetrate the intestinal epithelium and replicate within phagocytes at systemic sites within the host was potentiated by horizontal gene transfer events (6). Respectively, these activities are mediated through manipulations of the host cell machinery by type III secretion systems (T3SS) encoded by *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2) (7–10). Both SPI1 and SPI2 also encode transcription factors (HilA and SsrB, respectively) that are required for expression of the linked T3SS and the effectors they deliver into host cells (11, 12), indicating that these SPIs were acquired as functional units. Subsequent to the acquisition of SPI1 and SPI2, horizontal gene transfer events installed numerous additional effectors in the *Salmonella* genome, extending and refining how *Salmonella* interacts with host cells (13) (Fig. 1). Importantly, almost all of these require either HilA or SsrB for expression, indicating that they were also functional upon acquisition.

HilA and SsrB represent a significant paradigm in bacterial virulence: transcription factors acquired by horizontal gene transfer together with a complex multicomponent virulence system, allowing expression of the acquired traits. These allowed access to new niches in host organisms, freeing *Salmonella* from the competitive environment of the intestinal lumen and presenting a considerable opportunity for selectable advantage (6, 14, 15). However, simply placing an organism in a novel microenvironment would be of limited advantage unless that organism could thrive in such an environment. Thus, there would have been con-

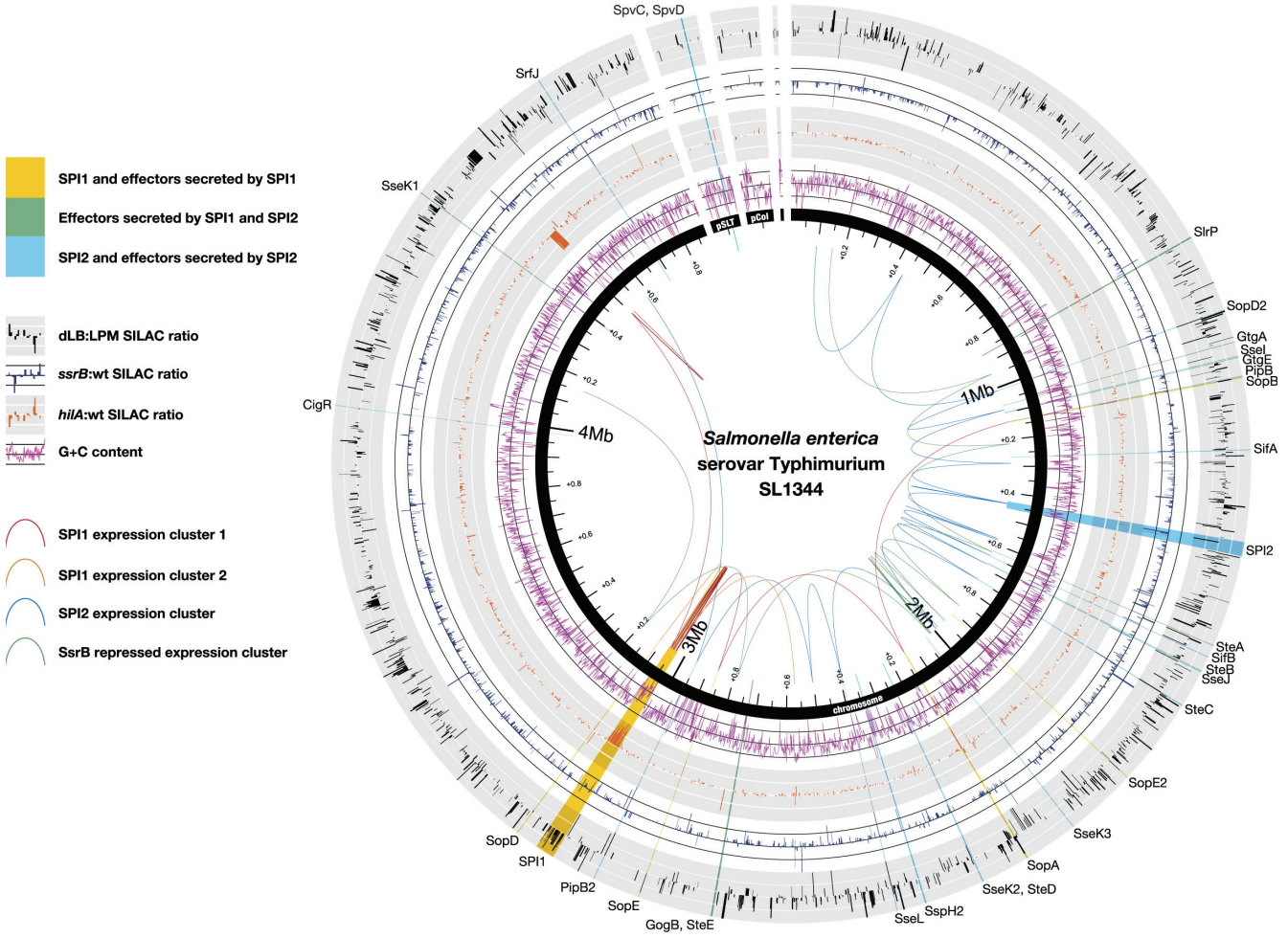


FIG 1 Proteomic data mapped onto a diagrammatic representation of the *Salmonella* Typhimurium SL1344 genome. The inner black broken circle represents the genome itself, with tick marks indicating scale, in megabase pairs. The chromosome, pSLT, and pCol are labeled, while pRSF is unlabeled due to space restrictions. Outside this are the G+C content of each gene plotted in purple on white, *hilA*:wt SILAC ratios in orange on gray, and *ssrB*:wt SILAC ratios in blue on white, followed by wt dLB/LPM SILAC ratios in black, from inside to outside. Layered below these data are radial highlights indicating the locations of SPI1, SPI2, and all effectors described to date. These are labeled with the protein (or pathogenicity island) name and colored according to the T3SS(s) with which they are associated. Inside the representation of the genome are colored lines linking all proteins belonging to the expression clusters in Fig. 5.

siderable selective pressure for *Salmonella* to evolve adaptations to this environment, ranging from basic metabolism to effectively utilize available nutrients to streamlining more complex cellular functions that are energetically expensive. Indeed, contemporary *Salmonella* is well adapted to the intracellular environment (16), providing support for this model. Given the role of HilA and SsrB in expressing traits that put *Salmonella* in these niches, we hypothesized that they may play a role in such adaptations. Evidence of *cis* regulatory evolution upstream of *srfN*, which is ancestral to SPI2 acquisition, leading to control by SsrB (17) directly supports this hypothesis. We used quantitative proteomics to define the HilA and SsrB regulons to test this hypothesis on a systems biology scale.

RESULTS

Data for our quantitative analysis of the cellular *Salmonella* proteome were generated according to the stable isotope labelling of amino acids in cell culture (SILAC) approach (18) described in Fig. 2. Each experiment was independently performed three times. Analyzing the spectra with MaxQuant (19, 20), we were able to identify 17,372 peptides from 2,149 proteins (see Table S1 in the supplemental material for the quantitative data set), representing over 45% of the protein-coding potential of the genome. This compares reasonably well with efforts to define the complete proteome of *Escherichia coli*, a species closely related to *Salmonella*, where 22,196 peptides from 2,602 proteins were expressed in LB medium (21).

The primary quantitative comparisons of interest were between the wild type and each isogenic transcription factor mutant grown under the respective inducing culture condition, with comparison of SPI1-inducing culture and SPI2-inducing culture being secondary. Complete data sets for each comparison are shown in Fig. 3. It can be seen that mutation of either *hilA* or *ssrB* significantly affects the abundance of relatively few proteins and that most of these decrease in abundance, consistent with the known role of each as an activator of transcription. In contrast, a larger proportion of the proteome is affected by growth medium, which likely reflects responses to the extensive differences in the

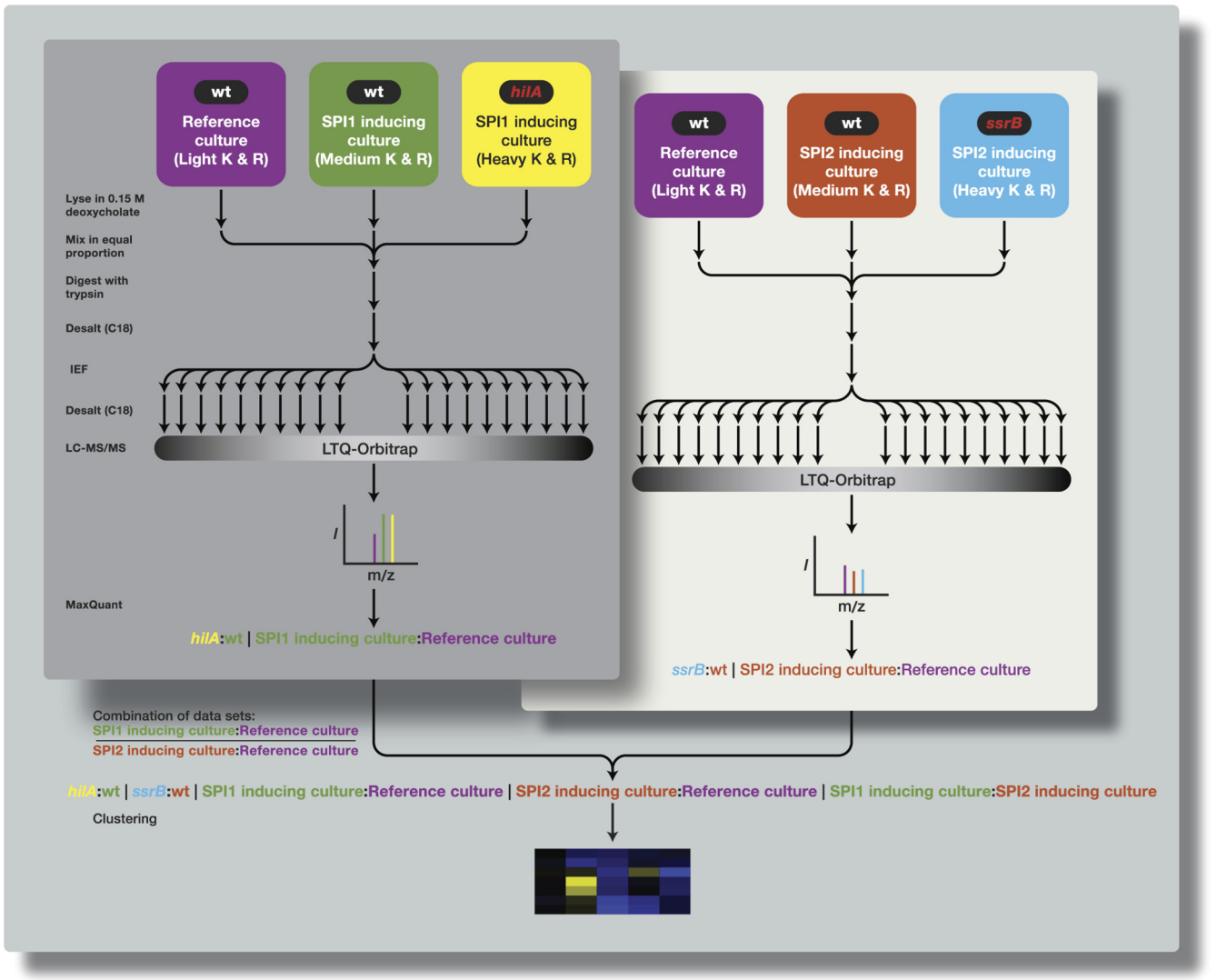


FIG 2 Proteomic data set generation and analysis pipeline. Two parallel experiments were performed, one focused on the HilA regulon, the other on the SsrB regulon. Both experiments included wt *Salmonella* cultured to stationary phase in LB medium as a common reference condition. For each experiment, the SILAC ratios of interest were transcription factor mutant: wt, with both cultured under the respective inducing condition, and wt inducing culture versus wt reference culture. The wt SILAC ratio for the SPI1-inducing culture versus the SPI2-inducing culture was calculated by dividing the SPI1-inducing culture/reference culture ratio by the SPI2-inducing culture/reference culture ratio, whereby the reference culture condition serves as a common denominator. The resulting five SILAC ratios were subjected to clustering by Euclidean distance.

chemistry (nutrient abundance, pH, osmolarity, etc.) between the SPI1-inducing medium and the SPI2-inducing medium.

In the case of *hilA*, the master SPI1 transcription factor, 1,914 proteins were quantified, of which 42 decreased and 16 increased significantly in the *hilA* mutant. Twenty-six of the 42 proteins decreased in the *hilA* mutant are encoded within SPI1 or effectors translocated by T3SS-1 and have previously been shown or predicted to be regulated by HilA (see Table S2 in the supplemental material). All proteins known to be regulated by HilA that were detected were significantly decreased, providing confidence in the data set. To broadly capture the functional significance of these proteins, the level 3 biological process gene ontology (GO) terms were used to annotate these proteins (Table 1). The GO terms "pathogenesis" and "protein secretion" (a level 7 term descended from the level 3 terms "establishment of localization," "macromolecule localization," and "cellular localization") were overrepresented in the proteins that decrease in the *hilA* mutant by Fisher's exact test (see Table S3 in the supplemental material). All of this is consistent with the known role of SPI1 encoding a T3SS that potentiates virulence through direct manipulation of host processes.

For the SPI2 master transcription factor mutant, the *ssrB* mutant, 1,503 proteins were detected and allowed quantitative comparison with the wild type (wt), of which 65 decreased and 26 increased significantly in the *ssrB* mutant. The public availability of a full SsrB chromatin immunoprecipitation (ChIP) data set (22) enabled further analysis of the SILAC data to infer whether SsrB acted directly on the encoding genes. A plot of SILAC versus ChIP values is shown in Fig. 4, indicating that proteins with decreased SILAC values can quite clearly be split into two groups:

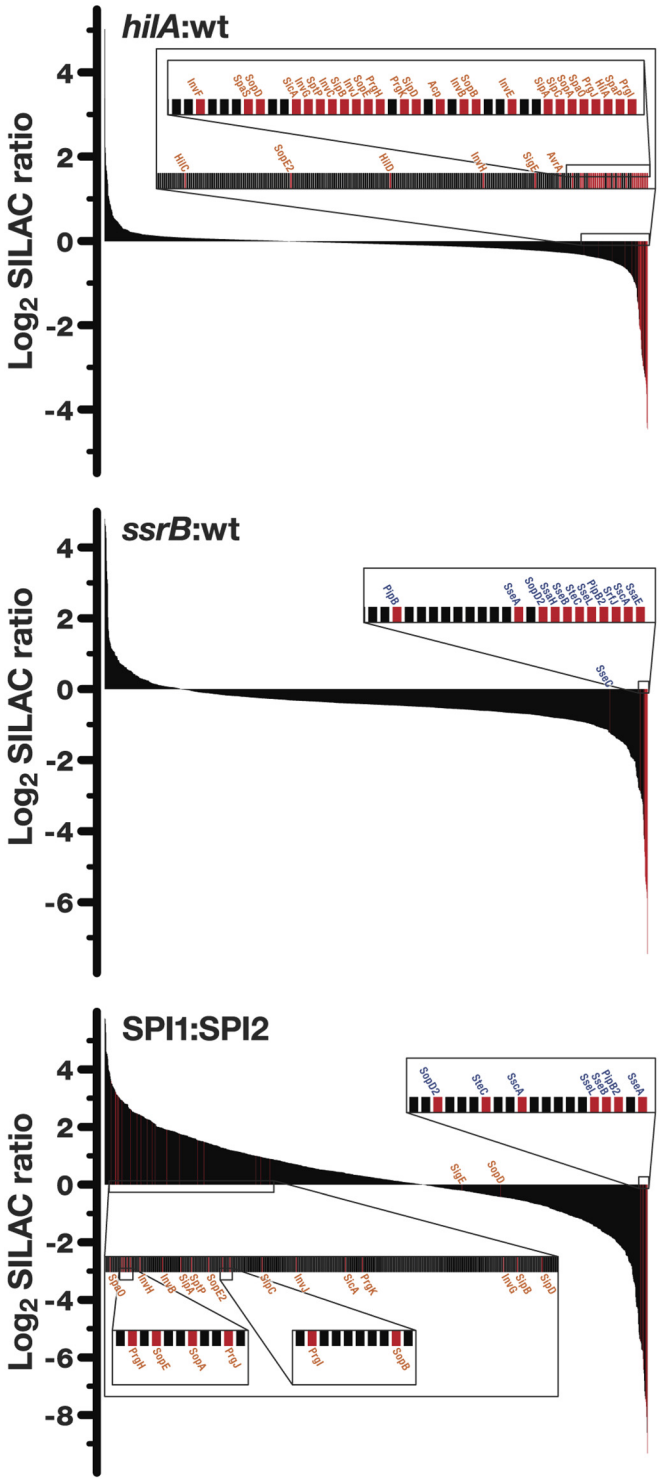


FIG 3 Distribution of SILAC data from highest to lowest ratios. In the graph showing the *hilA*/wt ratios, all T3SS-1 components and effectors are labeled in orange and the ratios are indicated in red, while in the graph showing the *ssrB*/wt ratios, all T3SS-2 components and effectors are labeled in blue and the ratios are indicated in red. The SPI1:SPI2 graph plots the SPI1-inducing culture/SPI2-inducing culture SILAC ratios calculated as described in Fig. 2 and in Materials and Methods, with the T3SS-1 and -2 proteins indicated in the same fashion.

TABLE 1 Level 3 gene ontology terms associated with proteins that significantly change in abundance in the *hilA* mutant

Abundance	Gene ontology term	No. of proteins
−	Pathogenesis	25
−	Establishment of localization	14
−	Primary metabolic process	12
−	Macromolecule localization	11
−	Cellular metabolic process	10
−	Cellular localization	9
−	Macromolecule metabolic process	7
−	Biosynthetic process	6
−	Oxidation-reduction process	2
+	Cellular metabolic process	7
+	Primary metabolic process	7
+	Biosynthetic process	6
+	Macromolecule metabolic process	5

those likely to be directly regulated by SsrB and those whose regulation is likely to be indirect. Consistent with this, SILAC and ChIP values were found to correlate for the directly regulated genes (Spearman $r = -0.5144$; $P = 0.0085$) but not for the indirectly regulated genes (Spearman $r = -0.1687$; $P = 0.2982$). On the other hand, for proteins that increased in the *ssrB* mutant, little could be found to support a role for SsrB in directly repressing their expression.

From Fig. 4 it can also be seen that many proteins that are unaffected by SsrB have high ChIP values. This might be explained by a significant false discovery rate in the ChIP data set, extensive posttranscriptional regulation, SsrB binding DNA in a manner unrelated to transcription, or some combination of these. In any case, the proportion of proteins that increase in abundance and have significant ChIP values does not significantly differ from the proportion of proteins that were unaffected by SsrB in Fisher's exact test ($P = 0.4578$). Conversely, the proportion of proteins with significant ChIP values that decrease in abundance does differ from those unaffected in Fisher's exact test ($P < 0.0001$). For these reasons, we divided the SsrB data set into four groups for analysis: (i) those that decreased in abundance and had significant ChIP scores (likely directly regulated), (ii) those that decreased in abundance and did not have significant ChIP scores (likely indirectly regulated), (iii) those that increased in abundance, and (iv) those that were unaffected by *ssrB*.

All 12 of the T3SS-2 components and effectors quantified showed significantly decreased expression ratios, and 11 of them had a significant ChIP peak upstream, supporting the validity of the data set (see Table S2 in the supplemental material). For the proteins that decreased in abundance in the *ssrB* mutant, numerous level 3 biological process GOs related to pathogenesis and protein secretion were associated with genes with significant SsrB ChIP scores, whereas metabolic processes dominated those that did not have significant ChIP scores (Table 2). GO terms that were significantly overrepresented in the 25 decreased proteins with significant ChIP scores by Fisher's exact test were "pathogenesis" and "protein secretion by the type III secretion system" (a level 8 term that is descended from "establishment of localization"; see Table S4 in the supplemental material). These are consistent with the known functions of SsrB-activated genes. No GO terms were overrepresented in the 40 proteins that decreased but did not have significant ChIP scores. For proteins that increased in abundance, numerous level 3 GO terms related to motility were found (Ta-

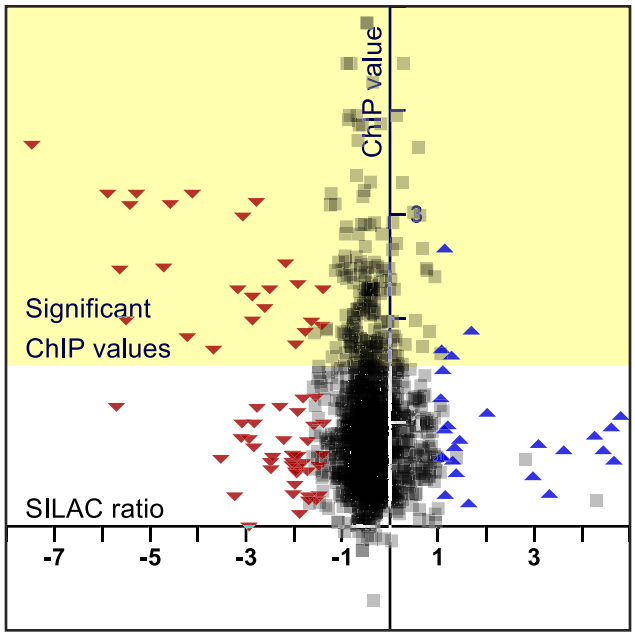


FIG 4 Plot of *ssrB*/wt SILAC ratios along the *x* axis and the highest ChIP peak value from the study by Tomljenovic-Berube et al. (22) for each protein in our data set. All points on the graph have transparency applied to indicate the number of similar data points in particularly dense areas of the graph. Points corresponding to SILAC values that are significantly decreased are indicated with red arrowheads, those that significantly increase are indicated with blue arrowheads, and all others are indicated with black squares. The area above the threshold of significance (3 standard deviations from the mean) for the ChIP data are indicated as a pale yellow background.

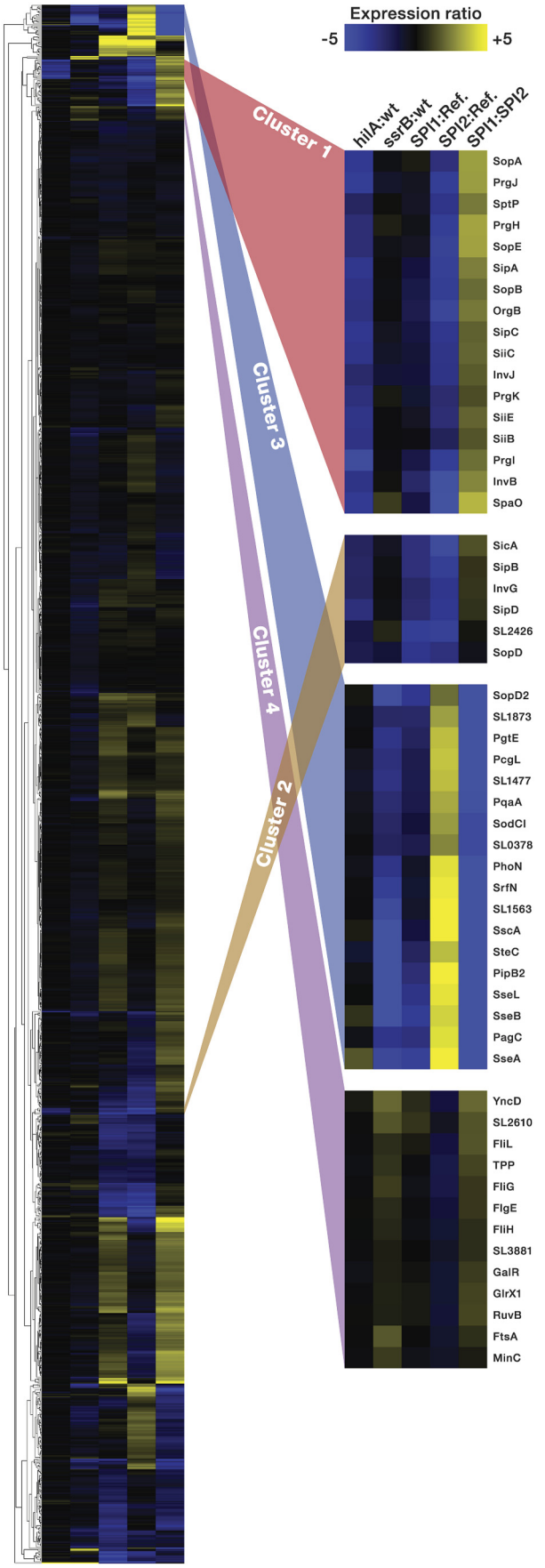
ble 2), and in terms of overrepresentation, "locomotion" (a level 2 term that is the parent of "taxis") was significantly enriched in the group of proteins that increased in abundance in *ssrB* (see Table S5 in the supplemental material).

Given that SPI1, SPI2, and effector genes have been acquired by *Salmonella* from horizontal gene transfer events, we tested the GC contents of the groups above to see if any differed from the data set mean, indicating horizontal transfer. Both the group of proteins that decreased in the *hilA* mutant (46.5% ± 6.27%) and the group of proteins that decreased in the *ssrB* mutant and had significant SsrB ChIP scores (45.6% ± 5.57%) had mean GC contents that were significantly below the mean for all genes in the data set (52.9% ± 4.60%), as determined by Dunnett's multiple comparison applied to a one-way analysis of variance (ANOVA). This is consistent with each of these groups containing the numerous, horizontally acquired, known targets of these transcription factors. On the other hand, neither of the groups that increased in the *hilA* (53.8% ± 4.98%) or *ssrB* (53.6% ± 3.59%) mutant nor the group that decreased in the *ssrB* mutant and did not have significant SsrB ChIP scores (51.2% ± 5.14%) had values that differed significantly from the mean for all proteins in the data set.

Comparison with array studies of mRNA expression. We were also interested in comparing our proteomic data set with other systems-level analyses studying the effect of HilA or SsrB on gene expression. To our knowledge, one such data set is available for HilA (23) and two for SsrB (22, 24). We compared the data available from these studies (significantly increased and/or decreased genes) with our corresponding data points (see Table S6 in the supplemental material). Despite the limited number of data points (17 data points shared between our data and reference 23, 30 with reference 22, and 18 with reference 24), significant ($P <$

TABLE 2 Level 3 gene ontologies associated with proteins that significantly change in abundance in the *ssrB* mutant

Abundance	Significant SsrB ChIP score	Gene ontology term	No. of proteins
−	+	Pathogenesis	10
−	+	Establishment of localization	9
−	+	Primary metabolic process	5
−	+	Cellular metabolic process	5
−	+	Cellular localization	4
−	+	Macromolecule localization	4
−	+	Response to stress	3
−	+	Macromolecule metabolic process	3
−	−	Cellular metabolic process	18
−	−	Primary metabolic process	17
−	−	Macromolecule metabolic process	12
−	−	Nitrogen compound metabolic process	10
−	−	Biosynthetic process	8
−	−	Small molecule metabolic process	8
−	−	Oxidation-reduction process	5
+	NA	Cellular metabolic process	10
+	NA	Regulation of biological process	5
+	NA	Establishment of localization	5
+	NA	Response to chemical stimulus	4
+	NA	Taxis	4
+	NA	Response to external stimulus	4
+	NA	Localization of cell	4
+	NA	Cellular component movement	4
+	NA	Cell cycle	2
+	NA	Cell division	2
+	NA	Cellular response to stimulus	2
+	NA	Cell communication	2



0.05) correlations were found in each case (Spearman $r = 0.82$, 0.67 and 0.48, respectively). While this supports the validity of our data, the limited number of data points precludes any additional conclusions about quantitation of expression at mRNA versus protein levels.

Expression clustering. In all, the abundance of 1,368 proteins was quantified under all five sets of conditions examined, and the data were clustered by Euclidean distance (Fig. 2). Of the 22 SPI1 proteins that were quantified under all conditions, 14 were coexpressed with the SPI4-encoded proteins SiiB, SiiC, and SiiE and formed cluster 1 (Fig. 5; also, see the links in Fig. 1). SPI4 encodes a secretion system and an adhesin important for attachment to the intestinal epithelium (25), and their expression has previously been shown to be coordinated with that of SPI1 and influenced by HilA (26), affirming our experimental and analysis approach. A second cluster (cluster 2) contained five SPI1 proteins and a hypothetical protein (SL2426) and was distinguished from cluster 1 primarily by lower abundance in SPI1-inducing medium (Fig. 5; also, see the links in Fig. 1).

Only seven T3SS-2 proteins and effectors were quantified under all conditions examined and included in the analysis. These seven proteins formed cluster 3 with SrfN, which is also known to be regulated by SsrB (17), and ten proteins whose functions have not been linked to SPI2 function, including known virulence factors PagC and SodCI (Fig. 5; also, see the links in Fig. 1). The coexpression of numerous proteins that are unrelated to T3SS-2 contrasts with our results for HilA and indicates a significant role for SsrB in coordinating expression of proteins that have not previously been linked to T3SS-2 functions.

Cluster 4 was notable for a moderate degree of repression by SsrB (Fig. 5; also, see the links in Fig. 1). This group of proteins included four flagellar proteins and when annotated using GO, showed a significant association with the terms "bacterial-type flagellum basal body" and "motor activity." This suggests that through SsrB, *Salmonella* actively down-regulates flagella, adding more weight to the hypothesis that SsrB coordinates more than just direct interactions with the host cell.

While our present understanding of SsrB is that it regulates, almost exclusively, direct interactions with the host through SPI2, the proteomics above indicated that other functions, not involving host interaction, also come under the control of SsrB. We decided to focus on two such functions. The first hypothesis we wished to test was that SsrB enhances the ability of *Salmonella* to detoxify reactive oxygen species (ROS). The second hypothesis was that SsrB decreases flagellar motility as well as expression of flagella during infection of host cells.

Within the *Salmonella*-containing vacuole (SCV), *Salmonella* is exposed to superoxide, a ROS generated by phagocyte oxidase as part of the host's antimicrobial response. SodCI, which we found to be coexpressed with SPI2 proteins, is the *Salmonella* superoxide dismutase that plays a major role in virulence by converting superoxide to hydrogen peroxide (27). We therefore predicted that *ssrB Salmonella* would be more sensitive to superoxide than the wt and tested these strains' resistance to superoxide generated by xanthine oxidase activity with hypoxanthine as a substrate. As can

FIG 5 Clustering data points quantified under all conditions according to Euclidian distance. (Left) High-density heatmap of all data points, with a tree. (Right) Individual clusters.

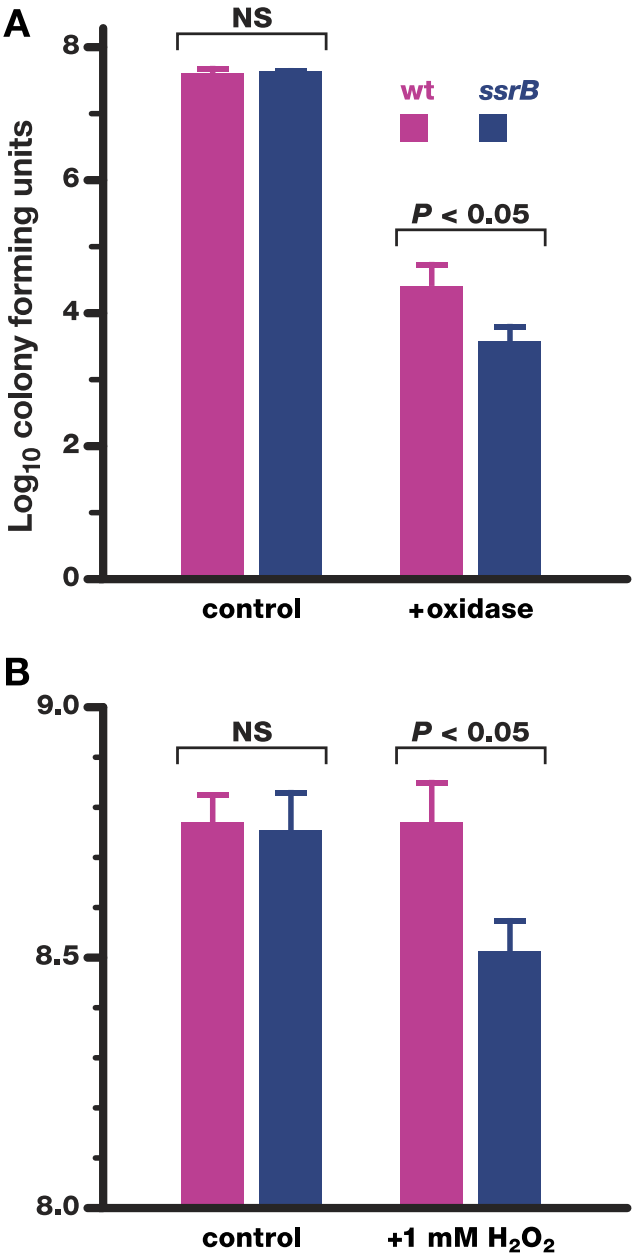


FIG 6 SsrB enhances resistance to ROS. Results from assays of resistance to superoxide (A) and hydrogen peroxide (B) are plotted. Data are arithmetic means and standard errors of the means from three experiments. The degrees of resistance were significantly different between the *ssrB* mutant and the wt according to a paired *t* test.

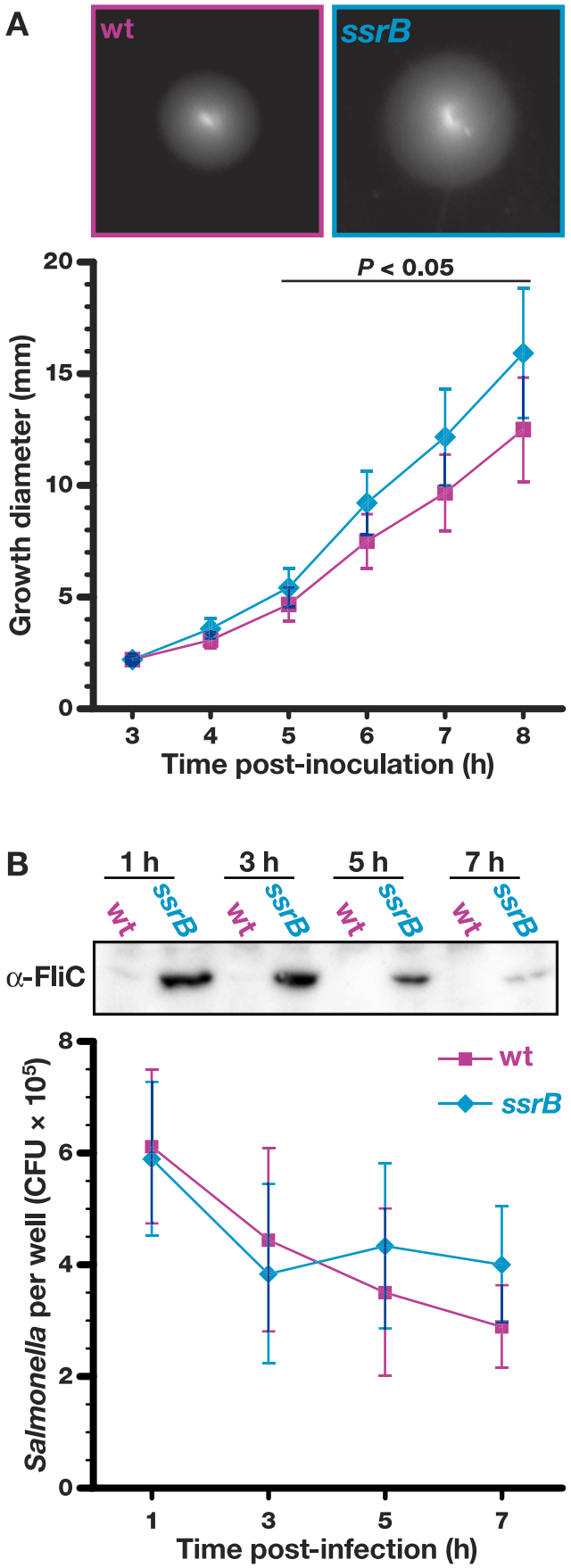
be seen in Fig. 6A and consistent with our hypothesis, the *ssrB* mutant was significantly more sensitive to superoxide. While SodCI provides a degree of resistance to ROS by converting superoxide to hydrogen peroxide, hydrogen peroxide itself is a ROS, which at concentrations of up to 1 mM is primarily toxic via the Fenton reaction with ferrous iron, which leads to DNA damage (28). Sequestration of ferrous iron effectively negates the Fenton reaction (28), and in *Salmonella*, bacterioferritin (Bfr), which in our proteomic data is reduced almost 3-fold (see Table S1 in the supplemental material), is the major iron storage protein and protects against hydrogen peroxide toxicity (29). Owing to this, we predicted that *ssrB Salmonella* would be more sensitive to 1 mM hydrogen peroxide than the wt, and as can be seen in Fig. 6B, this was indeed the case. Collectively, these results demonstrate that SsrB acts to regulate multiple levels of resistance to ROS, to which it is exposed in the SCV.

Salmonellae are highly motile bacteria due to high expression of flagella in environments such as the host's intestinal lumen. However, once the bacterium is enclosed within the SCV inside a macrophage, there is no known role for flagellar motility, and for reasons of energetic efficiency, it is likely that a benefit may exist for reducing flagellar expression in the SCV. The abundances of almost all flagellar proteins in the proteomic data set show modest increases in the *ssrB* mutant, many of which are supported by statistics (Table 2; also, see Tables S1 and S5 in the supplemental material), and many of which cluster together (Fig. 5, cluster 4). Given that SsrB is primarily active while *Salmonella* is resident within the SCV, we hypothesized that SsrB has a role in down-regulating flagellar expression in this environment. Our first experiment to test this examined the motility of the *ssrB* mutant on motility agar plates containing the SPI2-inducing low-phosphate, low-magnesium (LPM) medium. Compared to wt controls, the *ssrB* mutant had significantly increased motility (Fig. 7A), and this led us to investigate flagellin expression in the more physiologically relevant environment of the SCV in RAW264.7 cells. Over a time course of the first 7 h of infection, down-regulation of flagellin expression was delayed in the *ssrB* strain relative to the wt, and at any given time point, the abundance was much greater in the *ssrB* strain. Together, these results make clear the existence of a role for SsrB in decreasing flagellar expression following entry into host phagocytes.

DISCUSSION

Using the quantitative proteomic approach of SILAC, we were able to quantify the abundance of over 1,900 and 1,500 proteins, respectively, in the *hilA* and *ssrB* strains compared to the abundance in the wt during growth in inducing culture medium. Overall, HilA appears to have little influence outside expression of SPI1 and SPI4, which are the central mechanisms employed by *Salmonella* to interact with the intestinal epithelium. In contrast, the data herein indicate that SsrB appears to regulate a much broader range of functions than reported in previous studies, which had essentially focused on T3SS-2 and its effectors. In particular, the additional functions seem to represent adaptations to the SCV, which is established by the actions of T3SS-2 and its effectors.

It seems appropriate to describe SsrB as the master regulator of a multifaceted approach used to defend *Salmonella* against the phagocyte respiratory burst. One of the first distinctive phenotypes ascribed to SPI2 was manipulating infected host cells to avoid deposition of the NADPH oxidase on the SCV, thereby reducing the concentration of ROS to which *Salmonella* is exposed (30). While this finding has been followed by some controversy (31, 32), it at least seems that the SPI2-mediated inhibition of NADPH oxidase is incomplete, as exemplified by the increased sensitivity of gp97 phox$^{-/-}$ mice to wt *Salmonella* infection (33). Now the work herein has shown that SsrB positively influences two additional defenses against the oxidative burst (SodCI and Bfr), each of which protects against distinct ROS. Both SodCI and Bfr have significant SsrB ChIP peaks upstream (22), indicating that SsrB regulation is likely direct. So SsrB regulates *Salmonella*'s defense against the oxidative burst by subverting the burst itself, as



well as detoxifying residual ROS from the inhibited respiratory burst. The repression of flagellar components by SsrB was an unexpected finding of this study. While the magnitude of repression of flagellar proteins during culture in general was modest, the effect on motility was nonetheless significant, and flagellin expression during infection of RAW264.7 macrophages was striking. Considering the primary role of SsrB in coordinating interactions with host cells from within the SCV, down-regulation of flagella by SsrB seems efficient and logical, since flagellar motility would be of little use in such an environment. However, this may not be so straightforward. A previous study concluded that flagella (as well as T3SS-1) were expressed at the same time as T3SS-2 during growth within HeLa cells (34). While the results from this study are clear, there are alternative interpretations that merit consideration. Within epithelial cell lines, salmonellae replicate not only in SCVs but also directly in the cytosol, where, at early times following invasion, replication exceeds that within SCVs (35–38). The *in vivo* relevance of this niche remains to be demonstrated, but it is clear that determining gene expression by *Salmonella* within HeLa cells encompasses at least two very distinct microenvironments where *Salmonella* will certainly respond by expressing distinct traits. It is noteworthy that the *Salmonella* transcriptome within macrophages, a relevant cell type where salmonellae do not replicate in the cytosol, indicates that *Salmonella* does not express flagella within macrophages (16). Our data obtained with RAW264.7 cells are consistent with this, and we propose a role for SsrB in down-regulating flagella while salmonellae reside in an SCV. An additional possible benefit of this SsrB-mediated down-regulation is that it should reduce stimulation of the NLRC4 inflammasome, which has been shown to lead to an effective host response against *Salmonella* (39–42).

To conclude, we found that the HilA regulon does not extend beyond SPI1 and SPI4, which both directly interact with host cells, whereas the SsrB regulon extends beyond SPI2 to control many adaptations of the bacterial cell to existence within the SCV. This should be expected. The anatomical location in the host where SPI1 and SPI4 function is the intestinal lumen, which is an environment that the evolutionary ancestor of *Salmonella* had previously adapted to, and therefore it had regulatory systems that responded to relevant environmental cues. In contrast, upon acquisition of SPI2, *Salmonella* gained the capacity to manipulate host cell membrane traffic to establish a replicative niche inside host cells. This microenvironment is distinct from any in *Salmonella*'s immediate evolutionary history, and therefore, considerable advantage was to be gained by coordinating general cellular functions to increase bacterial numbers in the SCV. We have demonstrated that these functions include resistance to ROS and

FIG 7 SsrB inhibits motility and flagella production. (A) Motility in LPM medium. Cropped images from the same LPM motility plate incubated for 8 h are shown, indicating a typical difference in the zone of motility. The graph shows the arithmetic means and standard errors of the means of motility zone diameters from six experiments, plotted against time. Means from 5 to 8 h were significantly different according to a Wilcoxon signed rank test. (B) Flagellin expression during infection of RAW264.7 cells. Western blots of whole-cell lysates from infected cells made at the indicated times postinfection were probed for FliC. A representative blot from 3 replicates is shown. To control for bacterial numbers in the infected cells, we performed viable count assays at the same times, and a graph plotting the arithmetic mean and standard error of the mean is shown.

down-regulation of flagella, which serve no known purpose in such an environment.

MATERIALS AND METHODS

Strains and growth conditions. *Salmonella* strains used in this study are all derived from SL1344 (43). To efficiently label lysine and arginine in SILAC experiments, a *lysA argH* lysine arginine doubly auxotrophic strain was used (44). The *hilA* and *ssrB* strains have been described previously (45–47), and these kanamycin-marked mutations were moved into the *lysA argH* strain by P22 transduction. Where appropriate, kanamycin was added to all media at 50 μg ml^{-1}. All *Salmonella* strains were routinely cultured overnight at 37°C in lysogeny broth (LB) (48, 49). For the stationary-phase LB culture analyzed by mass spectrometry, *lysA argH Salmonella* from an overnight LB culture was used to inoculate 10 ml of LB with a 1:10,000 inoculum, followed by 24-h incubation at 37°C with shaking. The SPI1-inducing culture used in this study is a variation of the logarithmic-phase LB culture that is often used for this purpose (50). Being a complex medium, LB cannot be used to label amino acids, and we therefore designed a simple medium, termed defined lysogeny broth (dLB), roughly corresponding to the composition of LB. It consisted of 5 mM glucose, 0.2 M NaCl, 80 μM $CaCl_2$, 10 μM $FeCl_3$, 0.2 mM $MgSO_4$, 5 mM KH_2PO_4, 10 mM 3-(*N*-morpholino)propanesulfonic acid, and amino acids at the concentrations shown in Table S7 in the supplemental material. The medium pH was 7.1. Using an overnight culture grown in dLB, dLB cultures for mass spectrometry analysis were inoculated 1:33 and incubated at 37°C with shaking for 3 h. For SPI2-inducing conditions, a variant of LPM medium (pH 5.8) (47) was used where amino acids were added at concentrations shown in Table S7 in place of Casamino Acids, which are typically used with this medium. Using an overnight culture grown in LPM medium, LPM medium cultures for mass spectrometry analysis were inoculated 1:100 and incubated at 37°C with shaking for 8 h. $^{2}H_4$-lysine and $^{13}C_6$-arginine were used as "medium" labels and $^{13}C_6{}^{15}N_2$-lysine and $^{13}C_6{}^{15}N_4$-arginine were used as "heavy" labels where appropriate.

Sample preparation. Salmonellae were harvested from culture media by centrifugation at a relative centrifugal force (RCF) of 3,000 for 10 min at 4°C. They were then resuspended and lysed in 1 ml of 150 mM sodium deoxycholate, 50 mM $NaHCO_3$ at 99°C with vigorous shaking for 5 min The lysate was cleared by centrifugation at an RCF of 500 for 2 min, following which 0.5 mg of protein from each sample for SILAC comparison was combined in a clean tube and subject to trypsin digestion, desalting, and isoelectric focusing into 20 fractions for mass spectrometry analysis as previously described (51).

Mass spectrometry. Approximately 2 μg of tryptic peptides per isoelectric focusing fraction were analyzed on an LTQ-OrbitrapXL mass spectrometer (Thermo, Fisher Scientific) coupled to an Agilent 1100 series high-performance liquid chromatograph with a nanospray electrospray ionization source (Proxeon Biosystems), as previously described (52).

Phenotypic assays. For the assessment of the motility phenotype, 25 ml of LPM medium containing 0.35% agar was poured per 100-mm petri dish. Inocula of wt and *ssrB Salmonella* were stabbed 5 cm apart in the same LPM motility plate and incubated at 37°C. The zone of motility was measured twice (at right angles) and averaged hourly from 3 to 8 h. This experiment was conducted six times.

Salmonellae for superoxide resistance assays were grown in LPM medium for 8 h from a 1:100-diluted overnight LB culture. These salmonellae were then diluted 1:100 in a buffer which was identical to LPM medium except that carbon sources were excluded and it additionally contained 0.1 U ml^{-1} xanthine oxidase and 250 μM hypoxanthine (27). Controls lacking xanthine oxidase and hypoxanthine were also included. Following incubation at 37°C for various times, salmonellae were enumerated by plating dilutions. These experiments were performed six times.

Peroxide resistance assays were a modification from a previous procedure (53). For each strain to be examined in a peroxide resistance assay, two 3-ml cultures were each inoculated with 30 μl from an overnight LB culture and incubated at 37°C with shaking for 5 h. At this time, H_2O_2 was added to one culture to a concentration of 1 mM while the other was left as a control, and the cultures were further incubated for 1 h before viable counts were determined. Dilutions were plated on lysogeny agar plates treated with 2 U of catalase to remove residual H_2O_2.This experiment was conducted three times.

Data analysis and bioinformatics. Mass spectrometry data were analyzed and quantified using MaxQuant 1.3.0.5, and statistics were determined using Perseus 1.3.0.4 (19, 20, 54). To determine whether the expression of a protein was significantly affected by mutation of *hilA* or *ssrB*, we set a threshold of a magnitude change of at least 2 and a Benjamini-Hochberg false discovery rate (FDR) of less than 0.05 when applied to the MaxQuant significance B score (19). Proteins were functionally annotated according to GO using Blast2GO, with enrichment of terms determined using Fisher's exact test comparing the proteins of interest with all other proteins quantified under the respective condition (55, 56). A Benjamini-Hochberg false discovery rate cutoff of 0.05 was used when ratios different from the population mean were determined in Perseus, and when enrichment of GO terms in Blast2GO was determined. Expression data were clustered according to Euclidean distance based on average linkage using the TM4 microarray software suite (57, 58). Correlation determination did not assume Gaussian distributions and used the method of Spearman with a 95% confidence interval. For superoxide and peroxide resistance assays, means were compared using a paired *t* test. Means from motility assays at each time point were compared using a Wilcoxon signed rank test. All statistical tests used a confidence interval of 95%.

The ChIP data set (22) was compared with our proteomic data set using a visual basic script that will be provided upon request. It was run on a spreadsheet sorted by location in the genome and sought to identify orientation of the coding sequence for a protein in our data (or a protein of interest) and then the first gene in an operon based on the maximum gap between genes in an operon being 50 bp (59, 60), followed by defining a scan range extending from 2,000 bp upstream of the first gene in the operon up to the start of the coding sequence for the protein of interest. The scan region was defined in this way to allow for potential SsrB inputs within the operon but upstream of the gene encoding the protein of interest. The highest ChIP score in this scan region was recorded in the spreadsheet.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01727-14/-/DCSupplemental.

Table S1, XLSX file, 0.5 MB.
Table S2, XLSX file, 0.1 MB.
Table S3, XLSX file, 0.1 MB.
Table S4, XLSX file, 0.05 MB.
Table S5, XLSX file, 0.04 MB.
Table S6, XLSX file, 0.04 MB.
Table S7, XLSX file, 0.04 MB.

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