Guanosine tetraphosphate relieves the negative regulation of *Salmonella* pathogenicity island-2 gene transcription exerted by the AT-rich ssrA discriminator region

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The repressive activity of ancestral histone-like proteins helps integrate transcription of foreign genes with discrepant AT content into existing regulatory networks. Our investigations indicate that the AT-rich discriminator region located between the −10 promoter element and the transcription start site of the regulatory gene *ssrA* plays a distinct role in the balanced expression of the *Salmonella* pathogenicity island-2 (SPI2) type III secretion system. The RNA polymerase-binding protein DksA activates the *ssrAB* regulon post-transcriptionally, whereas the alarmone guanosine tetraphosphate (ppGpp) relieves the negative regulation imposed by the AT-rich *ssrA* discriminator region. An increase in the GC-content of the *ssrA* discriminator region enhances *ssrAB* transcription and SsrB translation, thus activating the expression of downstream SPI2 genes. A *Salmonella* strain expressing a GC-rich *ssrA* discriminator region is attenuated in mice and grows poorly intracellularly. The combined actions of ppGpp and DksA on SPI2 expression enable *Salmonella* to grow intracellularly, and cause disease in a murine model of infection. Collectively, these findings indicate that (p)pGpp relieves the negative regulation associated with the AT-rich discriminator region in the promoter of the horizontally-acquired *ssrA* gene, whereas DksA activates *ssrB* gene expression post-transcriptionally. The combined effects of (p)pGpp and DksA on the *ssrAB* locus facilitate a balanced SPI2 virulence gene transcription that is essential for *Salmonella* pathogenesis.

Nontyphoidal *Salmonella enterica* serovar Typhimurium is a common cause of gastroenteritis in immunocompetent individuals and a life-threatening disseminated complication in immunocompromised hosts unable to mount CD4+ T cell immunity or IFNγ host responses1,2. This intracellular pathogen replicates within *Salmonella*-containing vacuoles (SCV) of epithelial and phagocytic cells in part due to the activity of a type III secretion system that is encoded within the horizontally-acquired *Salmonella* pathogenicity island-2 (SPI2)3–5. Effector proteins translocated through the SPI2 type III secretion system minimize contact of SCV with lysosomes and cell host vesicles harboring NADPH phagocyte oxidase or inducible nitric oxide synthase (iNOS)6–10. By redirecting SCVs to the trans-Golgi network and exocytic pathway, the SPI2 type III secretion system also aids *Salmonella* in overcoming the nutritional restrictions found in vesicles of the degradative pathway11,12. *Salmonella* initiate SPI2 gene transcription as the transforming SCV microenvironment acidifies and becomes limiting for iron and other divalent cations13–18. These signals activate the EnvZ and PhoQ sensor kinases, which catalyze phosphotransfer reactions to their cognate response regulators OmpR and PhoP, respectively15,18. PhoP competes with histone-like proteins for binding to the *ssrA* promoter, counter-silencing the repressive activity of these nucleoid-structuring proteins19,20. The sensor kinase encoded by the *ssrA* gene senses acidification via

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several histidine residues in the periplasmic domain. Activated SsrA phosphorylates its cognate SsrB response regulator, which in turn recruits the RNA polymerase to SPI2 genes encoding components of the secretion apparatus, chaperones, and effectors. The negative regulation of SPI2 genes is also an important aspect in Salmonella pathogenesis. For example, ElaA, which prevents binding of SsrB to promoters of SPI2 target genes, is required for Salmonella virulence. Also, the inactivation of SsrB via oxidation or S-nitrosation of Cys contributes to Salmonella virulence. Moreover, binding of the histone-like proteins H-NS and YdgT to AT-rich SPI2 genes represses SPI2 transcription during non-inducing conditions. The absence of these histone-like proteins attenuates Salmonella in spite of SPI2 overexpression.

The AT-rich composition of the discriminator region, which is located between the −10 element and transcription start site, could be an additional repressive element to transcription of horizontally-acquired genes. Promoters with AT-rich discriminator regions often produce stable, long-lived, open complexes that become saturated with RNA polymerase, aborting initiation of transcription. The stringent response is controlled by the RNA polymerase-binding protein DksA and the nucleotide alarmones guanosine tetra/pentaphosphate [ppGpp] that are synthesized in Salmonella by the RelA and SpoT proteins. DksA binds to the secondary channel of the RNA polymerase, whereas two molecules of ppGpp bind between the ω and β′ subunits and at the interface of RNA polymerase and DksA. DksA and ppGpp exert transcriptional regulation by reducing the half-life of RNA polymerase-DNA open complexes. The stringent response generally activates or represses gene transcription from promoters with AT- or GC-rich discriminator regions, respectively. The preservation of AT-rich discriminator regions in horizontally-acquired genes suggests that the negative control associated with AT-rich discriminators provides a selective advantage to bacterial pathogens.

Microarrays and differential RNA sequencing indicate that the stringent response regulators DksA and ppGpp play additional roles during the intracellular growth of Salmonella. We measured the replication of ΔdksA and ΔrelA ΔspoT Salmonella in J774A.1 macrophage-like cells. Wild-type Salmonella began to replicate 8 h post-infection, reaching over 100-fold higher bacterial burdens 16 h after the initial infection (Fig. 1A). In contrast, ΔdksA Salmonella grew poorly (Fig. 1A). Under the experimental conditions tested, our J774A.1 cells do not generate detectable amounts of reactive oxygen species in response to Salmonella. These findings suggest that the growth defect of ΔdksA Salmonella in this population of J774A.1 cells cannot be attributed to its reported hypersusceptibility to oxidative stress. Treatment of J774A.1 cells with IFN-γ arrested growth of both wild-type and ΔdksA Salmonella (Fig. 1B). Wild-type bacteria, but not the ΔdksA mutant, grew in IFN-γ-activated macrophages treated with the iNOS specific inhibitor L-lysine (L-NIL) (Fig. 1B). As expected, L-NIL inhibited NO synthesis (Fig. S1A). Together, these investigations suggest that DksA can aid in the intracellular replication of Salmonella independently of its promotion of antioxidative defenses.

Since DksA and ppGpp often coregulate the RNA polymerase, we also tested the intracellular growth of a ΔrelA ΔspoT strain. A ΔrelA ΔspoT Salmonella strain exhibited profound intracellular growth defects (Fig. 1C). The failure of ΔdksA and ΔrelA ΔspoT Salmonella strains to replicate intracellularly resembles phenotypes reported for strains deficient in SPI2 genes. Accordingly, an isogenic strain lacking the SPI2 effector sifA, whose product is necessary for maintaining integrity of the SCV, grew as poorly in J774A.1 cells as ΔdksA and ΔrelA ΔspoT Salmonella controls (Fig. 1C). The growth defect of ΔdksA and ΔrelA ΔspoT Salmonella could be complemented by dksA and spoT alleles expressed in the chromosome (Fig. 1D). We next tested whether the stringent response regulators DksA and ppGpp contribute to the intracellular expression of sifA. Compared to wild-type controls, both ΔdksA and ΔrelA ΔspoT Salmonella expressed low levels of the SPI2 effector sifA in J774A.1 macrophage-like cells (Fig. 1E). As shown previously, wild-type Salmonella grown for 3 h in 8 mM MgCl2 N9 medium expressed all SPI2 promoters tested; however, ΔdksA or ΔrelA ΔspoT Salmonella did not stimulate expression of any SPI2 genes examined (Fig. S1B).

Collectively, these observations raise the possibility that the stringent response regulators DksA and ppGpp help Salmonella grow in macrophages by controlling the expression of the SPI2 type III secretion system.

### Contributions of DksA, ppGpp, and SsrB to Salmonella pathogenesis

Since DksA and ppGpp play broad roles in gene transcription, we deemed it important to quantify the extent that these stringent response regulators rely on the SPI2 type III secretion system to promote Salmonella pathogenesis. When compared to wild-type controls, the number of ΔsirB, ΔdksA, or ΔΔsirB ΔdksA ΔsirB Salmonella was about 1,000-fold lower in spleens (Fig. 2A) and livers (Fig. S2A) of C57BL/6 mice. Strains unable to generate ppGpp were more attenuated than ΔsirB or ΔdksA Salmonella, as demonstrated by their complete elimination from spleens and livers 3 days after intraperitoneal inoculation (Figs 2A and S2A). To determine fitness of ΔrelA ΔspoT Salmonella, C57BL/6 mice were inoculated with 10^6 CFU of each ΔrelA ΔspoT and ΔrelA ΔspoT ΔsirB Salmonella. The ΔrelA ΔspoT ΔsirB mutant had a competitive index of ~1 when compared to ΔrelA ΔspoT Salmonella, but showed a 100-fold lower competitive index than ΔsirB Salmonella (Figs 2B and S2B). These data suggest that ppGpp can participate in Salmonella virulence in SPI2-dependent and -independent ways. To
better calculate the apparent codependency of SsrB and DksA, we used the method described by Beuzon and Holden to quantify virulence gene interactions in vivo. Groups of C57BL/6 mice were inoculated with 10^5 CFU of ΔssrB ΔdksA in combination with ΔssrB or ΔdksA Salmonella. The ΔdksA ΔssrB double mutant strain was isolated from spleen and liver tissue in similar numbers to ΔssrB or ΔdksA single mutants (Figs 2B and S2B), suggesting that the role played by this RNA polymerase-binding protein in Salmonella pathogenesis appears to be strongly co-dependent on the SPI2 master regulator SsrB.

**Requirement of DksA and (p)ppGpp for the activation of ssrAB transcription.** We examined whether DksA and (p)ppGpp participate in the transcriptional activation of the ssrA and ssrB genes that encode the master two-component regulatory system that activates SPI2 expression. Wild-type Salmonella up-regulated the expression of ssrA (Fig. 3A) and ssrB mRNA (Fig. 3B) 3 h after culture in 8 μM MgCl_2 N9 medium. We also observed that ΔdksA Salmonella induced excellent ssrA and ssrB expression upon culture in 8 μM MgCl_2 N9 medium (Fig. 3A, B). These findings indicate that DksA does not appear to regulate ssrA or ssrB gene transcription. Since ΔdksA Salmonella induced ssrB expression but failed to globally activate SPI2 transcription, Western blotting was used to visualize the amount of SsrB protein in wild-type and ΔdksA Salmonella. Wild-type Salmonella...
harbored low concentrations of SsrB protein when grown under non-inducing 10 mM MgCl₂ N9 medium, but harbored high concentrations of this response regulator 3 h after growth in 8 μM MgCl₂ N9 medium (Fig. 3C).

Compared to wild-type controls, ΔdksA Salmonella expressed much lower concentrations of SsrB protein upon culture in 8 μM MgCl₂ N9 medium. Expression of a dksA allele reestablished production of SsrB protein in ΔdksA Salmonella (Fig. S3A). In view of the abundant ssrB mRNA seen in ΔdksA Salmonella, deficient production of SsrB protein indicates that DksA may regulate the expression of this response regulator post-transcriptionally.

Compared to ΔdksA Salmonella and wild-type controls, ΔrelA ΔspoT Salmonella had significantly lower basal levels of ssrA and ssrB mRNA in non-inducing 10 mM MgCl₂ N9 medium (Fig. 3A,B). Growth of ΔrelA ΔspoT Salmonella in 8 μM MgCl₂ N9 medium did not stimulate ssrA or ssrB expression. As predicted from these transcriptional profiles, ΔrelA ΔspoT Salmonella contained extremely low amounts of the SsrB protein (Fig. 3C).

The lack of ssrA and ssrB expression in ΔrelA ΔspoT Salmonella raises the possibility that (p)pGpp may directly activate ssrAB gene transcription. To test this idea, serine hydroxamate (SHX) was added to Salmonella grown to log phase in M9 minimal media. Addition of SHX to rapidly growing bacteria inhibits seryl-tRNA synthetase; the resulting accumulation of deacylated tRNAs stimulates (p)pGpp synthesis from RelA 48. The expression of ssrA mRNA was induced after the addition of SHX (Fig. 3D). SHX, however, did not induce ssrA transcription in ΔrelA ΔspoT Salmonella. To further examine the possibility that (p)pGpp directly activates ssrA transcription, ppGpp was added to in vitro transcription reactions containing the pTIM-ssrA plasmid template (Fig. S4). ssrA transcripts were quantified by combining in vitro transcription reactions with a highly sensitive and specific qRT-PCR method 49,50. This approach revealed that ppGpp directly stimulates ssrA in vitro transcription in a concentration-dependent manner (Fig. 3E). These data indicate that (p)pGpp suffices to activate ssrA transcription in Salmonella.

The ssrA AT-rich discriminator region facilitates Salmonella virulence. Since (p)pGpp often activates gene transcription from AT-rich discriminator regions that form stable, long-lived, open complexes with RNA polymerase27,28,31, we focused our attention on the AT-rich PsrrA discriminator region. We reasoned that increasing the GC-content would modulate the negative regulation associated with the AT-rich discriminator region of PsrrA. To test this model, we engineered three substitutions at the native locus in the Salmonella chromosome that increased the GC content in the ssrA discriminator region, yielding the ssrA_Dsc Salmonella strain (Figs 4A and S5A). Transcription of ssrA (Fig. 4B) and ssrB (Fig. 4C) was markedly higher in ssrA_Dsc Salmonella than wild-type controls grown in LB broth to early stationary phase. Consistent with higher levels of ssrA and ssrB mRNA, the concentration of SsrB protein was higher in ssrA_Dsc Salmonella grown in stationary phase in LB broth than in wild-type isogenic controls (Fig. 4D). The concentration of SsrB was also higher in ssrA_Dsc Salmonella

Figure 2. Codependence of SsrB, ppGpp, and DksA in Salmonella pathogenesis. Competitive indexes of Salmonella strains recovered from spleens of C57BL/6 mice 3 days after infection. Mice were inoculated intraperitoneally with 10² (A) or 10⁵ (B) CFU of the indicated Salmonella strains. No detectable (nd) CFU were isolated for the ΔrelA ΔspoT strain under the experimental conditions used in panel A. Competitive index was determined according to the equation: (strain 1/strain 2)output/(strain 1/strain 2)input. Non-significant (ns), **p < 0.01.
than wild-type controls grown in N9 low Mg\(^{2+}\) media (Fig. S5B). Expression of ssrA\(_Dsc\) allele in the \(\Delta\)relA\(\Delta\)spoT background dramatically reduced the amount of intracellular SsrB protein in early stationary phase \(\text{Salmonella}\), suggesting that the derepression of \(\text{ssrAB}\) transcription associated with a GC-rich ssrA discriminatory region is dependent on (p)ppGpp. It should be noted that \(\Delta\)relA\(\Delta\)spoT ssrADsc \(\text{Salmonella}\) expressed more SsrB than \(\Delta\)relA\(\Delta\)spoT controls (Fig. 4D), but less than wild-type and ssrADsc controls. The concentration of SsrB protein (Fig. 4D) reflected \(\text{ssrB}\) mRNA levels (Fig. 4C,E). Transcription of the SsrB-regulated \(\text{ssaG}\) gene was also upregulated (\(p < 0.001\)) in ssrA\(_Dsc\) \(\text{Salmonella}\) compared to isogenic wild-type bacteria (Fig. 4F).

**Virulence of ssrA\(_Dsc\) \(\text{Salmonella}\).** Because some SPI2-dependent phenotypes, such as the one associated with an SsrB C203S variant, were revealed in a C3H/HeN model of oral salmonellosis\(^{24}\), we chose this model to test the virulence of ssrA\(_Dsc\) \(\text{Salmonella}\). Moreover, the oral mucosa is the natural route of \(\text{Salmonella}\) infection. We found that ssrA\(_Dsc\) \(\text{Salmonella}\) appear to be as attenuated as \(\Delta\text{ssrAB}\) isogenic bacteria when compared to wild-type \(\text{Salmonella}\) (Fig. 5A). These data suggest that the overexpression of SPI2 attenuates ssrA\(_Dsc\) \(\text{Salmonella}\) in a murine model of oral salmonellosis. To test this idea, we evaluated the virulence of a \(\text{Salmonella}\) strain overexpressing the SsrB protein (Fig. 5B). \(\text{Salmonella}\) expressing pWSK29-ssrB, not the empty vector, were attenuated when inoculated p.o. into C3H/HeN mice (Fig. 5C). \(\text{Salmonella}\) strains expressing the ssrA\(_Dsc\) allele (\(p < 0.05\)) or the
pWSK29-ssrB plasmid (p < 0.001) grew to lower densities in J774 macrophage-like cells than wild-type controls (Fig. 5D). Collectively, these findings indicate that overexpression of SsrB diminishes Salmonella virulence.

Discussion
Horizontally-acquired and ancestral genes often contain considerable differences in base composition, as exemplified by the SPI2 genes of Salmonella51. This enteric pathogen has resolved potential difficulties of regulating the SPI2 virulence program by silencing AT-rich promoters with histone-like proteins such as H-NS and YdgT25,26. The inhibitory effects of H-NS are counter-silenced by transcription factors such as PhoP and SsrB19,52. The AT-rich composition of the discriminator region located between the −10 element and transcription start site can also impose a considerable burden to transcriptional initiation by forming stable, long-lived, open complexes27,28. Despite the potential burden to transcription, maintenance of an AT-rich discriminator region on ssrA suggests that this regulatory element provides a selective advantage to Salmonella pathogenesis. Herein, we tested the intriguing possibility that the AT-rich discriminator region serves as a negative regulatory element that is essential for both the appropriate expression of SPI2 gene transcription and Salmonella virulence.

In order to investigate whether the ssrA AT-rich discriminator region serves as a negative regulatory element, we constructed an ssrA_{Dsc} Salmonella strain with increased GC-content in the discriminator region. Salmonella expressing ssrA_{Dsc} overexpressed ssrA and ssrB genes, which led to enhanced expression of SsrB protein and the downstream ssaG gene. The amount of SsrB protein recorded in ssrA_{Dsc} Salmonella was dramatically reduced when combined with ΔrelA ΔspoT mutations. These findings demonstrate that (p)ppGpp controls the ssrA discriminator region and that preservation of an AT-rich discriminator region places control of SPI2 gene transcription under the stimulatory effects of (p)ppGpp. Despite the potential burden to transcription, maintenance of an AT-rich discriminator region on ssrA suggests that this regulatory element provides a selective advantage to Salmonella pathogenesis. Herein, we tested the intriguing possibility that the AT-rich discriminator region serves as a negative regulatory element that is essential for both the appropriate expression of SPI2 gene transcription and Salmonella virulence.

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strongly argue that the derepression of srrB expression seen in the GC-rich srrA_Dsc Salmonella is dependent on (p)ppGpp rather than through the relief of HN-S, YdgT or StpA binding.

Although appropriate SPI2 expression enables the intracellular replication of Salmonella, misregulation of SPI2 transcription seems to diminish the virulence potential of this enteropathogen in a murine model of infection and in macrophage-like J774 cells (herein and 23–26). The attenuation of Salmonella overexpressing SPI-2 genes is analogous to attenuation of Salmonella expressing a constitutively active PhoP allele.35 At present, we don’t know why the overexpression of SsrB attenuates Salmonella. In addition to activating SPI-2 gene transcription, SsrB activates the expression of ancestral genes.34 The overexpression of ancestral or horizontally-acquired genes may be detrimental to Salmonella pathogenesis. Together, our investigations emphasize the importance that the repression associated with the AT-rich srrA discriminator region plays in Salmonella pathogenesis.

Our investigations indicate that ΔrelA ΔspoT Salmonella are about 100-fold more attenuated than a ΔssrB mutant strain, suggesting roles for (p)ppGpp that are independent of SPI2 gene transcription. This result might reflect the fact that (p)ppGpp regulates 34% of coding RNA transcripts, including SPI1-dependent invasion genes and adaptive stress response programs.34,46,55. Important ways by which this alarmone may contribute to reflect the fact that (p)ppGpp regulates 34% of coding RNA transcripts, including SPI1-dependent invasion gene expression post-transcriptionally via the small RNA DsrA.58 Further investigations are needed to elucidate whether DsrA or a small RNA contribute to the DksA-dependent activation of srrB. For example, DksA regulates σ7 post-transcriptionally via the small RNA DsrA.58 Further investigations are needed to elucidate whether DsrA or a small RNA contribute to the DksA-dependent activation of srrB.

Because DksA regulates approximately 10% of the Salmonella transcriptome, we were surprised by the remarkably high level of co-dependency between DksA and the SPI2 master regulator SsrB in Salmonella pathogenesis. DksA has also been shown to play a major role in the antioxidant and antiinflammatory defenses of Salmonella.40,41. By regulating the expression of gene products of central metabolism, cytosine and glutathione biosynthesis, and iron and redox homeostasis, DksA promotes resistance to oxidative and nitrosative stress.40–42. Our findings herein raise the possibility that the antioxidant and antiinflammatory defenses associated with DksA and Salmonella are not limited to the regulation of NADPH/NADP+ and GSH/GSSG redox homeostasis.40–42. Given the effects that the SPI2 type III secretion system has on vesicular trafficking of NADPH oxidase and iNOS
hemoproteins\textsuperscript{6,7,10}, it is possible that the regulation of SPI2 gene transcription is a sizable component by which DksA promotes antioxidant and antinitrosative defenses of intracellular \textit{Salmonella}.

Our investigations shed light into the molecular mechanisms by which the stringent response regulators DksA and (p)ppGpp activate the expression of bacterial virulence programs. The stringent response regulators control intracellular spread of \textit{Shigella flexneri}\textsuperscript{39}, motility of \textit{Pseudomonas putida}\textsuperscript{40}, adherence and virulence of \textit{Haemophilus ducreyi}\textsuperscript{41}, and avoidance of lysosomes by \textit{Legionella pneumophila}\textsuperscript{42}. DksA and/or (p)ppGpp also regulate the transcription of genes encoding type III secretion systems of \textit{Bordetella pertussis}, \textit{Erwinia amylovora}, \textit{L. pneumophila}, and \textit{Pseudomonas syringae}\textsuperscript{42-45}. In \textit{Salmonella}, DksA activates motility\textsuperscript{46} as well as SPI-1 and SPI-2 type III secretion systems\textsuperscript{48}. In most cases, the mechanisms by which these virulence programs are regulated remain unknown. Employing the broadly conserved stringent response regulator (p)ppGpp to overcome the inhibitory barrier imposed by the AT-rich discriminator region of horizontally-acquired pathogenicity islands provides new insights into the regulation of virulence programs in pathogenic bacteria.

**Experimental Procedures.** \textit{Ethics Statement.} All methods and experimental procedures were carried out in accordance to protocols approved by the University of Colorado School of Medicine (UCSOM) Institutional Biosafety Committee, authorization number 01-028. Mouse experiments were performed at Animal Care Facility of the UCSCOM in accordance to the guidelines established by the UCSCOM Institutional Animal Care and Use Committee (IACUC) protocol # 56413(07)1E.

**Bacterial strains and growth conditions.** \textit{Salmonella enterica} serovar Typhimurium strain 14028s (ATCC, Manassas, VA) and derivative strains are described in Table S1. A 1916-bp DNA fragment, including a 352-bp of the promoter region of the srbB gene, was amplified by PCR from genomic DNA of strain AV07104. The PCR product was directionally cloned into EcoRIV/PstI sites of pWSK29, generating the pWSK29-ssrB 3x-FLAG plasmid. \textit{E. coli} strain DH5\textalpha{} (ATCC) was used in molecular cloning. Mutations and plasmids were confirmed by sequencing. Unless specified, bacteria were grown in Luria-Bertani (LB) broth at 37°C with continuous shaking. When applicable, 20 μg/mL chloramphenicol, 100 μg/mL penicillin, 100 μg/mL ampicillin, 100 μg/mL streptomycin, 20 μg/mL tetracycline, or 50 μg/mL kanamycin were added to the cultures.

**Construction of ssrA\textsubscript{Δ}s, \textit{Salmonella}.** Segments of a 6.1-kb DNA fragment containing the srrAB operon and a chloramphenicol resistant cassette were amplified from \textit{Salmonella} ssrB-3xFLAG genomic DNA by PCR using the primers described in Table S3 and Figure S5A. PCR products were digested and ligated into pBluescript SK(+) to generate pSK-ssr-AB-3xFLAG::cm. To introduce the discriminator mutations into the srrA promoter, primer ssrA-F and ssrA-Dc-R containing the mutations in the discriminator region were used to generate part srrA\textsubscript{Δ}s-R1. The 5′ end of ssrA was amplified using primers srrA\textsubscript{Δ}s-F and ssrA4-R to generate part srrA\textsubscript{Δ}s-P2. The two srrA segments, srrA\textsubscript{Δ}s-P1 and srrA\textsubscript{Δ}s-P2, were stitched together by PCR elongation. This fragment was ligated into pSK-ssrAB-3xFLAG::cm after digestion with EcoRI and NdeI. The 6.1-kb DNA fragment was digested with EcoRI and SacI out of pSK-ssrAB-3xFLAG::cm and introduced into ΔssrAB::FRT \textit{Salmonella} by allelic replacement.

**Allelic replacement.** \textit{Salmonella} strains generated in this study followed the method previously described by Datsenko and Wanner\textsuperscript{47} (Table S1). To generate \textit{Salmonella} mutant strains, the plasmids pKD13 and pSK::cm containing a flippase recognition target (FRT)-flanked chloramphenicol cassette was used as a template to generate ampicillin with 60-base-pair-long primers containing 40-base-pair regions of homology to the gene locus. \textit{Salmonella} strains containing the plasmid pTP223, which expresses the \textlambda{} Red recombinase from an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter, were grown in LB broth containing 20 μg/mL tetracycline for 16h at 37°C in a shaker incubator. Cells were subcultured 1:100 in LB broth containing 20 μg/mL tetracycline and 1 mg/mL IPTG. Cells were grown for 3 h in a shaker incubator followed by incubation on ice for 30 min. Cells were washed 3 times with 10% glycerol. Approximately 100 ng of DNA were electroporated into bacterial strains using an ECM 399 Decoy Single Cell Electroporation System (BTX Harvard Apparatus Inc., Holliston, MA) at 1800 V for 5 milliseconds. Chromosomal genes were replaced by \textlambda{} Red homologous recombination of electroporated PCR products\textsuperscript{47}. Translational fusions containing the promoters of SPI2 genes and lacZ\textsuperscript{48} or luciferase\textsuperscript{46} reporter genes were transduced into ΔdksA and ΔrelA ΔspoT \textit{Salmonella} using P22 phage. The strain ΔrelA ΔspoT put::spoT was generated by amplifying the genetic locus encompassing spoT with spoT pSK primers (Table S3) and cloning into pSK::cm by digestion with Apal and Xhol. The construct was amplified with put::spoT primers (Table S3) and recombined into the \textit{Salmonella put} site by allelic replacement.

**Intracellular replication of \textit{Salmonella}.** \textit{J774A.1} macrophage-like cells (ATCC) were grown in RPMI\textsuperscript{+} 1 medium [RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), 15 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO)] at 37°C in a 5% CO\textsubscript{2} incubator. \textit{J774A.1} cells were infected with stationary phase \textit{Salmonella} that had been grown in LB broth for 20 h at 37°C in a shaker incubator. Selected groups of macrophages were treated with 200 μM IFN-γ 20 h before \textit{Salmonella} infection, and where specified, some of the cultures were treated with 960 μM of the iNOS inhibitor L-NIL (Cayman Chemical, Ann Arbor, MI) at the time of infection. \textit{J774A.1} cells were infected with stationary phase \textit{Salmonella} at an MOI of 2. Cells were then incubated in RPMI\textsuperscript{+} medium containing 10 μg/mL gentamicin. At 2 h and 18 h post infection, cells were lysed with 0.25% deoxycholic acid and intracellular \textit{Salmonella} were enumerated by dilution plating on LB agar.

**Quantification of intracellular sifA::luc expression.** \textit{J774A.1} macrophage-like cells were infected at an MOI of 20 with \textit{sifA::luc}-expressing \textit{Salmonella} grown to stationary phase in LB broth for 20 h at 37°C in a shaker incubator. Extracellular bacteria were removed from the monolayers 25 min after challenge by washing with pre-warmed
RPMI medium containing 50 μg/ml gentamicin. At 8 h post infection, the macrophages were treated with lysis buffer (Promega, Madison, WI) containing 5 mg/ml lysozyme. In parallel, selected macrophages were lysed with 0.25% deoxycholic acid and intracellular bacteria were enumerated on LB agar. Gene expression was measured by following luciferase activity according to the instructions provided by the One-Glo luciferase kit (Promega). Luciferase activity was measured by a Glomax multi-detection system after 5 sec integration in a Lumistar chemoluminometer (Promega). The amount of sifA::luc expression is represented as relative light units (RLU) per colony forming unit (CFU).

**SPI2 induction.** *Salmonella* SPI2 induction was performed as previously described. *Salmonella* strains grown in LB broth for 16 h at 37 °C in a shaker incubator were subcultured 1:100 in N9 medium [5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 38 mM glycerol, 0.1% casamino acids and 100 mM Tris-HCl], pH 7.6 supplemented with 10 mM MgCl₂ until they reached an OD₆₀₀ of 0.5. The specimens were washed 3 times with 8 μM MgCl₂ N9 medium, pH 5.8, and then diluted to an OD₆₀₀ of 0.25 in 8 μM MgCl₂ N9 medium, pH 5.8. After 3 h, cells were pelleted for quantification of SPI2 expression. Alternatively, SPI2 gene expression was induced as *Salmonella* entered into stationary phase in LB broth as previously described. Briefly, *Salmonella* grown in LB broth for 16 h at 37 °C in a shaker incubator were subcultured 1:100 into fresh LB broth and grown for 2.5 h or 5 h at 37 °C in a shaker incubator. Independently, SPI2 gene expression was measured in *Salmonella* grown in LB broth for 2.5 h or 5 h at 37 °C in a shaker incubator. Mouse, independently, SPI2 gene expression was induced as *Salmonella* entered into stationary phase in LB broth as previously described. Briefly, *Salmonella* grown in LB broth for 16 h at 37 °C in a shaker incubator were subcultured 1:100 into fresh LB broth and grown for 2.5 h or 5 h at 37 °C in a shaker incubator. Mouse, independently, SPI2 gene expression was induced as *Salmonella* entered into stationary phase in LB broth as previously described.

**Quantification of ssrA and ssrB transcripts by real-time qPCR.** *Salmonella* cultures growth in 8 μM MgCl₂ N9 medium for 3 h were mixed 1:5 (v/v) with an ice-cold solution containing 5% phenol and 95% ethanol. The specimens were placed on ice for 20 min for RNA stabilization. Isolation of bacterial RNA, synthesis of cDNA, and qRT-PCR was performed as previously described. Briefly, RNA was purified using the high pure RNA isolation kit (Roche) according to the instructions provided by the manufacturer. The micrograms of total RNA was used to generate cDNA in reactions that contained 100 U MMLV reverse transcriptase (Promega), 0.45 μM N6 random hexamer primers (ThermoFisher Scientific), and 20 U RNasin Plus RNase inhibitor (Promega). Reverse transcription was performed for 1 h at 42 °C. The primers and probes used for the qRT-PCR are listed in Table S4. Reactions prepared using TaqMan Gene Expression Master Mix (ThermoFisher Scientific) were incubated for 2 min at 50 °C, followed by 10 min at 95 °C, 40 cycles for 15 sec at 95 °C, and 57 °C for 1 min. Data are expressed as relative expression over the *rpoD* housekeeping gene copy number.

**ssrA in vitro transcription and quantitative RT-PCR.** To measure *ssrA* in vitro transcription, we combined *in vitro* transcription reactions with non-radioactive qPCR analysis. Briefly, 5 nM pT7M-ssrA plasmid was mixed with increasing concentrations of ppGpp (Trilink) in reaction buffer (40 mM HEPES, pH 7.4, 2 mM MgCl₂, 60 mM potassium glutamate, 0.05% NP-40, 200 μM ATP, 200 μM GTP, 200 μM CTP, 200 μM UTP, and 1 mM DTT). Upon addition of 5 nM E. coli RNA polymerase σ₇₀ holoenzyme (NEB, Ipswich, MA) to a 10 μl reaction mixture, the *in vitro* transcription reaction was carried out at 37 °C for 10 min, and then terminated at 70 °C for 10 min. DNA-free DNA Removal kit (ThermoFisher) removed template DNA andDNaseI (ThermoFisher). The resulting materials were used as templates to generate cDNA with 100 U M-MLV reverse transcriptase (Promega), 0.45 μM N6 random hexamer primers (ThermoFisher), and 20 U RNase inhibitor (Promega). The amount of cDNA synthesized for 1 h at 42 °C was quantified by real-time PCR (qRT-PCR) using the primers and probe described in Table S4. The *ssrA* specific transcripts were normalized to the standard curve generated with known *ssrA* gene copy concentrations.

**Western blotting.** *Salmonella* expressing *ssrB* with a C-terminal FLAG epitope were cultured in SPI2-inducing 8 μM MgCl₂ N9 medium as described above. After 3 h, cultures were centrifuged at 10,000 g for 5 min and bacterial pellets were stored at −80 °C. Samples were lysed by sonication in 125 mM NaCl Tris buffer, pH 7.0. Cellular debris was pelleted upon centrifugation at 16,000 g for 5 min. The protein concentration was determined with a Pierce 660 nm Protein Assay Reagent (ThermoFisher Scientific). Total soluble proteins (500 ng) resolved in 12% SDS-PAGE gels were transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 5% milk, and immunoblotted with a 1:500 dilution of mouse IgG1 anti-FLAG M2 (Sigma-Aldrich) or a 1:2500 dilution of mouse IgG anti-DnaK (MBL International Corporation, Woburn, MA) monoclonal antibodies. The membranes were probed with 1:5,000 of HRP-conjugated sheep anti-mouse IgG secondary antibody (GE Healthcare). The signals in the membranes, developed with an Amersham ECL Prime Western antibody (GE Healthcare), were visualized with a Molecular Imager ChemiDoc XRS + system (Bio-Rad).

**Competitive index assay.** The relative contribution of *ssrB, dksA*, and (p)ppGpp to *Salmonella* virulence was quantified by recording the competitive index of mutant and wild-type isogenic controls. Briefly, C57BL/6 J (The Jackson Laboratory, Mount Desert Island, ME) mice bred in our animal facility according to institutional guidelines were infected i.p. with about 10⁶ or 10⁷ CFU of *Salmonella* grown to stationary phase in LB broth for 20 h at 37 °C in a shaker incubator. The bacteria used for inoculation were prepared in PBS. Spleens and livers collected 3 days after infection were macerated in PBS, and the amount of *Salmonella* present in the tissues was enumerated by dilution replica-plating on LB agar containing the appropriate antibiotics. The competitive index was calculated as:

\[(3) \text{(strain 1/(strain 2))}_{\text{output}}/(3) \text{(strain 1/(strain 2))}_{\text{input}}\]
Mouse survival. The virulence of ssrA<sub>H37Rv</sub>-expressing Salmonella was investigated in C3H/HeN mice (The Jackson Laboratory) that were bred in the CU Anschutz animal facility. Briefly, C3H/HeN mice were infected orally with $10^7$ CFU of the indicated Salmonella strains that had been grown in LB broth for 20 h at 37°C in a shaker incubator. The bacteria used for inoculation were prepared in PBS. Mice survival was monitored for 28 days. The data are from 10 mice.

Statistical Analysis. Statistical analysis and graphing were performed using GraphPad Prism 4.0 software. Determination of statistical significance between two comparisons was achieved using an unpaired t-test. Determination of statistical significance between multiple comparisons was done using a one-way analysis of variance (ANOVA) followed by Bonferroni or Dunnett's multiple comparison post-test with respective isogenic strain as control. To determine statistical significance for competitive indexes, one-way ANOVA or Mann-Whitney tests were used. Statistical significance for C3H/HeN mice survival curves was determined using log-rank test, comparing mutant Salmonella strain to wild-type controls.

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Author Contributions
T.T. and A.V.T. wrote the main manuscript text; T.T., J.S.K., M.A.C., L.F., L.L., and J.J.C. performed experiments; T.T., J.S.K., L.L., and A.V.T. prepared the figures and tables. All authors reviewed the manuscript.

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