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SpoT Induces Intracellular Salmonella Virulence Programs in the Phagosome

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ABSTRACT Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), together named (p)ppGpp, regulate diverse aspects of Salmonella pathogenesis, including synthesis of nutrients, resistance to inflammatory mediators, and expression of secretion systems. In Salmonella, these nucleotide alarmones are generated by the synthetase activities of RelA and SpoT proteins. In addition, the (p)ppGpp hydrolase activity of the bifunctional SpoT protein is essential to preserve cell viability. The contribution of SpoT to physiology and pathogenesis has proven elusive in organisms such as Salmonella, because the hydrolytic activity of this RelA and SpoT homologue (RSH) is vital to prevent inhibitory effects of (p)ppGpp produced by a functional RelA. Here, we describe the biochemical and functional characterization of a spoT-Δctd mutant Salmonella strain encoding a SpoT protein that lacks the C-terminal regulatory elements collectively referred to as “ctd.” Salmonella expressing the spoT-Δctd variant hydrolyzes (p)ppGpp with similar kinetics to those of wild-type bacteria, but it is defective at synthesizing (p)ppGpp in response to acidic pH. Salmonella spoT-Δctd mutants have virtually normal adaptations to nutritional, nitrosative, and oxidative stresses, but poorly induce metal cation uptake systems and Salmonella pathogenicity island 2 (SPI-2) genes in response to the acidic pH of the phagosome. Importantly, spoT-Δctd mutant Salmonella replicates poorly intracellularly and is attenuated in a murine model of acute salmonellosis. Collectively, these investigations indicate that (p)ppGpp synthesized by SpoT serves a unique function in the adaptation of Salmonella to the intracellular environment of host phagocytes that cannot be compensated by the presence of a functional RelA.

IMPORTANCE Pathogenic bacteria experience nutritional challenges during colonization and infection of mammalian hosts. Binding of the alarmone nucleotide guanosine tetraphosphate (ppGpp) to RNA polymerase coordinates metabolic adaptations and virulence gene transcription, increasing the fitness of diverse Gram-positive and Gram-negative bacteria as well as that of actinomycetes. Gammaproteobacteria such as Salmonella synthesize ppGpp by the combined activities of the closely related RelA and SpoT synthetases. Due to its profound inhibitory effects on growth, ppGpp must be removed; in Salmonella, this process is catalyzed by the vital hydrolytic activity of the bifunctional SpoT protein. Because SpoT hydrolyase activity is essential in cells expressing a functional RelA, we have a very limited understanding of unique roles these two synthetases may assume during interactions of bacterial pathogens with their hosts. We describe here a SpoT truncation mutant that lacks ppGpp synthetase activity and all C-terminal regulatory domains but retains excellent hydrolase activity. Our studies of this mutant reveal that SpoT uniquely senses the acidification of phagosomes, inducing virulence programs that increase Salmonella fitness in an acute model of infection. Our investigations indicate that the coexistence of RelA/
SpoT homologues in a bacterial cell is driven by the need to mount a stringent response to a myriad of physiological and host-specific signatures.

**KEYWORDS** Salmonella, bacterial pathogenesis, genetics, innate immunity, macrophages, stringent response, transposons

The alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), together named (p)ppGpp, are synthesized in the adaptation of nearly all bacterial species to nutritional starvation during a program commonly known as the stringent response (1). The stringent response is characterized by the transcriptional repression of rRNA, tRNA, and ribosomal protein genes (2, 3), and the concomitant activation of amino acid biosynthetic genes (1). Guanosine tetraphosphate also promotes the expression of alternative sigma factors, such as σ^5, and their regulons (4–6). Bacteria unable to mount the stringent response are amino acid auxotrophs, cannot adapt to nutrient downshifts, and are highly sensitive to the antimicrobial activity associated with oxidative, nitrosative, or acid stress (7–10).

Gammaproteobacteria such as *Salmonella* express RelA and SpoT homologues (RSH), which synthesize pppGpp or ppGpp through the transfer of a pyrophosphate moiety from ATP to the 3’ –OH group of GTP or GDP, respectively (11). Highly conserved RSH proteins are roughly 80 kDa in size and are expressed at low levels in most bacteria. In betaproteobacteria and gammaproteobacteria, the *relA* and *spoT* paralogs originated by duplication and have since diverged for specialized roles (12). RelA is activated in response to amino acid shortages, whereas SpoT synthesizes (p)ppGpp in response to the intracellular depletion of iron, phosphate, nitrogen, or fatty acids (13–17). In addition to its weak synthetic activity, SpoT is endowed with a strong and predominant (p)ppGpp phosphatase activity (18). The N terminus of RSH enzymes contains both (p)ppGpp-hydrolytic and (p)ppGpp-synthetic domains (HD and SYN, respectively) but, due to steric hindrance, HD or SYN activities are mutually exclusive at any given time in bifunctional enzymes such as SpoT (19). Interactions of domains in the C terminus with the HD and SYN domains influence which active site is formed (17). The RSH C terminus harbors three regulatory elements named the TGS (ThrRS, GTPase, and SpoT), INT (intermediate), and ACT (aspartate kinase, chorismate mutase, and TyrA) domains (Fig. 1). TGS and ACT regulatory domains, which were discovered in metabolic enzymes that do not synthesize or degrade (p)ppGpp, regulate enzymatic activity via allosteric binding to metabolites (12, 20). Although it is unclear whether the TGS and ACT domains of RSH homologues bind small molecules, it is apparent that the TGS domain of *Escherichia coli* SpoT facilitates protein-protein interactions. Specifically, the TGS domain mediates interactions of SpoT with acyl-carrier protein, thus inducing (p)ppGpp synthesis in response to fatty acid starvation (17). In addition, the SpoT TGS domain interacts with ObgE, a GTPase that regulates ribosome biogenesis (21, 22). The INT domain, which links the TGS and ACT domains, is highly conserved among RSH homologues and contains stretches of α-helices interrupted by short, intrinsically disordered segments (23). The *E. coli* RelA INT domain is responsible for binding to the ribosome (24), while the INT domain of *Mycobacterium smegmatis* Rel protein binds (p)ppGpp (25).

In most gammaproteobacteria species, Δ*spoT* mutants are not viable in the presence of functional *relA* alleles. This observation has led to the widely accepted idea that (p)ppGpp-hydrolysis is essential in (p)ppGpp-producing gammaproteobacteria (1). The inability to generate stable *spoT* deletion mutants has substantially hampered the study of *spoT* in bacterial physiology and pathogenesis. Compared to Δ*relA* isogenic controls, Δ*relA ΔspoT* mutant *Salmonella* is much more attenuated in murine models of salmonellosis (26), suggesting that (p)ppGpp synthesized by SpoT assumes an important role in the regulation of *Salmonella* virulence in mice. However, it is also plausible that RelA or SpoT each responds to distinct stimuli and synthesizes (p)ppGpp to regulate specific aspects of *Salmonella* virulence programs. In support of this view, our recent investigations have identified unique roles for RelA in the antinitrosative defenses of *Salmo-
nella (10), raising the intriguing possibility that SpoT may also adopt dedicated functions in the adaptation of Salmonella to specific stresses. The investigations here indicate that SpoT-derived (p)ppGpp plays a decisive role in the early activation of transcription of Salmonella pathogenicity island 2 (SPI-2) genes and metal cofactor acquisition genes in response to acidic cues encountered by Salmonella in the host cell phagosome.

RESULTS

Transposon mutagenesis identifies unique roles for spoT in the intracellular fitness of Salmonella. We examined the growth of a Salmonella enterica serovar Typhimurium barcoded transposon (Tn) library with ∼37,353 unique mutants in J774 macrophage-like cells. This screen showed critical roles for purine and pyrimidine biosynthesis, lipopolysaccharide biosynthesis, lipid III, itaconate, and Salmonella pathogenicity island 2 genes. We also found that the PhoPQ two-component system, DnaJ and DksA, promotes intracellular growth of Salmonella (27, 28). De novo nucleotide and amino acid biosynthesis and uptake genes were important for growth of Salmonella in J774 cells (Fig. 2; see Fig. S1 and S2 in the supplemental material). The Tn screen also showed that integrations into the spoT gene, but not into relA, reduced the intracellular fitness of Salmonella in J774 cells. The mere existence of transposons in the spoT gene was surprising to us because null spoT alleles should not be viable. Nevertheless, the

![Diagram of the Salmonella spoT gene.](image)
separate Salmonella transposon library of ~230,000 mutations identified 51 unique transposon integrations in the spoT gene (Table S1). The spoT transposon mutants in the library are viable, stable, and only experience mild negative selective pressure under laboratory conditions (29). Mapping of the sites of transposon integration into the spoT gene showed that the library did not contain any transposons in the HD- and SYN-encoded domains of spoT (Fig. 1A). However, the 3′-end 972 bases of spoT comprised a large number of transposon integration sites, consistent with the number expected for random integration. In contrast, the entire relA locus contained 161 Tn integrations spread throughout the gene (not shown). The lack of transposons in the 1,100 bases of the 5′ end of spoT encoding the SYN and HD catalytic domains suggests that such integrations render the dominant SpoT hydrolase activity nonfunctional, resulting in nonviable phenotypes in otherwise relA/H11001 bacteria. These data indicate that stable mutations can be generated in the C terminus of the Salmonella SpoT protein.

The following investigations exploited this observation to molecularly characterize the contributions of spoT to Salmonella virulence.

**SpoT plays unique roles in Salmonella virulence.** One of the spoT transposon insertions mapped at base 1131 within the spoT gene, at the 5′-end of the TGS domain in the C-terminal domain (CTD) regulatory region, indicating that the region of 932 bases downstream was dispensable for survival in vitro. To better understand the biochemical characteristics of the SpoT variant lacking the regulatory C-terminal region, an in-frame stop codon was introduced at base 1131 followed by the cat gene (Fig. 1B), yielding the spoT-Δctd mutant that lacks all C-terminal regulatory domains. This strain was virulent in C57BL/6 mice but attenuated in C3H/HeN mice that express a functional natural resistance-associated macrophage protein (NRAMP) divalent cation transporter in phagosomal membranes (Fig. 3A). Analysis of the competitive index showed that the spoT-Δctd mutant is over 100,000 times more attenuated than wild-type controls in C3H/HeN mice 13 to 17 days after oral delivery (Fig. 3B; see Fig. S3 in the supplemental material). We have also found that ΔrelA mutant Salmonella is attenuated in a C3H/HeN oral infection model (10). Collectively, these findings demonstrate that the spoT gene plays unique roles in Salmonella pathogenesis that cannot be performed by relA alone, and vice versa.

**Capacity of the spoT-Δctd allele to metabolize (p)ppGpp.** We next examined the ability of spoT-Δctd mutant Salmonella to synthesize and degrade (p)ppGpp. To induce (p)ppGpp synthesis, bacterial cultures were treated with serine hydroxamate (SHX), an irreversible inhibitor of seryl-tRNA synthetases that activates RelA in response to deacylated tRNAs erroneously loaded into the A site of the ribosome (30). In contrast to ΔrelA isogenic controls, Salmonella expressing the spoT-Δctd allele produced as much (p)ppGpp after serine hydroxamate treatment as wild-type bacteria (Fig. 4A). These findings indicate that RelA is functional in spoT-Δctd mutant Salmonella. To examine the (p)ppGpp-hydrolytic activity associated with the spoT-Δctd allele, tetracy-
cline was added to Salmonella pretreated with serine hydroxamate. Tetracycline binds to the A site of the ribosome, thereby not only blocking tRNA loading but also inhibiting RelA-mediated (p)ppGpp synthesis (13). Wild-type and spoT-Δctd mutant Salmonella treated with serine hydroxamate accumulated (p)ppGpp with similar kinetics, and the addition of tetracycline resulted in similar rates of (p)ppGpp hydrolysis (Fig. 4B). The estimated half-life of (p)ppGpp was <1 min in both strains (Fig. 4C). These findings suggest that the (p)ppGpp hydrolytic activity is fully functional in spoT-Δctd mutant Salmonella.

**Growth of spoT-Δctd mutant Salmonella in minimal media.** The growth of wild-type and spoT-Δctd mutant Salmonella was similar in LB broth, but growth of the spoT-Δctd mutant was slightly delayed when tested in E salts minimal medium supplemented with glucose and citric acid (Fig. 5A), suggesting that SpoT contributes to the growth of Salmonella in glucose and citric acid as the sole carbon sources. To examine the phenotype of spoT-Δctd mutant Salmonella during nutritional downshifts,
bacteria pregrown to the mid-exponential phase in LB broth were washed and transferred to M9 minimal medium containing glucose as the sole carbon source. Compared to wild-type controls, spoT-Δctd mutant Salmonella required slightly longer times to grow after this nutritional downshift. The growth defect of spoT-Δctd mutant Salmonella was less pronounced than that experienced by ΔrelA mutant Salmonella. A ΔrelA ΔspoT mutant Salmonella strain failed to grow in M9 minimal medium (Fig. 5B). These results confirm the importance of (p)ppGpp for growth during the transition to low-nutrient medium and strongly suggest that (p)ppGpp synthesized by both RelA and SpoT supports an efficient transition.

The growth defects exhibited by spoT-Δctd mutant Salmonella during a nutritional downshift suggest that the SpoT-Δctd variant is defective at producing (p)ppGpp under some conditions. To test this hypothesis further, the spoT-Δctd allele was transduced into a ΔrelA mutant Salmonella background. The ΔrelA spoT-Δctd double mutant grew as well as the ΔrelA mutant controls in LB broth, but exhibited extreme growth delays in EG minimal medium (Fig. 5C). LB broth is rich in amino acids and short peptides, supporting robust growth of Salmonella independently of (p)ppGpp synthesis. EG minimal medium fails to sustain growth of ΔrelA ΔspoT mutant Salmonella (Fig. 5C), because the inability of this strain to synthesize (p)ppGpp results in functional amino acid auxotrophies. The extreme growth defects of the ΔrelA spoT-Δctd double mutant in EG minimal medium provide genetic proof of the defective (p)ppGpp synthetic capacity of the SpoT-Δctd enzyme.

**Resistance of spoT-Δctd mutant Salmonella to oxidative and nitrosative stresses.** Macrophages use reactive oxygen and nitrogen species generated by NADPH oxidase and inducible nitric oxide (NO) synthase to impede intracellular Salmonella replication (31–33). Salmonella counteract oxidative and nitrosative stress via a RelA-mediated stringent response (10, 28). We assessed whether spoT also contributes to the antioxidant and antinitrosative defenses of Salmonella. These studies showed that spoT-Δctd mutant Salmonella was as resistant as the wild-type bacterium to either nitric oxide or H2O2 (see Fig. S4A, S4B in the supplemental material). Collectively, these data suggest that the (p)ppGpp-synthetase activity of SpoT is dispensable for Salmonella resistance to reactive nitrogen and reactive oxygen species in culture.

**spoT-Δctd mutant Salmonella replicates poorly in macrophages and exhibits defects in SPI-2 gene transcription.** Salmonella requires (p)ppGpp to replicate in macrophages; however, the source of (p)ppGpp remains unknown (26). Our initial transposon screen suggested that spoT transposon mutants are defective for intracellular replication in macrophages. Accordingly, spoT-Δctd mutant Salmonella exhibited poor growth in J774 cells compared to ΔrelA or wild-type controls (Fig. 6A), suggesting that (p)ppGpp produced by SpoT is important for the intracellular replication of Salmonella. Notably, the ΔrelA ΔspoT strain grew even more poorly than spoT-Δctd mutant Salmonella in macrophages, suggesting that RelA-derived (p)ppGpp can partially compensate for the lack of (p)ppGpp synthesis in spoT-Δctd mutant Salmonella.

The replication of intracellular Salmonella within phagocytes is greatly dependent on expression of SPI-2 genes encoding a type III secretion system (T3SS) that allows remodeling of the Salmonella-containing vesicle into an environment suitable for bacterial growth (34). Expression of the SPI-2 type III secretion system relies on the kinetic effects (p)ppGpp exerts on the stable open complex associated with the AT-rich discriminator region of the ssrAB locus (35). To examine SPI-2 gene transcription in spoT-Δctd mutant Salmonella, we followed the activation of an sifA::lacZY reporter fusion. Remarkably, spoT-Δctd mutant Salmonella expressed low levels (P > 0.001) of sifA::lacZY compared to wild-type or ΔrelA mutant controls in response to a Mg2++ and pH downshift (Fig. 6B). Real-time PCR confirmed the poor expression of sifA in spoT-Δctd mutant Salmonella (Fig. 6C). Consistent with previous reports, ΔrelA ΔspoT mutants were unable to induce expression of this β-galactosidase SPI-2 reporter (36).

Expression of sifA requires the SPI-2 type III secretion system master regulator SsrB (37). To examine whether the poor sifA::lacZY expression in spoT-Δctd mutant Salmonella could be due to inadequate SsrB expression, an ssrB-FLAG epitope-tagged allele
was transduced into spoT-Δctd mutant Salmonella. Compared to wild-type controls, spoT-Δctd mutant Salmonella exhibited defective transcription (Fig. 6D) and translation (Fig. 6E) of ssrB upon Mg²⁺ and pH downshifts. Salmonella expressing the spoT-Δctd allele was still capable of inducing ssrA and ssrB gene transcription if (p)ppGpp synthesis was stimulated with the RelA inducer serine hydroxamate (Fig. 6F). These data suggest that (p)ppGpp synthesis from SpoT optimizes SPI-2 type III gene transcription through expression of the ssrB master regulator, when stimulated with changes in magnesium and pH levels.

SpoT induces gene transcription in intracellular Salmonella. To determine whether SpoT contributes to SPI-2 gene expression in macrophages, transcription was studied in intracellular Salmonella isolated from J774 cells 8 h after infection. Consistent with the in vitro analysis, spoT-Δctd mutant Salmonella expressed lower levels of the ssrB gene encoding the SPI-2 master regulator (Fig. 7A). In addition, the spoT-Δctd strain expressed lower levels of spiC, ssaV, and sifA, which encode components of the SPI-2 secretion apparatus or effectors (Fig. 7A). Because the attenuation of spoT-Δctd mutant Salmonella was noted in C3H/HeN mice expressing a functional NRAMP locus, we tested whether (p)ppGpp synthesized by SpoT may also promote transcription of loci associated with cation uptake systems (Fig. 7B). The znuA gene, encoding a high-affinity zinc-binding periplasmic cassette, was expressed 5-fold less in spoT-Δctd mutant than in wild-type Salmonella. In contrast, the divergently regulated znuC component encoding a cytosolic ATPase was similarly expressed by wild-type and spoT-Δctd mutant Salmonella. In contrast to mntH and mntP loci encoding manganese uptake and homeostasis, the manganese uptake system encoded within the sit operon was expressed to lower levels in spoT-Δctd mutant Salmonella than in wild-type controls. We
found that SpoT is also needed for optimal expression of ferrous and ferric iron transport systems encoded by feo and fhu genes, but not for expression of fepB and fepD. The organization of these genes in the genome can be seen in Fig. S5 in the supplemental material. Together, these findings indicate that (p)ppGpp synthesized by SpoT activate transcription of both the SPI-2 genetic program and some metal cofactor acquisition systems in intracellular Salmonella.

SpoT activates gene transcription in response to the acidification of the phagosome. We modified the downshift growth conditions to gain more knowledge about the signals that activate the synthetase activity of SpoT intracellularly. Salmonella grown in N9 medium activated sifA::lacZY expression in a SpoT-dependent manner in response to acidification alone but did not significantly activate expression in response to Mg2+/H11001 downshift alone (Fig. 8A). However, acidity and low Mg2+ concentrations synergized to generate a strong induction of sifA::lacZY transcription. These data suggest that SpoT synthetase activity is turned on in response to low pH. To test this idea, we monitored the production of (p)ppGpp in Salmonella grown in morpholinepropanesulfonic acid (MOPS)-glucose medium (pH 7 or 5.5) containing all amino acids (Fig. 8B). MOPS medium was chosen because the Casamino Acids present in N9 medium reduce the incorporation of 32P into the nucleotide pool. In contrast to spoT-Δctd mutant Salmonella, wild-type controls produced ppGpp upon culture in MOPS-glucose medium (pH 5.5) for 5 min. However, the spoT-Δctd Salmonella strain produced as much ppGpp as wild-type controls when exposed to H2O2 (Fig. S5), which stimulates ppGpp synthesis through RelA (28). The intracellular growth advantage associated with the expression of full-length SpoT was lost in J774 cells treated with the vacuolar ATPase inhibitor bafilomycin (Fig. 8C). Bafilomycin treatment also dramatically reduced the expression of SPI-2 genes, znuA, sitA, and feoC in intracellular Salmonella (Fig. 8D). The inhibitory effects were more dramatic in wild-type Salmonella than in spoT-Δctd mutant controls. No transcriptional differences were noted between wild-type and spoT-Δctd mutant Salmonella treated with bafilomycin. Together, these investigations indicate that acidification of the phagosome activates (p)ppGpp production by SpoT, upregulating transcription of SPI-2 and metal cofactor acquisition systems that are needed for the intracellular fitness of Salmonella.

DISCUSSION

Despite the importance of the role (p)ppGpp plays in Salmonella virulence (26), the specific roles of the (p)ppGpp-synthetases RelA and SpoT have not been adequately characterized. The conditional essentiality of the spoT gene and its interrelationship with relA have made examination of SpoT function challenging. Here, we identified a SpoT variant with apparently normal hydrolase activity but defective (p)ppGpp syn-
thetic activity. The latter feature of the spoT-Δctd allele is phenotypically analogous to the SpoT SYN point mutants described in E. coli (38), making the SpoT-Δctd variant a potentially powerful tool for further studying the biology of (p)ppGpp synthesized by SpoT.

The Salmonella spoT-Δctd mutant is attenuated in a model of acute nontyphoidal Salmonella infection and grows poorly in phagocytic cells, indicating that (p)ppGpp synthesized by SpoT supports Salmonella pathogenesis (Fig. 9). Specifically, spoT-Δctd mutant Salmonella expresses low levels of ssrA and ssrB, which encode the master regulatory system that controls global SPI-2 gene transcription. Guanosine tetraphosphate promotes SPI-2 expression by relieving the kinetic constraint the AT-rich region of the ssrA promoter imposes on RNA polymerase (35). Defective expression of SPI-2 genes is likely to contribute to the intracellular growth defect and attenuation of spoT-Δctd mutant Salmonella. In addition, a functional SpoT activates transcription of iron, manganese, and zinc uptake systems in response to acidification of the phagosome. Given the attenuation of spoT-Δctd mutant Salmonella in C3H/HeN mice expressing a functional NRAMP locus that has recently been implicated in the uptake of magnesium (39), it is also possible that ppGpp synthesized by SpoT play roles in the activation of magnesium uptake systems. Together, our investigations identify SpoT as

FIG 8 Sensing of phagosomal acidity activates SpoT-mediated gene transcription. (A) Expression of silA-lacZY fusion in Salmonella grown in N9 minimal medium adjusted to pH 7.0 or 5.8. Where indicated, the cultures contained 10 mM (H) or 8 μM (L) MgCl₂. N = 8; ns, not significant; ****, P < 0.0001 by two-way ANOVA. (B) Detection of the TLC autoradiogram of [³²P]-labeled nucleotides from wild-type (wt) and spoT-Δctd mutant Salmonella after 5 min treatment at pH 5.5. (C) Effect of the ATPase proton inhibitor bafilomycin on the growth of Salmonella in J774 cells. Some of the cultures were treated with 100 nM bafilomycin for 20 h before enumeration of intracellular bacterial burden. N = 12; ns, not significant; ****, P < 0.0001 by two-way ANOVA. (D) qRT-PCR-based intracellular expression of SPI-2 and metal cation uptake genes in wild-type and spoT-Δctd mutant Salmonella. J774 cells infected with Salmonella were treated for 8 h with 100 nM the ATPase inhibitor bafilomycin. Green and red bars represent downregulated and upregulated genes. The data are the mean ± SD from 3 or 4 independent experiments with 3 technical replicates each. The data were normalized using an average value of the rpoD gene. The vertical dotted line represents the 1.5-fold up- or downregulation considered to exhibit a significant change. Bafilomycin significantly downregulated all of the genes assayed in wt and spoT-Δctd mutant Salmonella, as measured by one-way ANOVA. No differences were found with bafilomycin treatment between wt and spoT-Δctd mutant Salmonella.

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a critical regulator of intramacrophage virulence. It remains likely that (p)ppGpp synthesized by SpoT in response to the acidification of the phagosome regulates global gene expression in intracellular *Salmonella*.

Our studies (here and in reference 10) indicate that (p)ppGpp synthesized by either RelA or SpoT can be sufficient for some aspects of *Salmonella* pathogenesis, including the survival in C57BL/6 mice. However, (p)ppGpp synthesized by either RelA or SpoT each supports unique aspects of *Salmonella* pathogenesis. Our recent work has demonstrated that RelA is required for resistance to nitrosative stress (10). NO induces functional auxotrophies for branch chain amino acids, eliciting (p)ppGpp synthesis by RelA near the A site of the ribosome (10). Here, we reported that *spoT*-Δctd mutant *Salmonella* express low levels of SPI-2 genes and SsrB protein in response to acidification, a relevant signal encountered by *Salmonella* in the phagosome of macrophages. A specific role for SpoT, but not for RelA, has also been documented in the adaptation of *Legionella* to its macrophage vacuole (40). However, the role played by SpoT in *Legionella* pathogenesis was mapped to its hydrolytic activity. It remains unknown if the hydrolytic activity of SpoT contributes to *Salmonella* pathogenesis.

Our results demonstrate that SpoT synthesizes (p)ppGpp in response to acidic pH. The other effector of the stringent response, the transcriptional regulator DksA, changes its activity as the pH decreases (41). It is not clear if SpoT can similarly sense changes in cytosolic pH. Fatty acid starvation leads to SpoT-mediated (p)ppGpp synthesis through the direct interaction of acyl carrier protein and the SpoT TGS domain. It is possible that sensing of acid pH by SpoT occurs indirectly through binding a protein partner. Future studies are required to examine exactly how phagosomal acidification mediates SpoT-derived (p)ppGpp synthesis in *Salmonella*.

Guanosine tetraphosphate generated by RelA or SpoT can regulate some aspects of *Salmonella* virulence. However, there also seems to be a specialized division of labor among RelA and SpoT according to signals encountered during colonization and infection of the mammalian host (here and in reference 10).
**MATERIALS AND METHODS**

**Defined minimal media.** EG (57.4 mM KH₂PO₄, 1.7 mM MgSO₄, 9.5 mM citric acid, 16.7 mM H₃NaPO₄, 0.4% v/v glucose; pH 7.0 unless indicated otherwise), M9 (48 mM Na₂HPO₄·7H₂O, 22 mM KH₂PO₄, 8.56 mM NaCl, 18.69 mM NH₄Cl, 1 mM MgSO₄, 0.02% v/v glucose, and 50 μM FeSO₄·7H₂O; pH 7.1), N9–high Mg²⁺ (100 mM Tris·HCl, 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 1 mM KH₂PO₄, 38 mM glycerol, 0.1% Casamino Acids, and 10 mM MgCl₂; pH 8.0), N9–low Mg²⁺ (100 mM Tris·HCl, 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 1 mM KH₂PO₄, 38 mM glycerol, 0.1% Casamino Acids, 8 μM MgCl₂; pH 5.8), and MOPS (40 mM MOPS buffer, 4 mM tricine, 0.4% v/v glucose, 40 μg/ml of each amino acid except serine, 2 mM K₂HPO₄, 10 μM FeSO₄·7H₂O, 9.5 mM NH₄Cl, 276 μM K₂SO₄, 500 mM CaCl₂, 50 mM NaCl, 525 μM MgCl₂, 2.9 mM (NH₄)₂MoO₄·4H₂O, 400 mM H₃BO₃, 30 mM CoCl₂, 9.6 mM CuSO₄, 80.8 mM MnCl₂, and 9.74 mM ZnSO₄; pH 7.2) minimal media were used in the course of these investigations (42).

**Bacterial strains and genetics.** *Salmonella enterica* serovar Typhimurium 14028s and its mutant derivatives were maintained on LB broth (Lennox). Bacterial cultures were grown at 37°C in a shaking incubator. Gene deletions were performed with the λ Red recombinease system as previously described (43, 44) with minor modifications. Specifically, the cat gene was PCR amplified from pMD3 with the primers GCTACAACAGAGCGCCGGTAGTTCGTTTGAATTATCGAATAATATGAATATCCTCCTTAGTT and CAT TTTCGATATCCCGGCTATCCGTTGATTTATCGAATAATATGAATATCCTCCTTAGTT and a Bonferroni-corrected right-sided hypergeometric test with mid-

**DNA library preparation, sequencing and data analysis.** The methods for DNA library preparation, sequencing, and data analysis were previously described (29, 45). In brief, bacteria were recovered and grown in LB + 60 μg/ml kanamycin. Bacteria were pelleted, lysed, and subjected to PCR using primers directly flanking the N18 barcode. The frequency of each barcode was enumerated by Illumina sequencing of PCR-amplified flanking regions.

**ClueGO analysis.** Transposon mutants that were negatively selected in the output population (adjusted P value of less than 0.05 compared to the input), and that were contained within the coding region for the gene they interrupted, were analyzed via pathway analysis with ClueGO (46, 47) version 2.5.4 in Cytoscape (48, 49) version 3.7.1. The following S. Typhimurium 14028s ontologies were referenced in the pathway analysis: KEGG, GO Biological Process, 10.02.2015, 20h06, and GO Molecular Function, 10.02.2015, 20h06. Only pathways that had at least 10% of genes represented and a Bonferroni-corrected right-sided hypergeometric test with mid-P values less than or equal to 0.05 were displayed. Gene ontology (GO) levels 3 to 10 were used. Larger nodes represent more significant terms, while node shading is proportional to the percentage of genes represented for that term. Grouping of nodes was done with a perfuse directed layout based on a kappa score of 0.67. Group terms are based on the node with the highest percentage of genes per term compared to the cluster. Any groups sharing 40% of genes or 30% of terms were merged.

**Growth assays.** Overnight bacterial cultures grown in LB broth were diluted 1:1,250 into either fresh LB broth or M9 minimal medium. The optical densities at 600 nm (OD₆₀₀) of 200-μl aliquots of bacterial cultures were recorded with a Bioscreen C plate reader (Growth Curves USA, Piscataway, NJ) every 15 min for 4.5 h. The time (h) at which half of the maximal OD₆₀₀ was reached for each culture was calculated by exponential regression.

**Nutrient downshift assay.** *Salmonella* cells grown to the mid-exponential phase (OD₆₀₀ 0.4 to 0.6) in LB broth were collected, washed 3× with M9 minimal medium, and resuspended in M9 minimal medium (OD₆₀₀ ~ 0.1). Growth was tracked by measuring OD₆₀₀.

**NO recovery assays.** *Salmonella* cultures grown to the early exponential phase (OD₆₀₀ 0.2 to 0.3) in M9 minimal medium were challenged with 750 μM spermine NONOate. OD₆₀₀ measurements were recorded every 30 min for 5.5 h. *Salmonella* cultures were grown to the early exponential phase (OD₆₀₀ 0.2 to 0.3) in EG minimal medium supplemented with Casamino acids (EGCA) and were challenged with 400 μM of H₂O₂. OD₆₀₀ measurements were recorded every 30 min for 4.5 h. H₂O₂ killing. LB overnight cultures of *Salmonella* were diluted to approximately 10⁶ CFU/ml in phosphate-buffered saline (PBS). Cultures were challenged with 100 μM of H₂O₂ for 2 h at 37°C and 5% CO₂. The percent survival was calculated compared to time zero before H₂O₂ challenge.

**Measurement of (p)pGpp pools.** (p)pGpp pools were visualized as previously described (10, 50). *Salmonella* was grown in MOPS minimal medium supplemented with 0.4% v/v glucose, 40 μg/ml of each amino acid except serine, and low-concentration phosphate (0.4 mM). *Salmonella* cultures grown to an OD₆₀₀ of 0.2 were labeled with 10 μCi/ml of phosphorus-32 (³²P; PerkinElmer, Waltham, Massachusetts) for approximately 1.5 doubling times. Bacteria were treated with 0.4 mg/ml serine hydroxamate and 70 μg/ml tetracycline. For the acid challenge studies, the pH of the medium was adjusted to 5.5 with 1 M HCl. Aliquots of bacterial cultures (200 or 400 μl) were added to 80 or 160 μl of ice-cold 50% formic acid, respectively. Samples were thoroughly mixed and incubated on ice for at least 20 min. Samples were centrifuged at 13,000 rpm for 5 min and then returned to ice. Formic acid extracts (5 μl) were...
spotted along the bottom of polyethyleneimine (PEI)-cellulose thin-layer chromatography (TLC) plates (Millipore, Darmstadt, Germany). Samples were separated using 1.25 M KH₂PO₄ (pH 3.4) as a solvent system. TLC plates were air-dried in plastic, and exposed against K-screens overnight. K-screens were visualized using a phosphorimager (Bio-Rad, Hercules, California).

β-Galactosidase assays. SPI-2 gene expression was performed as previously described (51). Briefly, overnight LB broth cultures of *Salmonella* expressing the *sifA*:JacZ::Km single-copy chromosomal fusion (51) were diluted 1:100 into N9–high Mg²⁺ minimal medium and were grown for 3.5 to 4.5 h to an OD₆₀₀ of approximately 0.5. Cultures were split, collected, washed three times with either N9–high Mg²⁺ or N9–low Mg²⁺ minimal medium, and resuspended in either N9–high Mg²⁺ or N9–low Mg²⁺ minimal medium at an OD₆₀₀ of approximately 0.25. Cultures were grown for 3 h. The final culture OD₆₀₀ was recorded, and 100 µl of culture was diluted into 900 µl of Z buffer with 3.9 µl of β-mercaptoethanol, 25 µl of 0.1% SDS, and 50 µl of chloroform. Specimens were mixed, and the reactions were started upon the addition of 200 µl of 4 mg/ml ortho-nitrophenyl-β-galactosidase. Reactions were stopped with the addition of 0.5 ml 1 M sodium carbonate. The A₄₂₀ and A₅₅₀ of 200 µl of each reaction were recorded, and Miller units were calculated according to the following equation: 

\[
\text{IU} = \frac{A_{420} - \text{blank}}{\text{time of reaction (min)}} 
\times \frac{\text{volume of culture (ml)}}{A_{550} \text{ culture - blank}}. 
\]

Western blotting. Wild-type and *spoT-Δctd* mutant *Salmonella* expressing the ssB-FLAG:Km chromosomal single-copy epitope-tagged allele were grown exactly as described above in the SPI-2 β-galactosidase assay. After 3 h of Mg²⁺ downshift, 1 ml of culture was mixed with 150 µl ice-cold 100% trichloroacetic acid and incubated on ice for 15 min. Specimens were collected at 16,000 × g for 10 min at 4°C, and the pellets were resuspended in PBS with 50 mM NaOH and 6× Laemmli buffer and boiled for 10 min. Approximately 50 ng of protein for each sample was loaded into a 12% polyacrylamide SDS gel and was electrohoresed at 80 V for 15 min in SDS running buffer (25 mM Tris base, 192 mM glycine, and 0.1% sodium dodecyl sulfate). Once the samples had entered the separating gel, the current was increased to 110 V for approximately 80 min. Samples were transferred to nitrocellulose membranes at 25 V for 30 min on a Bio-Rad semidry transfer apparatus with transfer buffer (48 mM Tris base, 39 mM glycine, 20% methanol [vol/vol]). Membranes were blocked in 5% skim milk in TBST (20 mM Tris base, 150 mM NaCl, pH 7.6, and 0.025% Triton X-100 [vol/vol]), gently rocking for 2 h at room temperature. Membranes were probed with primary anti-DnaK and anti-FLAG murine antibodies diluted 1:2,000 and 1:500, respectively, in 5% skim milk TBST for 2 h at room temperature. Membranes washed 3× with TBST buffer were probed at room temperature for 1 h with goat anti-mouse antibodies conjugated to horseradish peroxidase diluted 1:10,000 in 5% skim milk TBST. Membranes were then washed 3× with TBST buffer. The blots were processed using the ECL Prime Western blotting detection reagent (GE Healthcare, Chicago, IL) mixed as directed and added to the membranes (approximately 1 ml per blot); excess liquid was wicked away, and chemiluminescence was visualized with a gel dock (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Transcriptional analysis of intracellular *Salmonella*. *J774* macrophage-like cells were seeded on the 150-mm plate to 90% confluence. The cells were washed once with prewarmed PBS and culture in excess liquid was wicked away, and chemiluminescence was visualized with a gel dock (Bio-Rad, Healthcare, Chicago, IL) mixed as directed and added to the membranes (approximately 1 ml per blot); excess liquid was wicked away, and chemiluminescence was visualized with a gel dock (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. First-strand cDNA generation from total RNA was generated using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, USA). Relative mRNA quantitation was done using the SYBR green quantitative real-time PCR (qRT-PCR) master mix (Roche, Germany) using the primers described in Table S3 in the supplemental material. Data evaluation of 3 or 4 biological replicates done in triplicate was performed using the threshold cycle (2⁻¹Ct) method. Genes that exhibited ≥2-fold up- or downregulation were considered to exhibit a significant change.

Intracellular replication. Stationary-phase *Salmonella* cultures diluted in RPMI+ medium were added to 10⁶ *J774* cells at an MOI of 2. Plates were centrifuged for 2 min at 4,000 rpm and returned to a 37°C CO₂ incubator for 30 min. The *Salmonella*-containing RPMI+ medium was replaced with RPMI+ medium supplemented with 50 µg/ml gentamicin for 1 h and then replaced again with RPMI+ medium supplemented with 10 µg/ml gentamicin for either 1 or 19 additional hours. *J774* cells were lysed with 0.1% Triton X-100 in PBS. Lysates were diluted and plated for CFU enumeration. To calculate fold replication, the number of intracellular *Salmonella* CFU recovered after 20 h of culture was normalized to the number of *Salmonella* CFU recovered after 2 h of infection.

Murine survival and infection. Mice were bred and maintained at the University of Colorado School of Medicine according to IACUC protocols. C57BL/6 or C3H/HeN mice (6 to 8 weeks old) were infected orally (p.o.) with 5 × 10⁶ or 10⁷ CFU of *Salmonella*, respectively. Survival of mice after infection was monitored for 28 days. Mice showing signs of disease were humanely euthanized by CO₂ inhalation and cervical dislocation. In addition, some C3H/HeN mice were inoculated p.o. with 10⁷ CFU containing equal numbers of wild-type and *spoT-Δctd* mutant *Salmonella*. The mice were euthanized between 7 and 9 days.
after infection, when signs of disease first appeared. Bacterial burden was quantified in livers and spleens, and the competitive index was assessed as described (52).

Statistical analysis. 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’s multiple comparison posttest. Statistical significance for mouse survival curves was determined using the log rank test. A P value of <0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, DOCX file, 1.1 MB.

FIG S2, DOCX file, 1.2 MB.

FIG S3, DOCX file, 0.2 MB.

FIG S4, DOCX file, 0.2 MB.

FIG S5, DOCX file, 0.7 MB.

FIG S6, DOCX file, 0.3 MB.

TABLE S1, DOCX file, 0.1 MB.

TABLE S2, DOCX file, 0.05 MB.

TABLE S3, DOCX file, 0.1 MB.

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