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RESEARCH ARTICLE

Salmonella Reprograms Nucleotide Metabolism in Its Adaptation to Nitrosative Stress

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ABSTRACT The adaptations that protect pathogenic microorganisms against the cytotoxicity of nitric oxide (NO) engendered in the immune response are incompletely understood. We show here that salmonellae experiencing nitrosative stress suffer dramatic losses of the nucleoside triphosphates ATP, GTP, CTP, and UTP while simultaneously generating a massive burst of the alarmone nucleotide guanosine tetraphosphate. RelA proteins associated with ribosomes overwhelmingly synthesize guanosine tetraphosphate in response to NO as a feedback mechanism to transient branched-chain amino acid auxotrophies. Guanosine tetraphosphate activates the transcription of valine biosynthetic genes, thereby reestablishing branched-chain amino acid biosynthesis that enables the translation of the NO-consuming flavohemoglobin Hmp. Guanosine tetraphosphate synthesized by RelA protects salmonellae from the metabolic stress inflicted by reactive nitrogen species generated in the mammalian host response. This research illustrates the importance of nucleotide metabolism in the adaptation of salmonellae to the nutritional stress imposed by NO released in the innate host response.

IMPORTANCE Nitric oxide triggers dramatic drops in nucleoside triphosphates, the building blocks that power DNA replication; RNA transcription; translation; cell division; and the biosynthesis of fatty acids, lipopolysaccharide, and peptidoglycan. Concomitantly, this diatomic gas stimulates a burst of guanosine tetraphosphate. Global changes in nucleotide metabolism may contribute to the potent bacteriostatic activity of nitric oxide. In addition to inhibiting numerous growth-dependent processes, guanosine tetraphosphate positively regulates the transcription of branched-chain amino acid biosynthesis genes, thereby facilitating the translation of antinitrosative defenses that mediate recovery from nitrosative stress.

KEYWORDS *Salmonella*, adaptive resistance, animal models, cellular redox status, guanosine tetraphosphate, nitric oxide, nitrosative stress, nucleotide metabolism, stringent response

Salmonella enterica serovar Typhimurium is a causative agent of zoonotic nontyphoidal salmonellosis. Although typically self-limiting in healthy individuals, this syndrome can be life threatening in immunocompromised patients with gamma interferon signaling defects or HIV or malaria comorbidities (1–4). Far from alleviating symptoms, antibiotic therapy can prolong the shedding of salmonellae in immunocompetent individuals (5) and often select for increasingly resistant *Salmonella* populations (6, 7). The World Health Organization has called for a greater focus on the development of novel treatments against fluoroquinolone-resistant salmonellae (8). Basic knowledge of bacterial pathogenesis provides new targets for the development of next-generation antibiotic therapies. Growth in macrophages is a key step in *Salmonella* pathogenesis. Interactions of salmonellae with professional phagocytes activate the translation of

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inducible nitric oxide synthase (iNOS), a flavohemoprotein that synthesizes nitric oxide (NO) from L-arginine, O_2, and NADPH (9, 10). NO and its nitrosative and oxidative congeners exert bacteriostasis against salmonellae. The concerted actions of low-molecular-weight thiols, cytochrome *bd*, and the flavohemoglobin Hmp protect salmonellae against the nitrosative stress encountered in mammalian hosts (11–13). Despite these antinitrosative defenses, NO attacks redox-active moieties in dihydroxy-acid dehydratase and lipoamide-dependent lipoamide dehydrogenase, thus inhibiting the biosynthesis of branched-chain amino acids, methionine, and lysine (14, 15). The molecular mechanisms that help salmonellae resolve the resulting functional amino acid auxotrophies remain inadequately understood.

The alarmones guanosine tetraphosphate (ppGpp) and pentaphosphate (pppGpp) [collectively referred to as (p)ppGpp] are synthesized by bacteria in their adaptive response to nutritional starvation (16). The enzymes RelA and SpoT synthesize (p)ppGpp by transferring pyrophosphate from ATP to the 3′-OH group of ribose in GDP or GTP, yielding AMP and ppGpp or pppGpp, respectively (17). Typically, both ppGpp and pppGpp are produced at once in roughly equimolar concentrations (18), depending on the source of stress. RelA proteins respond to amino acid starvation by directly sensing unaminoacylated tRNAs erroneously placed in the A site of the ribosome (19, 20). Cytosolic SpoT proteins synthesize (p)ppGpp in response to drops in physiological concentrations of iron, fatty acids, phosphate, or nitrogen (16). SpoT is a bifunctional enzyme that can synthesize and hydrolyze (p)ppGpp (21–23). Transcription is the best-characterized target of (p)ppGpp, which binds to a hydrophobic pocket between the β' and ω subunits of the RNA polymerase (RNAP) and at the interface of DksA and the rim of the β' subunit (24, 25). Binding of (p)ppGpp to these sites destabilizes the RNAP holoenzyme-DNA open complex, repressing genes encoding rRNAs, tRNAs, and ribosomal proteins while activating amino acid biosynthesis operons (16). In addition, (p)ppGpp can inhibit translation, ribosome biogenesis, protein stability, and lipid and cell wall biosynthesis (16, 26–28). By doing so, (p)ppGpp influences major aspects of bacterial cell physiology.

Many bacterial pathogens have incorporated (p)ppGpp into regulatory circuits to control the expression of virulence determinants (29). Functional RelA and SpoT proteins are required for the contextual expression of *Salmonella* virulence programs associated with epithelial cell invasion and intracellular survival (30). Genetic evidence has also implicated (p)ppGpp in the antinitrosative defenses of salmonellae (31); however, the mechanisms by which these nucleotides enhance the resistance of salmonellae to NO are unknown. Our investigations described here indicate that (p)ppGpp aids with the recovery of salmonellae from nitrosative stress by activating the biosynthesis of branched-chain amino acids that fuel the translation of antinitrosative defenses.

RESULTS

Salmonellae experiencing nitrosative stress synthesize ppGpp. Salmonellae bearing mutations in *relA* and *spoT* are hypersusceptible to NO (31), suggesting that (p)ppGpp increases the fitness of salmonellae undergoing nitrosative stress. Accordingly, salmonellae treated for 5 min with a 500 μM concentration of the NO donor spermine NONOate abundantly accumulated ppGpp while experiencing marked depletions of ATP and GTP (Fig. 1A). NO treatment did not affect bacterial viability. The NO donor propylamine propylamine (PAPA) NONOate also stimulated the synthesis of ppGpp in salmonellae (see Fig. S1A in the supplemental material). Curiously, neither spermine NONOate nor PAPA NONOate triggered pppGpp synthesis. Kinetic analyses revealed that ppGpp was produced as early as 30 s after spermine NONOate treatment (Fig. 1B), lasting about 30 min thereafter (Fig. 1C; Fig. S1B).

Gammaproteobacteria such as salmonellae synthesize (p)ppGpp through the enzymatic activity of RelA or SpoT protein (Fig. 1D) (32). Δ*relA* mutant salmonellae failed to accumulate any measureable ppGpp after a NO challenge (Fig. 1E), implying that RelA may be solely responsible for the burst of ppGpp recorded in salmonellae experiencing

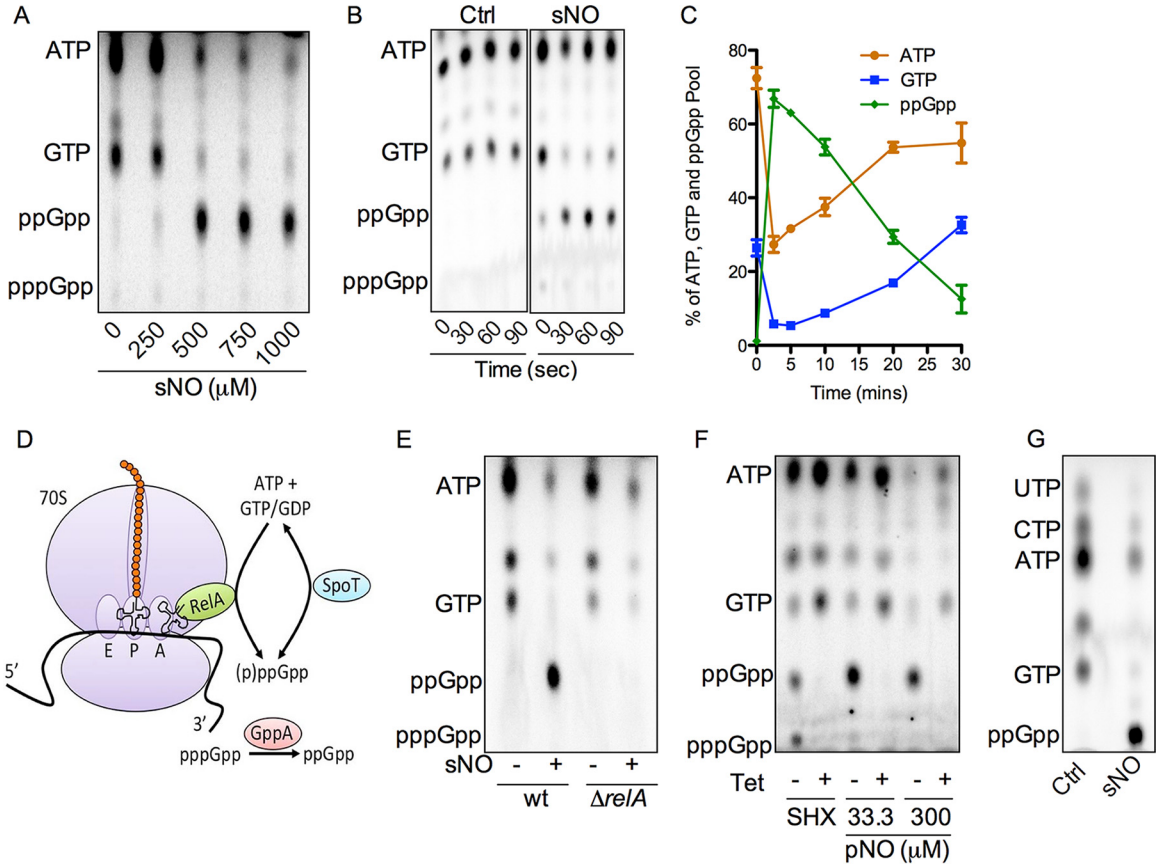

FIG 1 NO dramatically affects the nucleotide pools of salmonellae. (A) TLC autoradiogram of ^{32}P-labeled nucleotides extracted from log-phase salmonellae 5 min after exposure to increasing concentrations of spermine NONOate (sNO). (B) Nucleotides were examined 0 to 90 s after treatment with 750 μM sNO. (C) Relative abundance of ATP, GTP, and ppGpp in salmonellae treated with 750 μM sNO (see Fig. S1B for an example of the autoradiograms used in these analyses). $n = 4$. Data are shown as the mean value $\pm$ the standard error of the mean. (D) Model of (p)ppGpp metabolism. RelA synthesizes (p)ppGpp from ATP and GTP or GDP in response to unaminoacylated tRNAs erroneously loaded in the A site of the ribosome. SpoT can both synthesize and hydrolyze (p)ppGpp, whereas GppA hydrolyzes pppGpp to ppGpp. (E) Nucleotides from wild-type (wt) and Δ*relA* mutant salmonellae treated for 5 min with 750 μM sNO. (F) Nucleotides in salmonellae treated with 70 μg/ml tetracycline for 3 min before the addition of 0.4 mg/ml serine hydroxamate (SHX) or 33.3 or 300 μM PAPA NONOate (pNO) for 2 min. All of the autoradiograms shown are representative of two to six independent experiments. (G) Nucleotides from salmonellae treated for 5 min with sNO were examined as described for panel A. Extracts in panels A, B, E, and F were separated with 1.25 M KH_2PO_4 buffer, pH 3.4. Specimens in panel G were separated with 0.9 M KH_2PO_4 buffer, pH 3.4. Ctrl, control.

nitrosative stress. RelA is classically activated during amino acid starvation in response to its direct contact with unaminoacylated tRNAs erroneously loaded into the A site of the ribosome (19). NO modifies cysteines and [4Fe-4S] clusters in lipoamide-dependent lipoamide dehydrogenase and dihydroxy-acid dehydratase, respectively, inhibiting methionine, lysine, and branched-chain amino acid biosynthesis (14, 15). The consequent accumulation of unaminoacylated tRNAs could activate RelA. To test this model, salmonellae were pretreated with tetracycline, an antibiotic that blocks the entry of tRNAs into the A site of ribosomes and thereby prevents classical RelA activation (18). Tetracycline completely prevented the synthesis of (p)ppGpp in response to serine hydroxamate (Fig. 1F), a classical RelA activator that irreversibly inhibits seryl-tRNA synthetases (26). Tetracycline also completely inhibited ppGpp synthesis in response to the NO donor PAPA NONOate. These data suggest that NO activates ppGpp production from RelA by increasing the amount of unaminoacylated tRNAs.

To examine the rates of SpoT-dependent (p)ppGpp decay, salmonellae were pretreated with either serine hydroxamate or PAPA NONOate before tetracycline was added to the cultures. The burst of (p)ppGpp decayed quickly (5 min) and with similar kinetics after the addition of serine hydroxamate or 33.3 μM PAPA NONOate (Fig. S1C) (estimated half-lives of 1.3 and 0.7 min, respectively). In contrast, the ppGpp pool in

salmonellae challenged with 300 μM PAPA NONOate diminished at a substantially lower rate (i.e., a half-life of 8.5 min), suggesting that high concentrations of NO inhibit the ability of SpoT to hydrolyze ppGpp.

In the canonical (p)ppGpp cycle, pppGpp is the primary nucleotide synthesized by RelA, but the 5′-monophosphatase activity of GppA converts pppGpp to ppGpp (Fig. 1D) (18, 33). According to this model, serine hydroxamate-treated Δ*gppA* mutant salmonellae synthesized nearly four times as much pppGpp as ppGpp, whereas serine hydroxamate stimulated nearly equimolar concentrations of pppGpp and ppGpp in wild-type salmonellae (Fig. S1D). As described for wild-type controls, NO stimulated the exclusive production of ppGpp in Δ*gppA* mutant salmonellae. These data demonstrate that NO selectively induces ppGpp synthesis independently of the enzymatic activity of GppA.

Because nitrosative stress diminishes ATP and GTP synthesis (see above), the thin-layer chromatography (TLC) solvent system was modified to determine the global effects of NO on nucleoside triphosphates (NTPs). NO-treated salmonellae suffered massive drops in ATP, UTP, CTP, and GTP (Fig. 1G), perhaps reflecting the fact that NTP synthesis requires ATP. Consistent with the patterns seen by TLC, firefly luciferase analyses revealed that NO reduces the ATP pool by roughly 60% in salmonellae (Fig. S1E). Using ATP as an internal standard, we estimate that the intracellular concentration of ppGpp reaches nearly 2 mM after NO treatment. Collectively, these data indicate that salmonellae dramatically reprogram nucleotide metabolism in response to NO, with overall decreases in NTPs and a parallel accumulation of ppGpp. The overall changes in nucleotides could have substantial consequences for bacterial physiology, NO toxicity, and the antinitrosative defenses of salmonellae.

Branched-chain amino acids reestablish the antinitrosative defenses of salmonellae. We next examined whether ppGpp synthesized in response to NO contributes to the antinitrosative defenses of salmonellae. Wild-type salmonellae grown to log phase in glucose minimal medium resumed growth at about 2.5 h after treatment with 750 μM spermine NONOate (Fig. 2A). On the other hand, Δ*relA* mutant salmonellae never recovered from the bacteriostasis induced by NO in the 5-h time frame examined. The hypersusceptibility of Δ*relA* mutant salmonellae to NO could be rescued by genetic complementation with wild-type *relA* but not by complementation with the catalytically deficient D275G or E335Q (22) *relA* allele (Fig. S2A). Addition of all amino acids to glucose minimal medium significantly increased the resistance of salmonellae to the antimicrobial activity of NO (Fig. 2A). These observations are consistent with previous studies (15, 31). The addition of amino acids to glucose minimal medium also stimulated the growth of Δ*relA* mutant salmonellae undergoing nitrosative stress, suggesting that NO induces transient, functional amino acid auxotrophies that are resolved by RelA-derived ppGpp.

NO and its oxidative and nitrosative congeners inhibit branched-chain amino acid (leucine, isoleucine, and valine), aromatic amino acid (phenylalanine, tyrosine, and tryptophan), methionine, and lysine biosynthetic pathways (14, 15). To gain insights into the amino acid biosynthetic pathways that might be targeted by NO, salmonellae were challenged with spermine NONOate in glucose minimal medium supplemented with different combinations of amino acids. The addition of branched-chain amino acids rendered both wild-type and Δ*relA* mutant salmonellae resistant to nitrosative stress (Fig. 2A; Fig. S2E). The addition of aromatic amino acids (Fig. S2B) or methionine and lysine (Fig. S2C) did not greatly affect the recovery of NO-treated salmonellae. Moreover, amino acid mixtures lacking aromatic amino acids or lysine and methionine still rendered log-phase salmonellae highly resistant to NO (Fig. S2B and C). These findings indicate that under the conditions examined here, NO exerts considerable pressure on the biosynthesis of branched-chain amino acids but not aromatic amino acids or lysine and methionine. Given previous findings (15), the apparent dispensability of lysine and methionine in the recovery of salmonellae from NO might seem surprising. The apparent inconsistencies between these studies could be due to the growth phase at which salmonellae were challenged with NO, as suggested by the fact that methionine

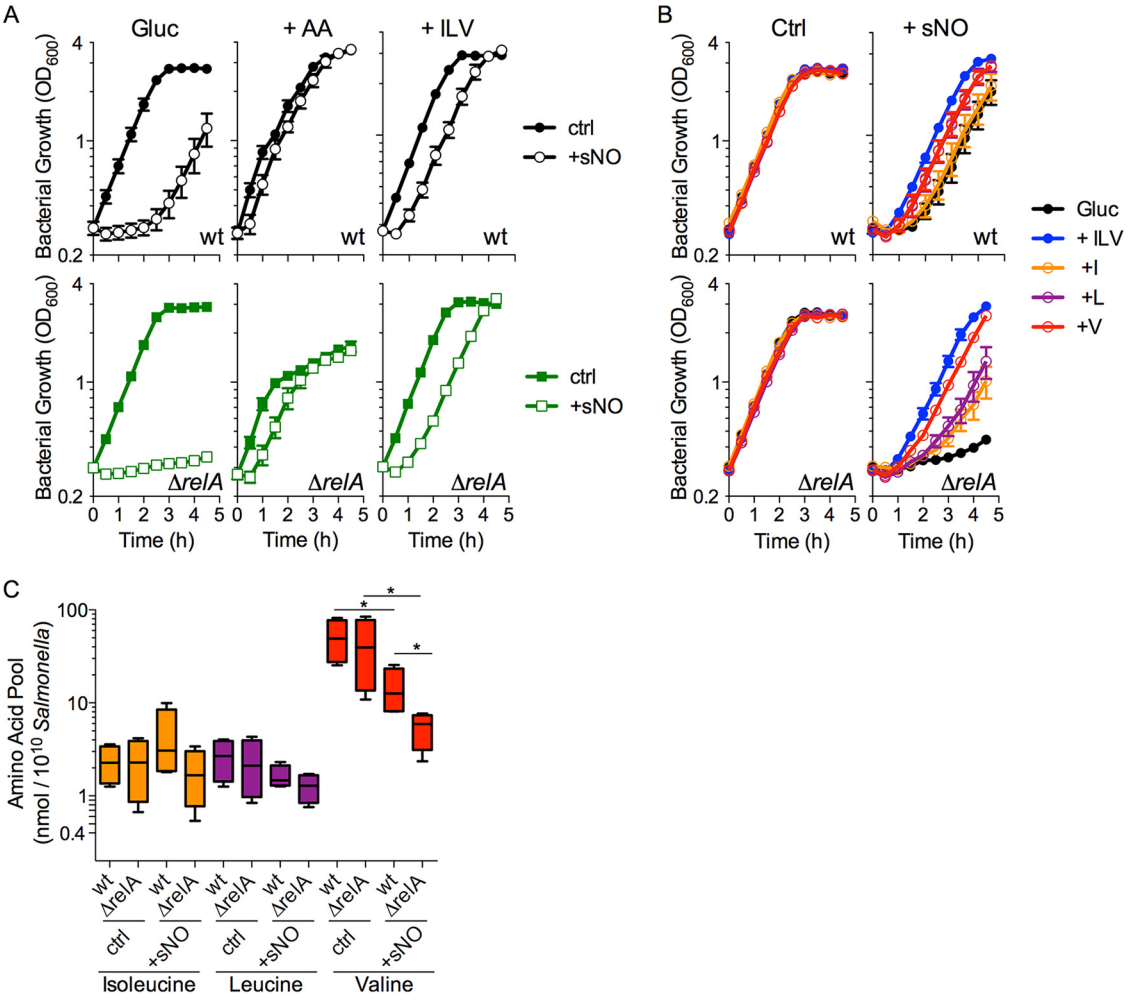

FIG 2 RelA and branched-chain amino acids accelerate the recovery of NO-treated salmonellae. (A) Growth of wild-type (wt) and Δ*relA* mutant salmonellae in MOPS glucose minimal medium (Gluc) in the presence or absence of 750 μM spermine NONOate (+sNO). Where indicated, all 20 amino acids (+AA) or branched-chain amino acids (+ILV) were added to the medium. $n = 4$ to 6. Data are shown as the mean value ± the standard error of the mean. (B) Growth of wild-type and Δ*relA* mutant salmonellae challenged with 750 μM sNO in MOPS glucose minimal medium supplemented with branched-chain amino acids (+ILV), isoleucine (+I), leucine (+L), or valine (+V). $n = 4$. Data are shown as the mean value ± the standard error of the mean. (C) Intracellular concentrations of isoleucine, leucine, and valine were determined by LC-MS in wild-type and Δ*relA* mutant salmonellae grown in M9 glucose minimal medium. Where indicated, 750 μM sNO was added to the cultures. $n = 4$. The data are represented as box-and-whiskers plots. *; $P < 0.05$ (determined by Mann-Whitney analysis). Ctrl, control.

and lysine, not branched-chain amino acids, aided the recovery of stationary-phase salmonellae from NO toxicity (Fig. S2D). Cumulatively, salmonellae seem to develop different transient, functional amino acid auxotrophies, depending on the growth phase in which they encounter NO.

To identify which one of the branched-chain amino acids contributes to the anti-nitrosative defenses of salmonellae, wild-type and Δ*relA* mutant salmonellae were challenged with NO in morpholinepropanesulfonic acid (MOPS) glucose minimal medium supplemented with valine, leucine, or isoleucine. Compared to isoleucine and leucine, valine had the greatest impact on the recovery of wild-type and Δ*relA* mutant salmonellae from the bacteriostatic activity of NO (Fig. 2B; Fig. S2F). Consistent with these observations, the intracellular valine pools were substantially reduced in both wild-type and Δ*relA* mutant salmonellae experiencing nitrosative stress (Fig. 2C). It should be noted that the diminution of valine was more pronounced in NO-treated Δ*relA* mutant salmonellae than in similarly treated wild-type controls. In contrast to valine, other amino acid pools were largely unaffected by the Δ*relA* mutation, a NO

challenge, or both (Fig. S3). The only exceptions were aspartate, which was reduced after a NO challenge in wild-type salmonellae; arginine, which was lower in Δ*relA* mutant salmonellae; and threonine, which was reduced in NO-treated Δ*relA* mutant salmonellae (Fig. S3). NO-mediated damage of IlvB would curtail the synthesis of all three branched-chain amino acids, yet it only seems to affect valine pools. This might be due to the high levels of valine, which is about 20-fold more abundant than isoleucine and leucine, maintained by salmonellae (Fig. 2C). Altogether, these data demonstrate that NO depletes the intracellular pools of valine in exponentially growing salmonellae and that ppGpp synthesized by RelA mitigates the NO-induced valine starvation, thereby helping resolve the auxotrophy of this branched-chain amino acid.

Branched-chain amino acid biosynthetic genes are reexpressed in a *relA*-dependent manner following a NO challenge. In the first step of valine biosynthesis, acetohydroxy acid synthase I decarboxylates pyruvate and transfers the active aldehyde to a second molecule of pyruvate (Fig. S4A) (34). However, the addition of pyruvate to glucose minimal medium did not alter the toxicity of NO against wild-type or Δ*relA* mutant salmonellae (Fig. S5), suggesting that pyruvate is not limiting for the recovery of salmonellae from nitrosative stress. We therefore focused on the expression of valine biosynthetic enzymes. Valine is synthesized by the sequential enzymatic activities of acetohydroxy acid synthase I, ketol-acid reductoisomerase, dihydroxy-acid dehydratase, and transaminase B (encoded by *ilvBN*, *ilvC*, *ilvD*, and *ilvE*, respectively) (Fig. S4B). Valine transamination is a reversible enzymatic reaction, and thus valine can be a substrate for leucine biosynthesis. The transcription of *ilvB* and *ilvD* was repressed in wild-type salmonellae after 30 min of NO treatment (Fig. 3A and C); that of *ilvD* was also repressed ($P < 0.001$) in NO-treaed Δ*relA* mutant salmonellae (Fig. 3C). Wild-type salmonellae recovered the transcription of *ilvB*, *ilvC*, *ilvD*, and *leuA* after 1 h of NO treatment. One hour after NO treatment, the transcription of these genes was significantly lower in Δ*relA* mutant controls than in wild-type salmonellae. Expression of IlvB-3×FLAG (Fig. 3E) and IlvD-3×FLAG (Fig. 3G) protein levels matched mRNA expression patterns. IlvC-3×FLAG (Fig. 3F) and LeuD-3×FLAG (Fig. 3H) did not appreciably change in NO-treated salmonellae. The NO-mediated repression of valine biosynthetic genes may explain why the addition of valine enhances the resistance of salmonellae to nitrosative stress. To study further the effects of ppGpp on the expression of branched-chain amino acid genes, we measured the *in vitro* transcription of *ivbL* and *ilvD* (Fig. 3I), whose promoters drive *ilvB* and *ilvD* expression, respectively (Fig. S4B). Although ppGpp by itself did not activate *ivbL* or *ilvD in vitro* transcription, this alarmone synergized with the RNAP-binding protein DksA (Fig. 3I). The synergism was most evident at low concentrations (i.e., 10 to 100 μM) of ppGpp.

The translation of antinitrosative defenses is dependent on ppGpp and branched-chain amino acids. The flavohemoglobin Hmp is the most potent antinitrosative defense yet described in salmonellae (12, 35). We examined whether RelA-derived ppGpp and branched-chain amino acids affect the expression of *hmpA*. A cocktail of all 20 amino acids or branched-chain amino acids, in this order of importance, enhanced the amount of Hmp translated in NO-treated salmonellae (Fig. 4A). NO-treated Δ*relA* mutant salmonellae expressed lower Hmp concentrations than did wild-type controls, perhaps explaining why Δ*relA* mutant salmonellae still recover more slowly than wild-type controls in the presence of all 20 amino acids or branched-chain amino acids (Fig. 2A; Fig. S2E). Wild-type salmonellae grown in glucose required longer times and expressed smaller amounts of Hmp than controls grown in all 20 amino acids or branched-chain amino acids. In contrast to wild-type salmonellae, Δ*relA* mutant bacteria grown in glucose minimal medium barely expressed Hmp 2 h after NO treatment (Fig. 4A). The inability of Δ*relA* mutant salmonellae to express Hmp cannot be explained by defects in *hmpA* transcription (Fig. 4B).

As expected (12), Δ*hmpA* mutant salmonellae were hypersusceptible to the bacteriostatic activity of NO (Fig. 4C). However, in contrast to Δ*relA* mutant salmonellae (Fig. 2A), the extreme susceptibility of Δ*hmpA* mutant salmonellae to nitrosative stress was not ameliorated by branched-chain amino acids (Fig. 4C). Cumulatively, these

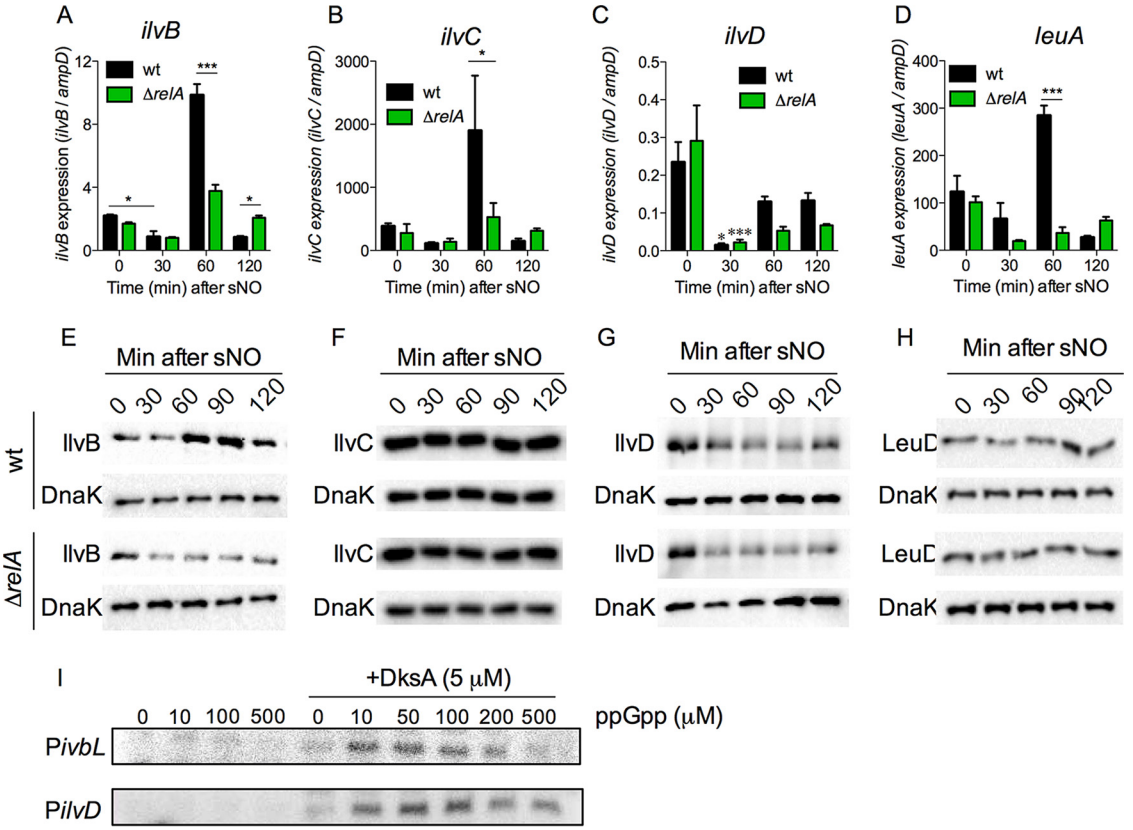

FIG 3 RelA regulates the expression of branched-chain amino acid biosynthetic loci in salmonellae undergoing nitrosative stress. (A to D) Transcription of *ilvB*, *ilvC*, *ilvD*, and *leuA* mRNAs in wild-type (wt) and Δ*relA* mutant salmonellae grown in MOPS glucose minimal medium was quantified by qRT-PCR. Bacterial cultures were treated with 750 μM spermine NONOate for the times indicated. The results were normalized to internal levels of the *ampD* housekeeping gene. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (determined by two-way ANOVA). $n = 4$. Data are shown as the mean value ± the standard error of the mean. (E to H) Western blot analysis of IlvB-3×FLAG, IlvC-3×FLAG, IlvD-3×FLAG, and LeuD-3×FLAG from the *Salmonella* strains indicated grown in MOPS glucose minimal medium after the addition of 750 μM sNO. Blots are representative of two or three independent experiments. (I) Transcription of *ivbL* (top) and *ilvD* (bottom) promoters by RNAP in reaction mixtures containing the concentrations of ppGpp indicated and/or 5 μM DksA. The results are representative of three independent experiments.

investigations suggest that defective expression of NO-consuming Hmp could contribute to the hypersusceptibility of Δ*relA* mutant salmonellae to NO. Consistent with this idea, Δ*relA* mutant salmonellae recovered from NO toxicity as well as wild-type controls upon switching to fresh glucose minimal medium (Fig. 4D).

Branched-chain amino acid biosynthesis and RelA-derived ppGpp support *Salmonella* pathogenesis. Our data here demonstrate that ppGpp enhances the recovery of salmonellae from NO-associated cytotoxicity, in part by reestablishing branched-chain amino acid biosynthesis. We found that Δ*relA* mutant salmonellae are attenuated in this murine model of infection ($P < 0.01$) (Fig. 5A). To determine if the attenuation of Δ*relA* mutant salmonellae is due to NO-dependent immunity, the drinking water of infected mice was supplemented with the iNOS inhibitor aminoguanidine. Δ*relA* mutant salmonellae became as virulent ($P = 0.937$) as wild-type controls in aminoguanidine-treated C3H/HeN mice (Fig. 5B).

Salmonella is equipped with two specialized branched-chain amino acid uptake systems, LIV-I and LIV-II, encoded by *livKHMGF* (and *livJ*) and *brnQ*, respectively (Fig. S6) (36, 37). To determine if either branched-chain amino acid biosynthesis or import is important for *Salmonella* pathogenesis, C3H/HeN mice were infected orally (p.o.) with wild-type or Δ*ilvD* or Δ*livKHMGF* Δ*brnQ* mutant salmonellae (Fig. 5C). Wild-type and Δ*livKHMGF* Δ*brnQ* mutant salmonellae killed C3H/HeN mice with similar kinetics ($P = 0.141$) (Fig. 5C). In contrast, Δ*ilvD* mutant salmonellae were attenuated ($P < 0.01$) (Fig. 5C). These data point to an important role for branched-chain amino acid biosyn-

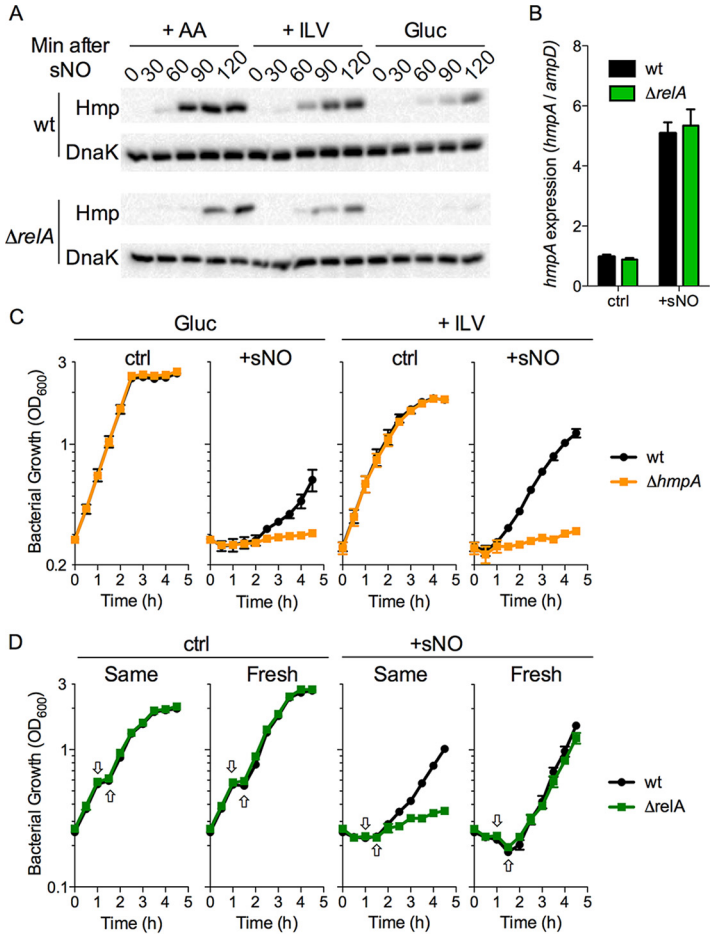

FIG 4 RelA aids the translation of the antinitrosative defense Hmp in salmonellae experiencing nitrosative stress. (A) Western blot analysis of Hmp-3×FLAG in wild-type (wt) or Δ*relA* mutant salmonellae grown in MOPS glucose minimal medium (Gluc) after the addition of 750 μM spermine NONOate (sNO). Where indicated, the medium was supplemented with all 20 amino acids (+AA) or branched-chain amino acids (+ILV). Blots are representative of five independent experiments. (B) Transcription of *hmpA* mRNA in salmonellae grown in MOPS glucose minimal medium 60 min after treatment with 750 μM sNO. (C) Growth of wild-type and Δ*hmpA* mutant salmonellae in M9 glucose minimal medium. Selected samples were treated with 750 μM spermine NONOate (sNO). Where noted, the medium was supplemented with branched-chain amino acids (+ILV). (D) Growth of wild-type and Δ*relA* mutant salmonellae in M9 glucose minimal medium. One hour after the addition of 750 μM sNO, bacteria were collected by centrifugation (down arrow) and the pellets were resuspended in either the same medium (up arrow) or fresh M9 medium lacking sNO. $n = 4$ to 6. Data are shown as the mean value ± the standard error of the mean. ctrl, control.

thesis, not import, in *Salmonella* pathogenesis. Nonetheless, a combination of Δ*relA* and Δ*livKHMGF* Δ*brnQ* mutations greatly attenuated salmonellae ($P < 0.001$) (Fig. 5C), suggesting that the LIV-I and LIV-II systems can partially complement the defects of Δ*relA* mutant salmonellae to maintain valine biosynthesis.

DISCUSSION

The NTPs ATP, GTP, CTP, and UTP power cellular energetics, serve as building blocks for DNA replication and RNA transcription, regulate key steps in translation and cell division, and are substrates in lipid and peptidoglycan biosynthesis. The nucleotide alarmone ppGpp inhibits many of these processes (16, 27). Thus, the massive depletion of NTPs, along with a simultaneous burst of ppGpp, likely contributes to the bacteriostasis recorded in NO-treated salmonellae (Fig. 6). Global reprogramming of nucleotide metabolism may allow salmonellae to avoid deleterious damage of DNA, lipids, and proteins by reactive nitrogen species. In addition to blocking numerous cellular processes, ppGpp offers a feedback mechanism to reestablish *Salmonella* homeostasis, as

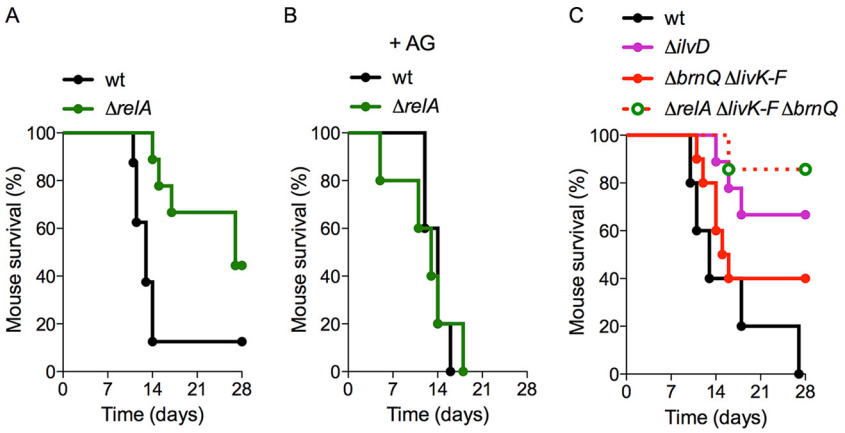

FIG 5 Importance of *relA* and *ilvD* in the pathogenesis of salmonellae. (A to C) C3H/HeN mice were infected p.o. with 5×10^6 CFU of the *Salmonella* strains indicated. Murine survival was monitored for 28 days. The drinking water in panel B was treated with a 2.5% solution of the iNOS inhibitor aminoguanidine (AG). $n = 5$ to 10 mice. Log rank analysis results: wild type versus Δ*relA* mutant, $P < 0.01$; wild type versus Δ*ilvD* mutant, $P < 0.01$; wild type versus Δ*ilvD-F* Δ*brnQ* mutant, $P = 0.141$; wild type versus Δ*relA* Δ*ilvD-F* Δ*brnQ* mutant, $P < 0.001$. No statistically significant difference between the wild type and the Δ*relA* mutant in AG-treated C3H/HeN mice was found ($P = 0.937$).

this alarmone activates branched-chain amino acid biosynthesis, which is a prerequisite for the translation of antinitrosative defenses.

Global transcriptional analyses had already indicated that NO elicits the stringent response (38). The RNAP-binding protein DksA is a *bona fide* sensor of reactive nitrogen species that coordinates the transcription of several *Salmonella* antinitrosative defenses

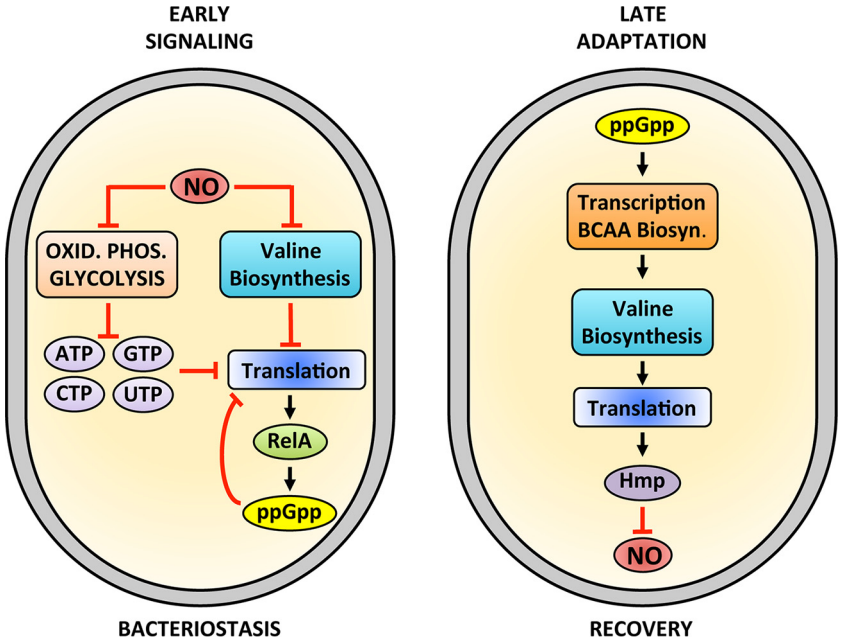

FIG 6 Integrated model of the role of nucleotide metabolism and branched-chain amino acids in the adaptation of salmonellae to nitrosative stress. Early signaling events in response to nitrosative stress. NO inhibits respiration and glycolysis. The depletion of ATP that follows the NO-mediated inhibition of substrate level and oxidative phosphorylation leads to drops in GTP, CTP, and UTP. NO also inhibits valine biosynthesis. In addition, drops in valine stimulate the accumulation of unaminoacylated $tRNA^{Val}$, stimulating the synthesis of ppGpp by RelA associated with ribosomes. Depletion of NTPs and valine and synthesis of ppGpp inhibit translation and bacterial growth. Late adaptive responses by which ppGpp aids in the recovery of salmonellae from nitrosative stress are shown on the right. The ppGpp produced by RelA activates the transcription of branched-chain amino acid (BCAA) biosynthetic genes, restoring valine levels that are needed for the translation of the NO-consuming flavohemoprotein Hmp.

(39–41). The phenotypes of *relA spoT* mutants had also suggested roles for (p)ppGpp in the adaptation of salmonellae to nitrosative stress (31). Here, we demonstrate fast accumulation of high concentrations of ppGpp in salmonellae responding to NO. This nucleotide activates the expression of valine biosynthesis as part of the adaptation that enables the translation of NO detoxification systems (Fig. 6). The indirect role ppGpp plays in the translation of Hmp protein acts in concert with the transcriptional derepression of *hmpA* initiated by nitrosylated NsrR (42).

As GTP is the most abundant and preferred substrate of RelA (18), amino acid starvation mostly induces pppGpp synthesis. The phosphatase activity of GppA is responsible for the nearly equimolar amounts of ppGpp and pppGpp synthesized in amino acid-starving bacteria (this study and reference 33). In contrast to classical nutritional starvation, RelA appears to favor ppGpp synthesis in NO-treated salmonellae. The depletion of the GTP pool could make GDP the more abundant substrate for RelA in NO-treated salmonellae. However, salmonellae exposed to low concentrations of NO still overwhelmingly produced ppGpp, despite abundant GTP pools in the cell. Investigations to identify the mechanism by which NO selectively induces ppGpp are beyond the scope of the present work. Regardless of the mechanism, the predominant production of ppGpp could have major implications for *Salmonella* physiology, as this nucleotide is more potent than its pppGpp analog (17).

GTPases are major targets of (p)ppGpp (27). These nucleotides can inhibit GTPases by indirectly depleting GTP or through direct competitive binding with GTP. Ribosomal GTPases such as EF-Tu, EF-G, EF-Ts, RF3, IF2, and CgtA/Obg can bind (p)ppGpp (43–46). The accumulation of 2 mM ppGpp coupled with a steep depletion of GTP (from ~1 to 0.22 mM) could drastically reduce, if not temporarily eliminate, translation. Rapid and thorough inhibition of translation by ppGpp could preserve small amounts of aminoacylation in tRNA pools in amino acid-starved bacteria. As seen in Δ*relA* mutant *Escherichia coli* for a variety of amino acids (47), Δ*relA* mutant salmonellae suffered a greater depletion of valine after NO treatment than did wild-type controls. Moreover, (p)ppGpp activates the hydrolysis of ribosomal proteins and antitoxin modules by Lon protease (48). Amino acids released in this process could counteract NO-mediated valine starvation. Together, these independent mechanisms may explain why NO-treated Δ*relA* mutant salmonellae do not translate Hmp as quickly or to the same levels as wild-type controls, even when all amino acids are readily available.

Hydrolysis of ppGpp is greatly diminished in salmonellae experiencing high fluxes of nitrosative stress. Hydrolysis of (p)ppGpp by SpoT relies on energy provided by the proton motive force (49), which is inhibited upon the nitrosylation of terminal cytochromes in the electron transport chain (50). Thus, repression of respiration by NO could prevent the hydrolysis of ppGpp by SpoT. Inhibition of ATP synthesis may also explain the overall depletion of NTPs in NO-treated salmonellae (Fig. 6).

Stationary-phase salmonellae undergoing nitrosative stress require methionine and lysine for recovery (this study and reference 15), while exponentially growing cells require branched-chain amino acids. We find it curious that salmonellae display specific amino acid auxotrophies, depending on the growth phase at which they are exposed to NO. Methionine and lysine are derived from the tricarboxylic acid (TCA) cycle intermediate succinyl coenzyme A. The TCA cycle is poorly expressed in stationary-phase salmonellae (51), a situation that can be further aggravated by the NO-dependent modification of aconitase [4Fe-4S] cluster and lipoamide-dependent lipoamide dehydrogenase catalytic cysteine residues (15, 52). NO also prevents the expression of *frdABCD* and *sdhCDAB* in the reductive branch of the TCA cycle in stationary-phase, but not log-phase, salmonellae (15, 51). These mechanisms might explain why the addition of lysine and methionine is inconsequential to exponentially growing salmonellae but helps stationary-phase bacteria recover from nitrosative stress. Nonetheless, NO seems to put pressure on lysine, methionine, and branched-chain amino acid biosynthetic pathways during *Salmonella* infection in mice. MetD, LIV-I, and LIV-II transport systems help salmonellae overcome shortages of methionine and branched-chain amino acids during periods of nitrosative stress in vertebrate hosts, especially if the generation of these amino acids is

hampered by mutations in biosynthetic genes or *relA* (this study and reference 15). Taken together, these investigations suggest that *Salmonella* must resist the antimicrobial activity of NO in periods of both active and inactive growth.

Branched-chain amino acids enable immunity (53). Dietary restrictions of branched-chain amino acids increase the susceptibility of mice to salmonellae (54). Human mononuclear phagocytes use branched-chain aminotransferase 1 to generate TCA cycle intermediates to support iNOS expression in response to lipopolysaccharide (55). Given the reliance of the immune system on branched-chain amino acids and the fact that host-derived NO induces branched-chain amino acid auxotrophies in salmonellae, we were surprised to find that the LIV-I and LIV-II transport systems appear to be dispensable for *Salmonella* pathogenesis. Nonetheless, LIV-I and LIV-II contribute to the pathogenesis of Δ*relA* mutant salmonellae. It is possible that in the absence of the ppGpp-mediated upregulation in the biosynthesis of branched-chain amino acids, scavenging of branched-chain amino acids becomes an important mechanism by which salmonellae resist the antimicrobial activity of iNOS.

Dinitrosyl-iron complexes in the [4Fe-4S] cluster of dihydroxy-acid dehydratase induce functional auxotrophies for branched-chain amino acids (14). Repair of the [Fe-4S] cluster of dihydroxy-acid dehydratase is a major aspect of the regeneration of branched-chain amino acid biosynthesis. According to this model, we noted that nitrosative stress diminishes the valine pools of salmonellae. Our investigations also add a new perspective to this model and suggest that ppGpp-mediated activation of the transcription of *ilvB*-encoded acetohydroxy acid synthase I is a previously unknown aspect that facilitates the recovery of salmonellae from nitrosative stress. Our data suggest that ppGpp acts in concert with DksA for activation of the *ivbL* and *ilvD* promoters, an observation that is consistent with the auxotrophies of *dksA* and *relA spoT* mutants for leucine and valine (56). Salmonellae experiencing nitrosative stress translate *ilvB* faster than *hmpA*, suggesting that this enteropathogen prioritizes amino acid biosynthesis over the enzymatic detoxification of NO. Amino acid biosynthesis genes are enriched for starvation-resistant codons, whose cognate tRNAs maintain significant levels of aminoacylation during amino acid starvation (57). However, valine codons are not notable for resistance to starvation, and neither the *ilvB* nor the *hmpA* gene is enriched in starvation-resistant codons (Table S1). Additional regulation by small RNAs and the RNA-binding protein Hfq could contribute to the delayed translation of *hmpA* (58, 59).

Salmonellae employ (p)ppGpp in the regulation of numerous virulence programs. Poor transcription of genes encoding SPI1 and SPI2 type III secretion systems likely contributes to the attenuation of Δ*relA* Δ*spoT* mutant salmonellae and their inability to invade epithelial cells or grow intracellularly (30). As is the case in other pathogenic bacteria (29), Δ*relA* single mutant salmonellae have minor phenotypes, as illustrated by the fact that Δ*relA* mutant salmonellae can invade HeLa cells and replicate intracellularly in J774 macrophages (not shown). This has led to the idea that (p)ppGpp synthesized by either RelA or SpoT is sufficient for most aspects of bacterial pathogenesis. The investigations presented here have identified a unique role for RelA in the antinitrosative defenses of salmonellae that cannot be compensated by SpoT. Our work sheds light on the specialized roles (p)ppGpp and (p)ppGpp synthetases can play in bacterial pathogenesis.

In summary, salmonellae undergoing nitrosative stress suffer radical changes in their nucleotide pools. Because nucleotides serve as energy sources, substrates for macromolecule synthesis, and signaling molecules, changes in nucleotide metabolism will have a major impact on the response of salmonellae to NO. As part of the nucleotide metabolic reprogramming, ppGpp generated by RelA enhances the expression of antinitrosative defenses in salmonellae by activating valine biosynthesis, which fuels the translation of antinitrosative defenses.

MATERIALS AND METHODS

Bacterial strains. *S. enterica* serovar Typhimurium 14028s and its derivatives were maintained on LB (Lennox broth). Genetic manipulations of salmonellae were made with the λ Red recombinase system as previously described (60, 61). Mutations were transduced between strains with P22 phage (62), and antibiotic resistance genes were excised by FLP-mediated recombination (63). Wild-type and *relA* point

mutant alleles were moved into the *relA* locus of Δ*relA* mutant *Salmonella* strain AV0663 with the λ Red recombinase system. Tables S2 to S4 list the primers, plasmids, and strains used in the course of our investigations.

Minimal media. *Salmonella* strains were grown either in MOPS minimal medium [40 mM MOPS buffer (pH 7.2), 4 mM Tricine, 2 mM K_2HPO_4, 10 μM $FeSO_4 \cdot 7H_2O$, 9.5 mM NH_4Cl, 276 μM K_2SO_4, 500 nM $CaCl_2$, 50 mM NaCl, 525 pM $MgCl_2$, 2.9 nM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 400 nM H_3BO_3, 30 nM $CoCl_2$, 9.6 nM $CuSO_4$, 80.8 nM $MnCl_2$, 9.74 nM $ZnSO_4$] (64) supplemented with 0.4% glucose (22.2 mM) or in M9 minimal medium (49.31 mM Na_2HPO_4, 22.04 mM KH_2PO_4, 8.56 mM NaCl, 0.997 mM $MgSO_4$, 135.16 μM $CaCl_2$) supplemented with 11.1 mM glucose and 10 μM $FeSO_4 \cdot 7H_2O$. Where indicated, defined amino acid mixtures (each amino acid at 40 μg/ml) were added to the minimal media.

^{32}P-labeled nucleotide pools. Nucleotides were examined as originally described by Cashel, with minor modifications (65). Briefly, salmonellae grown overnight in MOPS glucose minimal medium supplemented with all amino acids and 2 mM K_2HPO_4 were diluted 1:100 in fresh medium supplemented with 0.4 mM K_2HPO_4. The cultures were grown to early exponential phase (optical density at 600 nm [OD_{600}] of ~0.2). One-milliliter culture aliquots were labeled with 10 μCi of ^{32}P-labeled orthophosphate for approximately 2.5 doubling times (roughly 1 h to an OD_{600} of 0.5). Salmonellae were treated with NaOH (vehicle control for the NO donors), spermine NONOate, PAPA NONOate, serine hydroxamate, or tetracycline before 0.4 ml of ice-cold 50% formic acid was added to the cultures. Extracts were incubated on ice for at least 20 min before the specimens were centrifuged at 16,000 × *g* for 5 min. A 5- or 10-μl volume of ice-cold extracts was spotted along the bottom of polyethyleneimine-cellulose TLC plates (20 by 20 cm; EDM Millipore, Billerica, MA). The spots were air dried, and the TLC plates were put into a chamber containing 1.25 M K_2HPO_4, pH 3.4. To resolve all NTPs, 0.9 M K_2HPO_4, pH 3.4, was used as the solvent. ^{32}P-labeled nucleotides in the TLC plates were visualized with phosphor screens and a phosphorimager (Bio-Rad, Hercules, CA), and relative nucleotide levels were quantified with the ImageJ software (NIH, Rockville, MD).

Luciferase ATP assay. Intracellular pools of ATP were quantified with the luciferase-based ATP determination kit (Molecular Probes, Eugene, OR). Briefly, wild-type salmonellae grown to early exponential phase (OD_{600} of 0.2 to 0.4) were challenged for 5 min with 750 μM spermine NONOate in MOPS glucose minimal medium supplemented with each amino acid at 40 μg/ml. Aliquots were set aside for serial 10-fold plate dilutions, and 0.5-ml samples of cultures were thoroughly mixed with 0.6 ml of ice-cold, freshly prepared 380 mM formic acid containing 17 mM EDTA. Samples stored at −80°C were centrifuged for 1 min at 16,000 × *g*. Supernatants were diluted 25-fold into 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.4. Ten-microliter volumes of samples or ATP standards were mixed with 90 μl of reaction master mix (8.9 ml of water, 500 μl of 20× buffer, 500 μl of 10 mM D-luciferin, 100 μl of 100 mM dithiothreitol [DTT], 2.5 μl of 5-mg/ml firefly luciferase). Luminescence was recorded in an LMax 1.1L luminometer, and the data were analyzed with SoftMax Pro software (Molecular Devices, Sunnyvale, CA). ATP concentrations in the lysates were calculated by linear regression using known standards. The intracellular concentration of ATP was calculated by taking into account the ATP concentrations in the lysates, the number of CFU per milliliter, and an estimated bacterial cell volume of 1 fl.

NO recovery assays. Salmonellae grown overnight in either MOPS or M9 glucose minimal medium were subcultured 1:100 into the same medium and grown until they reached early exponential phase. The cultures were diluted to an OD_{600} of 0.2, and selected specimens were challenged with 750 μM spermine NONOate. The OD_{600} was recorded in a 96-well microtiter plate every 30 min for 4.5 h. The time required for each culture to double the initial OD_{600} was calculated by exponential regression. Where indicated, stationary-phase salmonellae grown overnight in LB broth were diluted in MOPS glucose minimal medium and challenged with spermine NONOate.

Liquid chromatography (LC)-mass spectrometry (MS) of amino acid pools. Amino acids were extracted from 10^{10} *Salmonella* cells/ml of ice-cold lysis buffer (5:3:2 ratio of methanol-acetonitrile-water [Fisher Scientific, Pittsburgh, PA] containing a 3 μM concentration of a mixture of amino acid standards [Cambridge Isotope Laboratories, Inc., Tewksbury, MA]). Samples were vortexed for 30 min at 4°C in the presence of 1-mm glass beads. Insoluble proteins and lipids were pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C. The supernatants were collected and dried with a SpeedVac concentrator. The pellets resuspended in 0.1% formic acid were analyzed in a Thermo Vanquish ultrahigh-performance liquid chromatography (UHPLC) device coupled online to a Thermo Q Exactive mass spectrometer. The UHPLC-MS methods and data analysis approaches used were described previously (66).

Total RNA isolation, cDNA synthesis, and quantitative real-time (qRT)-PCR. Salmonellae were grown to early exponential phase in MOPS glucose minimal medium. Bacterial cultures were challenged with 750 μM spermine NONOate for 0, 30, 60, or 120 min, and 5-ml aliquots were incubated on ice for at least 10 min after being mixed with 1 ml of ice-cold RNA stabilization solution (95% ethanol, 5% aqueous phenol). The specimens were centrifuged at 16,000 × *g* for 10 min at 4°C, and the pellets were saved at −80°C until extraction. Total RNA was extracted and purified with the High Pure RNA Isolation kit (Roche, Basel, Switzerland). RNA samples were stored at −80°C until cDNA synthesis. Briefly, 1 μg of RNA was mixed with 1.3 μl of 10 mM random DNA hexamers in a final volume of 16.5 μl. The RNA solution was heated to 70°C for 5 min and then cooled for 5 min at 4°C. An 8.5-μl volume of master mix (5 μl of 5× Moloney murine leukemia virus [MMLV] buffer, 2.5 μl of 4 mM each deoxynucleoside triphosphate, 0.5 μl of RNasin, 0.5 μl of MMLV reverse transcriptase) was added to each sample, and cDNA was synthesized at 42°C for 1 h. The amounts of cDNA in the samples were quantified by real-time PCR. Briefly, 25-μl reaction mixtures were prepared in 96-well Hard-Shell plates in a CFX Connect real-time system with Bio-Rad CFX Manager 3.1 software (Bio-Rad, Hercules, CA). Each reaction mixture contained 5 μl of water, 12.5 μl of 2× SYBR Select master mix (Applied Biosystems, Thermo Fisher,

Waltham, MA), 1 μl of each gene-specific primer at 10 μM, 0.5 μl of gene-specific Probe (IDT, Coralville, IA) at 10 μM, and 5 μl of cDNA or gene-specific PCR product. The quantification cycle was calculated by exponential regression by using PCR product standards. Gene expression was normalized to the internal housekeeping gene *ampD* (67).

***In vitro* transcription.** *pivbL* and *pivlD* PCR templates were purified with the GeneJET gel extraction kit (Thermo Scientific). RNA synthesized in *in vitro* transcription reactions was labeled with [α-^{32}P]UTP after incubation of 1 nM template DNA, 5 nM *E. coli* RNA polymerase (holoenzyme; NEB), and 5 μM DksA in reaction buffer (40 mM HEPES [pH 7.4]; 2 mM $MgCl_2$; 60 mM potassium glutamate; 0.1% NP-40; 200 μM each ATP, GTP, and UTP; 8 U of RiboLock RNase inhibitor [Thermo Scientific]; 1 mM DTT) in the presence or absence of ppGpp (40). DksA was purified as previously described (41). The specimens were incubated at 37°C for 10 min, and reactions were terminated by heating at 70°C for 10 min. Products of *in vitro* transcription reactions were separated on 7 M urea–6% polyacrylamide gels and visualized by phosphorimaging.

Western blotting. *Salmonella* strains expressing 3×FLAG epitope-tagged alleles were grown in MOPS glucose or M9 minimal medium to an OD_{600} of 0.2 to 0.5. Bacterial cells were challenged with 750 μM spermine NONOate. At regular time intervals after the addition of spermine NONOate, 1-ml samples were mixed with 111 μl of ice-cold 100% trichloroacetic acid and the specimens were placed on ice for at least 10 min. Trichloroacetic acid-treated samples were centrifuged at 16,000 × *g* for 10 min, and pellets were stored at −80°C until analysis. Pellets were resuspended with either 10 mM Tris buffer (pH 7.4) or phosphate-buffered saline containing 50 mM NaOH. Samples were mixed with 6× Laemmli loading buffer (416 mM sodium dodecyl sulfate [SDS], 0.9 mM bromophenol blue, 47% [vol/vol] glycerol, 60 mM Tris [pH 6.8]) containing 5% β-mercaptoethanol, boiled for 10 min, and loaded onto reducing 10% polyacrylamide gels. Proteins were separated by SDS-PAGE (85 V for 25 min, followed by 110 V for 50 min) in SDS running buffer. Proteins were transferred onto nitrocellulose membranes with transfer buffer (50 mM Tris base, 40 mM glycine, 1 mM SDS, 20% [vol/vol] methanol) at 25 V for 30 min on a semidry transfer apparatus (Bio-Rad, Hercules, CA). Membranes were blocked overnight at 4°C in 5% skim milk in TBST buffer (25 mM Tris [pH 7.6], 150 mM NaCl, 0.1% [vol/vol] Tween 20). Membranes were then probed with 1:500 anti-FLAG or 1:2,942 anti-DnaK mouse antibody diluted in 5% skim milk in TBST buffer while gently rocking for 2 h at room temperature. After washing with TBST buffer, membranes were probed for 1 h at room temperature with 1:4,000 goat anti-mouse horseradish peroxidase-labeled secondary antibody prepared in 5% skim milk in TBST buffer. Chemiluminescence signals were visualized after the addition of horseradish peroxidase detection solution (GE Healthcare Life Sciences, Pittsburgh, PA) on a Gel Doc (Bio-Rad, Hercules, CA).

Murine infections. C3H/HeN mice were housed and bred at the University of Colorado Denver Animal Care and Use Committee guidelines. Six- to 8-week-old mice were infected by oral gavage with approximately 5 × 10^6 CFU of either wild-type or mutant salmonellae. Murine survival was monitored daily for 28 days. Sick mice were humanely euthanized by CO_2-mediated asphyxiation, followed by cervical dislocation. For selected studies, the drinking water was treated daily with a 2.5% solution of the iNOS inhibitor aminoguanidine.

Statistical analysis. The Student *t* test, the Mann-Whitney *t* test, one-way analysis of variance (ANOVA), two-way ANOVA, and log rank analyses were performed with GraphPad Prism version 5.0b. Differences were considered significant when the *P* value was <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00211-18.

FIG S1, PDF file, 3.9 MB.
FIG S2, PDF file, 3 MB.
FIG S3, PDF file, 2.3 MB.
FIG S4, PDF file, 2.9 MB.
FIG S5, PDF file, 0.5 MB.
FIG S6, PDF file, 1.7 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.04 MB.
TABLE S3, PDF file, 0.03 MB.
TABLE S4, PDF file, 0.1 MB.

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