Magnesium homeostasis protects *Salmonella* against nitrooxidative stress

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The PhoPQ two-component regulatory system coordinates the response of *Salmonella enterica* serovar Typhimurium to diverse environmental challenges encountered during infection of hosts, including changes in Mg2+ concentrations, pH, and antimicrobial peptides. Moreover, PhoPQ-dependent regulation of gene expression promotes intracellular survival of *Salmonella* in macrophages, and contributes to the resistance of this pathogen to reactive nitrogen species (RNS) generated from the nitric oxide produced by the inducible nitric oxide (NO) synthase of macrophages. We report here that *Salmonella* strains with mutations of phoPQ are hypersensitive to killing by RNS generated in vitro. The increased susceptibility of ∆phoQ Salmonella to RNS requires molecular O2 and coincides with the nitrotyrosine formation, the oxidation of [4Fe-4S] clusters of dehydratases, and DNA damage. Mutations of respiratory NADH dehydrogenases prevent nitrotyrosine formation and abrogate the cytotoxicity of RNS against ∆phoQ Salmonella, presumably by limiting the formation of peroxynitrite (ONOO−) arising from the diffusion-limited reaction of exogenous NO and endogenous superoxide (O2•−) produced in the electron transport chain. The mechanism underlying PhoPQ-mediated resistance to RNS is linked to the coordination of Mg2+ homeostasis through the PhoPQ-regulated MgtA transporter. Collectively, our investigations are consistent with a model in which PhoPQ-dependent Mg2+ homeostasis protects *Salmonella* against nitrooxidative stress.

Mutations in phoPQ attenuate *Salmonella* virulence by at least 10,000-fold1–3. The attenuated phenotype of phoPQ mutants has been associated with poor intracellular survival in macrophages, defective activation of *Salmonella* pathogenicity island 2 (SPI2) transcription, and hypersensitivity to defensins, antimicrobial peptides, divalent cations, iron, acid and bile salts1,4–10. PhoPQ signaling also boosts antioxidant defenses through the positive regulation of the sodCI-encoded superoxide dismutase, the posttranslational stabilization of the alternative σ factor, and the limitation in the availability of free iron7,11,12. In addition, PhoPQ lessens the cytotoxicity of reactive nitrogen species (RNS) generated by inducible nitric oxide synthase (iNOS) in the innate immune response of mononuclear phagocytic cells13.

The antimicrobial activity of NO is best demonstrated in IFN-γ-activated phagocytes; however, very little anti-*Salmonella* activity is derived from iNOS expressed through the innate recognition of *Salmonella* lipopolysaccharide by host-cell Toll-like receptor 414–19. There are several possible explanations underlying the marked resistance of *Salmonella* to the nitrosative species synthesized by iNOS during the innate response of professional phagocytes. The low NO fluxes generated in the innate response dramatically limit the synthesis of autooxidative products such as dinitrogen trioxide (N2O3), which has been associated with sustained anti-*Salmonella* activity of IFN-γ-primed macrophages17. On the other hand, the SPI2 type III secretion system, the Hmp flavohemoprotein, and low-molecular weight thiols protect *Salmonella* against moderate NO rates generated in the innate immune response20–22. As just mentioned, we have recently shown that PhoPQ signaling enhances the intracellular fitness of *Salmonella* by antagonizing the innate host response associated with NO13. The mechanism by which the PhoPQ two-component regulatory system defends *Salmonella* against the antimicrobial actions of NO congeners remains unknown. The investigations presented herein have revealed that the PhoPQ two-component regulatory

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system enhances the resistance of *Salmonella* against the nitrooxidative stress generated in the interaction of exogenous NO with endogenously produced $\text{O}_2^{{+•}}$ through its regulation of intracellular $\text{Mg}^{2+}$ concentrations.

**Results**

**PhoPQ-deficient *Salmonella* are hypersusceptible to NO.** The PhoPQ signaling cascade coordinates important aspects of the antioxidant and antinitrosative defenses of *Salmonella* [12,13]. The PhoPQ two-component regulatory system is involved in *Salmonella* defense against Fenton-mediated oxidative stress [14], however, it is unclear how PhoPQ signaling promotes resistance to RNS. To learn more about the role of PhoPQ in resistance of *Salmonella* to RNS, we investigated the survival of a Δ*phoQ* mutant exposed to the NO generator spermine NONOate (sperNO). Most wild-type *Salmonella* survived 6 h after challenge with 250 µM sperNO, while ~99% of Δ*phoQ* *Salmonella* were killed upon sperNO treatment (Fig. 1A). The NO-mediated killing of Δ*phoQ* *Salmonella* was already noted after 4 h of challenge. The susceptibility of Δ*phoQ* *Salmonella* to sperNO appears to rely on the generation of NO because the polyamine spermine control lacked antimicrobial activity (Fig. 1B). A dose-dependent inhibition of growth by sperNO was observed for both Δ*phoP* or Δ*phoQ* strains when inoculated in LB broth or minimal E salts medium supplemented with malic acid (Fig. 1C and Fig. S1), which is consistent with the notion that the PhoP response regulator boosts the antinitrosative potential of *Salmonella* in conjunction with its cognate PhoQ sensor kinase. The growth of Δ*phoQ* *Salmonella* in E salts was completely inhibited by 250 µM sperNO, which corresponds to the dose of sperNO that resulted in significant lethality when cells were challenged in PBS (Fig. 1 and Fig. S1). The hypersensitivity of Δ*phoQ* *Salmonella* to sperNO does not appear to be due to defects in viability, as wild-type and Δ*phoQ* *Salmonella* strains grew with similar kinetics in LB and various minimal E salts media in the absence of sperNO (Fig. S2). Complementation of Δ*phoQ* *Salmonella* with a plasmid encoding a wild-type allele of *phoQ* (pPhoQ) restored wild-type levels of growth following sperNO treatment (Fig. 1C). Collectively, these data indicate that the PhoPQ two-component regulatory system contributes to the protection of *Salmonella* against the cytotoxic activity associated with RNS.

**Oxygen is required for the NO-dependent killing of *phoQ*-deficient *Salmonella*.** RNS including nitrogen dioxide ($\text{NO}_2^{{+•}}$), $\text{N}_2\text{O}_3$, and ONOO$^{-}$ produced in the reaction of NO with $\text{O}_2$ and $\text{O}_2^{{+•}}$ indirectly mediate NO cytotoxicity [21]. To determine whether killing of Δ*phoQ* *Salmonella* by sperNO is mediated by NO itself or by a variety of RNS, *Salmonella* were exposed to 250 µM sperNO in the presence or absence of $\text{O}_2$. To generate a hypoxic environment, PBS was flushed with $\text{N}_2$ for 10 min and the experiments were carried out in sealed tubes. The viability of wild-type *Salmonella* was not ($P > 0.05$) affected by sperNO in either normoxic or hypoxic conditions (Fig. 2A). In contrast, the NO-dependent killing of Δ*phoQ* *Salmonella* was completely abrogated in hypoxic cultures (Fig. 2B). These findings suggest that the PhoPQ two-component regulatory system protects *Salmonella* against nitrooxidative products formed in the reaction of NO and $\text{O}_2$ metabolites.

The antioxidant defenses associated with PhoPQ signaling rely on the expression of a functional PmrAB two-component regulatory system and the CorA metal transporter [22,23]. The susceptibility of Δ*phoQ* *Salmonella* to NO appears, however, to be independent of *pmrA* (Fig. 2C), which is consistent with previous investigations that reported that a Δ*pmrA* mutant is as resistant to sperNO as wild-type controls [24]. The hypersusceptibility of *phoP* mutants to $\text{Fe}^{2+}$-mediated oxidative stress can be prevented by a mutation in the *corA* metal transporter. However, a mutation in *corA* did not prevent the killing of Δ*phoQ* *Salmonella* by sperNO (Fig. 2C). In addition...
Substantial nitrotyrosine formation was also detected within 30 min after exposure to sperNO (Fig. 2D and E). Interestingly, a strain of Salmonella phoQ lacks the RNS-dependent cytotoxicity than the mutant, suggesting that in the absence of PhoPQ the alternative sigma factor RpoS to the resistance of Salmonella to 250 µM sperNO is shown in (C, D and E), respectively. *P < 0.05 compared to WT. **P < 0.05 compared to the ΔphoQ strain.

Salmonella exposed to NO undergoes nitrooxidative stress. To determine whether wild-type and ΔphoQ Salmonella experience different degrees of nitrooxidative stress upon exposure to sperNO, we monitored the formation of N₂O₃ (a reactive species generated upon autooxidation of NO in the presence of O₂) and nitrotyrosine (an oxidative signature of the reaction of ONOO⁻ or other RNS with tyrosyl residues). Similar concentrations of N₂O₃ were generated after treatment of wild-type or ΔphoQ Salmonella with 250 µM sperNO (Fig. 3A). Substantial nitrotyrosine formation was also detected within 30 min after Salmonella were challenged with sperNO (Fig. 3B). Moreover, the profiles and kinetics of nitrotyrosine formation were similar in both wild-type and ΔphoQ Salmonella strains (Fig. 3B). Nitrotyrosine formation was not observed in low O₂ cultures (Fig. 3C), arising from the diffusion-limited reaction of exogenous NO with endogenous O₂. The formation of N₂O₃ (a reactive species generated upon autooxidation of NO in the presence of O₂) and nitrotyrosine (an oxidative signature of the reaction of ONOO⁻ or other RNS with tyrosyl residues) was indirectly measured by following the expression of a transcriptional fusion to the SOS response lacZY gene. The recA::lacZY transcriptional fusion was similarly induced in both wild-type and ΔphoQ Salmonella after exposure to 12 J/m² UV light or treatment with 2.5 mM sperNO (Fig. 3E).

Mutations in NADH dehydrogenases NDH-I and NDH-II protect ΔphoQ Salmonella from NO-dependent cytotoxicity and protein nitration. We examined in more detail the mechanism underlying the cytotoxicity of RNS against ΔphoQ Salmonella. NADH dehydrogenases of the electron transport chain can be a sizable source of oxidative stress in the cell. We tested whether the sperNO-mediated O₂⁻•− arising from the adventitious reduction of O₂ by NADH dehydrogenases of the electron transport chain. To test this hypothesis, the ΔphoQ::km mutant allele was introduced into the Δnuo Δndh mutant strain AV0438 lacking both NDH-I and NDH-II NADH dehydrogenases. As noted for H₂O₂ and ONOO⁻, the complex I-deficient Δnuo Δndh strain AV0438 was resistant to 250 µM sperNO (Fig. 4A). Strikingly, strain AV0810 harboring mutations in phoQ, nuo and ndh was also resistant to NO (Fig. 4A). Similar to the complex I-deficient isogenic strain AV0438, strain AV0810 lacking phoQ, nuo and ndh appear to be protected from ONOO⁻ as indicated by a lack of nitrotyrosine to contributing to iron homeostasis, PhoPQ can activate Salmonella’s antioxidant defenses through the positive regulation of SodCl expression and the stabilization of RpoS. However, neither SodCl nor RpoS appear to contribute to the increased susceptibility of ΔphoQ Salmonella under the experimental conditions tested here (Fig. 2D and E). Interestingly, a strain of Salmonella lacking both phoQ and rpoS was even more susceptible to the RNS-dependent cytotoxicity than the phoQ mutant, suggesting that in the absence of PhoPQ the alternative sigma factor RpoS assumes a critical role in the regulation of the antinitrosative defenses of Salmonella.
formation 6 h after exposure to 250 µM sperNO (Fig. 4B). Collectively, these data indicate that ONOO$^-$-dependent nitrooxidative stress engendered upon reaction of exogenous NO with O$_2•^-$ produced by NADH dehydrogenases of the electron transport chain contributes to the NO-mediated killing of $\Delta$phoQ Salmonella.

Exogenous Mg$^{2+}$ rescues $\Delta$phoQ Salmonella from RNS-dependent killing. Previously, Salmonella was shown to exhibit increased susceptibility to oxidative stress following disruptions in Mg$^{2+}$ uptake through mutations of phoP or the PhoPQ-regulated Mg$^{2+}$ transporters mgtA and mgtB$^7$. Therefore, we investigated whether the increased susceptibility of $\Delta$phoQ Salmonella to RNS was due to disruptions in Mg$^{2+}$ homeostasis. The increased susceptibility of the $\Delta$phoQ Salmonella strain to killing by 250 µM sperNO in PBS was prevented by the addition of 10 mM MgSO$_4$ but had no effect on the survival of the wild-type strain or the $\Delta$phoQ strain complemented with a pPhoQ plasmid (Fig. 5A). Moreover, the sperNO-dependent inhibition of growth of $\Delta$phoQ Salmonella was alleviated by the addition of 10 mM MgSO$_4$ when cultured in LB with 2.5 mM sperNO.
This protective effect of exogenous MgSO₄ was also observed in ∆phoQ Salmonella challenged with 250 µM sperNO in minimal E salts media supplemented with glucose, malic acid, or fumarate (Fig. S2). Moreover, the addition of MgCl₂ also restored the growth of ∆phoQ Salmonella challenged with sperNO, while the addition of CaCl₂ had no effect (Fig. S3). Collectively, these data suggest that the hypersensitivity of ∆phoQ Salmonella to RNS is due to disruptions in Mg²⁺ homeostasis.

We hypothesized that the increased susceptibility of the ∆phoQ Salmonella strain to RNS was linked to its inability to upregulate the expression of Mg²⁺ transporters encoded by mgtA and mgtB. Therefore, we compared the susceptibility of Salmonella strains lacking mgtA and/or mgtBC to killing by 250 µM sperNO. The ∆mgtA-deficient Salmonella strain showed a significantly increased susceptibility to killing by sperNO compared to both wild-type and ∆mgtBC strains (Fig. 5C). A strain harboring mutations in both mgtA and mgtBC was no more susceptible to killing by RNS as the ∆mgtA mutant strain suggesting that the PhoPQ-dependent regulation of mgtA promotes resistance to RNS. As was the case for the ∆phoQ Salmonella strain (Fig. 5A), the addition of 10 mM MgSO₄ prevented sperNO-dependent killing of the ∆mgtA Salmonella strain (Fig. 5D). These data suggested that the increased susceptibility of ∆phoQ Salmonella to RNS is due to disruptions in Mg²⁺ homeostasis.

To test this hypothesis, we introduced a pBAD/HisA plasmid encoding mgtA (pBmgtA) under an arabinose-inducible promoter into the ∆phoQ Salmonella strain, and compared its ability to grow in LB broth in the presence or absence of sperNO. The wild-type, ∆phoQ, and ∆phoQ pMgtA strains showed similar growth kinetics in LB broth (Fig. 5D). As described earlier, ∆phoQ Salmonella were unable to grow in LB broth in the presence of 2.5 mM sperNO (Figs 1C and 5D). In contrast, the introduction of a pMgtA plasmid to the ∆phoQ strain restored growth in LB broth in the presence of 2.5 mM sperNO, albeit with an increased lag compared to the wild-type control (Fig. 5D). This lag was eliminated by the addition of 10 mM MgSO₄, suggesting that the expression of mgtA from the pMgtA plasmid could only partially restore Mg²⁺ homeostasis in the ∆phoQ strain. Collectively, these data support the hypothesis that PhoPQ promotes resistance to nitrooxidative stress in Salmonella through the regulation of Mg²⁺ homeostasis.

**Discussion**

The PhoP regulon controls the antioxidant defenses of Salmonella, Yersinia pestis and Enterococcus faecalis, and work from our laboratory indicates that this two-component regulatory system also contributes to the antinitrosative defenses of Salmonella. Elegant investigations by Dr. Groisman’s group have elucidated that the
PhoP regulon defends *Salmonella* against oxidative stress engendered in the reduction of H$_2$O$_2$ by the Fenton catalyst Fe$^{2+}$. Little is known, however, about the nitrosative chemistry antagonized by this two-component regulatory system. We therefore deemed it important to investigate the newly described function of PhoPQ in the antinitrosative defenses of *Salmonella*. The investigations presented here are consistent with a model in which the PhoPQ two-component regulatory system antagonizes the antimicrobial activity of ONOO$^-$.

**Figure 5.** PhoPQ-dependent regulation of Mg$^{2+}$ homeostasis protects *Salmonella* from RNS cytotoxicity. The survival of WT, ΔphoQ, and ΔphoQ pWSK29::phoQ (pPhoQ) *Salmonella* strains was determined after incubation of strains with 250µM sperNO in the presence or absence of 10mM MgSO$_4$ in PBS at 37°C for 6h (A). The data, which are represented as mean ± S.D., are from 8 replicates collected from 2 separate experiments. *P < 0.01 compared to sperNO-treated WT controls. The OD$_{600nm}$ was measured over time as described in Fig. 1 to determine the growth of *Salmonella* strains cultured in LB + 2.5 mM sperNO, or LB + 2.5 mM sperNO + 10 mM MgSO$_4$ at 37°C. The survival of WT, ΔmgtA::km, ΔmgtBC, and ΔmgtBC ΔmgtA::km *Salmonella* strains was determined following incubation with 250µM sperNO in the presence or absence of 10mM MgSO$_4$ in PBS at 37°C for 6h (C). The data are represented as mean % survival ± SD from 8 replicates collected from 2 separate experiments. *P < 0.01 compared to sperNO-treated WT controls. The OD$_{600nm}$ was measured over time as described in Fig. 1 to determine the growth of *Salmonella* strains cultured in LB + 2.5 mM sperNO or LB + 2.5 mM sperNO + 10 mM MgSO$_4$ at 37°C (D). Data represent the mean OD$_{600nm}$ of 3 biological replicates.

PhoP regulon defends *Salmonella* against oxidative stress engendered in the reduction of H$_2$O$_2$ by the Fenton catalyst Fe$^{2+}$. Little is known, however, about the nitrosative chemistry antagonized by this two-component regulatory system. We therefore deemed it important to investigate the newly described function of PhoPQ in the antinitrosative defenses of *Salmonella*. The investigations presented here are consistent with a model in which the PhoPQ two-component regulatory system antagonizes the antimicrobial activity of ONOO$^-$. In support of this model, the sperNO-mediated killing of ΔphoQ *Salmonella* is restricted to aerobic cultures, and coincides with the formation of nitrotyrosine, and the inactivation of the TCA cycle enzyme aconitase. These observations can be explained if one takes into account that the generation of ONOO$^-$ requires the reaction of O$_2^-$/NO. O$_2^-$/NO is
formed adventitiously at the flavin or quinone-binding sites of NADH dehydrogenases of the electron transport chain and its production requires O2. The ONOO− produced in the reaction of endogenous O2•− and exogenous NO is a powerful nitrating and oxidizing agent that could explain the formation of nitrotyrosine residues in cytoplasmic proteins and the oxidation of the [4Fe-4S] clusters of dehydratases. The protection afforded by mutations in NADH dehydrogenases against ONOO−-dependent cytotoxicity could be explained by three independent and complementary mechanisms. First, the accumulation of NADH in Δndh Δnuo Salmonella effectively scavenges NO•− and OH• radicals caged in peroxynitrous acid (ONOO−OH), which is the dominant ONOO−-congener at the neutral pH of the bacterial cytoplasm27. Second, NADH fuels the enzymatic detoxification of ONOO− by the AhpCF alkylhydroperoxidases27,28. And third, a lack of NADH dehydrogenases diminishes ONOO− synthesis by limiting the flow of electrons through the respiratory chain that is required for the generation of O2•−. Our investigations suggest that ONOO− is necessary but not sufficient for the NO-mediated antimicrobial activity, because wild-type Salmonella and the phoQ mutant bacteria suffer a similar degree of nitrotyrosine formation and inactivation of aconitate upon exposure to sperNO, but are differently killed by the oxidative congeners of this diatomic radical.

[4Fe-4S] clusters of dehydratases can be directly nitrosylated by NO at a rate constant of 106 M−1 sec−12. Because the nitrosylation of [4Fe-4S] clusters is second order for NO, this chemistry is less likely to occur at the low NO fluxes sustained in the course of our investigations. The inactivation of [4Fe-4S] clusters by ONOO−, which occurs at the fast rate of 1.4 × 107 M−1 sec−112, is first order for ONOO−. The speed of the reaction indicates that the oxidation of [4Fe-4S] clusters by ONOO− is limited by the production of this RNS. NO and O2•− react with a second order rate constant of 109 M−1 sec−1 to form ONOO−32. However, high concentrations of NO readily consume ONOO−. Therefore, generation of ONOO− in the Salmonella cytoplasm is most likely to be maximal at the low rates of NO synthesis supported during the innate immune response. In the presence of a functional PhoPQ two-component regulatory system the ONOO− produced endogenously appears to be tolerated by Salmonella.

The Hmp-mediated and cytochrome bd-mediated detoxification of NO, the stringent response, low-molecular weight thiols, along with DNA repair systems minimize the cytotoxicity of NO produced in the innate response to Salmonella. Our investigations identify PhoPQ-dependent regulation of Mg2+ homeostasis as an additional antinitrosative defense that shields Salmonella from the cytotoxicity of low NO fluxes. While the hypersensitivity of ΔphoQ Salmonella to RNS is tied to disrupted Mg2+ homeostasis, this phenotype appears to be independent of both PmrAB-dependent and CorA-dependent resistance Fe2+ toxicity. This conclusion is supported by the fact that 1) pmrA or corA single mutant strains do not exhibit increased sensitivity to RNS compared to wild-type strains, and 2) phoQ pmrA or phoQ corA double mutant Salmonella strains were as sensitive to killing by sperNO as ΔphoQ Salmonella. Cellular concentrations of Mg2+ in Salmonella are capable of reaching 100 mM46, with the majority of Mg2+ bound to ribosomes and nucleotide triphosphates. Therefore, the reduced cytoplasmic Mg2+ concentrations in ΔphoQ Salmonella may increase the susceptibility to killing by RNS due to several factors including 1) reduced protein synthesis resulting from the dissociation of Mg2+ from ribosomes, and 2) leaching of Mg2+ from nucleotide triphosphates preventing their use as substrates for enzymatic reactions necessary to repair RNS-induced cellular damage. This is supported by the fact that the addition of 10 mM MgSO4, rescued both ΔphoQ and ΔmgtA Salmonella strains from RNS-dependent killing.

Independent of the regulation of antioxidant defenses or targets of nitrooxidative stress, a functional PhoPQ two-component regulatory system is likely to promote antinitrosative defenses through the activation of SPI2 transcription38–40, because the SPI2 type III secretion system has been shown to minimize fusion of Salmonella-containing vacuoles with vesicles harboring iNOS41. In contrast to the concerted and rich repertoire of antinitrosative defenses that protect Salmonella against the low NO fluxes produced in the innate response, no antinitrosative defenses are known to protect this facultative intracellular pathogen against the massive nitrosative stress unleashed in IFNγ-activated macrophages. Paradoxically, N2O3 and other high oxidation NO congeners generated by IFNγ-primed macrophages exert profound anti-Salmonella activity by repressing SPI2 transcription and PhoPQ signaling15,16,17. In turn, RNS-dependent repression of PhoPQ signaling and SPI2 transcription promotes the maturation of the Salmonella phagosome along the degradative pathway for fusion with lysosomes.

In summary, this study has revealed that in addition to its known roles in protecting Salmonella from acid pH, bile salts, antimicrobial peptides, and oxidative stress14–16, the PhoPQ two-component regulatory system contributes to the resistance of Salmonella against the nitrooxidative stress generated in the reaction of exogenous NO and endogenously produced O2•− by maintaining Mg2+ homeostasis (Fig. 6).

Methods

Bacterial Strains. Salmonella enterica serovar Typhimurium strain ATCC 14028s was used throughout this study as wild-type, and as a background for the construction of mutations and a recA::lacZY transcriptional fusion (Table 1). The mutations were generated following the one-step, λ-Red-mediated gene replacement method of Datsenko and Wanner42. Briefly, primers encoding 40–42 nucleotides homologous to the target gene followed by 20 nucleotides homologous to the pKD13 template plasmid were used for the PCR amplification of the Flp recombinant target (FRT)-flanked kanamycin resistance cassette. The resulting PCR products were DpnI-digested and electroporated into S. Typhimurium strain TT22236 carrying the pTP2223 plasmid that expresses the λ-Red recombinase under PtcA control. Mutations were moved between strains by P22-mediated transduction and pseudolysogens eliminated by streaking on Evans blue uranine agar plates. Nonpolar deletions were generated by recombing the two FRT sites flanking the kanamycin resistance cassette with the Flp recombinase encoded by the pCP20 plasmid43. The mutations were confirmed by PCR analysis. A recA::lacZY transcriptional fusion was constructed by the pCP20-mediated integration of pCE36 encoding a promoterless lacZY operon into the unique FRT scar engineered immediately downstream of the recA stop codon. The pMgtA plasmid was generated by cloning a wild-type copy of the magnesium transporter mgtA into the pBAD/HisA vector using
the primers listed in Table 2. The pMgtA plasmid was electroporated into Salmonella strain AV0475 carrying a ΔphoQ::FRT allele to produce the TJB1301 strain.

**Susceptibility of Salmonella to reactive nitrogen species.** Salmonella strains were inoculated from frozen stocks and grown in Luria-Bertani (LB) broth with shaking at 325 r.p.m. at 37 °C for 20 h. Strains were then diluted in PBS to a concentration of ~5 × 10⁵ cells ml⁻¹. The bacteria were challenged at 37 °C with 250 μM of the NO donor spermine NONOate (Cayman Chemical, Ann Arbor, MI). Selected groups of bacteria were challenged at 37 °C with 250 μM spermine NONOate in PBS that had been depleted of O₂ after 10 min of flushing with N₂. Percent survival was calculated by recording the number of bacteria capable of forming a CFU on LB agar plates. Alternatively, stationary phase cultures of the Salmonella strains were subcultured 1:200 in fresh LB broth in the presence or absence of 2.5 mM spermine NONOate. Bacterial suspensions were seeded in 96-well plates, and were grown at 37 °C with shaking at 282 r.p.m. with the optical density measured at 600 nm (OD₆₀₀nm) using a Cytation 5 multi-mode plate reader (BioTek, Winooski, VT).

**Aconitase enzymatic assay.** Salmonella strains grown in Luria-Bertani (LB) broth with shaking at 325 r.p.m. at 37 °C for 20 h were pelleted by centrifugation and resuspended in PBS to an OD₆₀₀nm of 0.5. Soluble cytoplasmic proteins were isolated from bacteria incubated at 37 °C for 6 h in PBS in the presence or absence of 250 μM spermine NONOate. Briefly, the bacteria were washed in 20 mM Tris-citrate buffer pH 8.0, and the cytoplasmic proteins extracted by sonication. Bacterial debris was removed by centrifugation at 14,000 RPM in a microcentrifuge for 30 s. Aconitase activity contained in the cytoplasmic extracts was estimated spectrophotometrically at 240 nm by following the formation of cis-aconitate in Tris-citrate buffer containing 20 mM isocitrate. The Aconitase activity is expressed as the mean OD₂₄₀nm/min/mg protein ± SD of 2 independent experiments.

**Estimation of DNA damage.** The accumulation of single strand and double strand DNA damage was indirectly estimated by measuring the expression of a lacZY transcriptional fusion of the SOS response recA gene. Selected groups of bacteria were irradiated with 12 J/m² UV using a TL-2000 Ultraviolet Translinker (Ultraviolet Products, Upland, CA), or treated with 2.5 mM spermine NONOate at 37 °C for 6h. Expression of the recA::lacZY transcriptional fusion was quantified spectrophotometrically as β-galactosidase enzymatic activity using the substrate o-nitrophenyl-β-D-galactopyranoside. β-galactosidase activity is expressed as Miller units using the equation $1000 \times [(OD_{420nm} - 1.75 \times OD_{550nm})/(T_{(min)} \times V_{(ml)} \times OD_{600nm})]$. The protein concentration in the cytoplasmic extracts was measured by the BCA protein assay (Pierce, Rockford, IL). Aconitase activity is expressed as the mean OD₂₄₀nm/min/mg protein ± SD of 2 independent experiments.

**N₂O₃ quantification.** The generation of N₂O₃ in Salmonella strains exposed to 250 μM spermine NONOate was determined indirectly by following the formation of the N-nitrosophenyl derivative of 2,3-diaminonaphthalene (Sigma-Aldrich) as described. A 100 mM stock of 2,3-diaminonaphthalene prepared in dimethylformamide was used at a final concentration of 200 μM in PBS. Accumulation of N-nitrosophenol was recorded for 30 min following treatment of Salmonella strains with spermine NONOate. Fluorescence was measured on a Synergy HT fluorometer (BioTek) set at λ_exc = 375 nm and λ_em = 460 nm.

**Detection of nitrotyrosine formation by Western blot analysis.** Salmonella strains grown in Luria-Bertani (LB) broth with shaking at 325 r.p.m. at 37 °C for 20 h were pelleted by centrifugation, and resuspended in PBS to an OD₆₀₀nm of 0.5. Bacteria were incubated at 37 °C in the presence or absence of 250 μM spermine...
| Strains | Description | Reference |
|---------|-------------|-----------|
| Salmonella enterica serovar Typhimurium strain 14028 s | Wild-type | ATCC |
| TT22236 | LT2 Salmonella carrying pTP2223 | (Price-Carter et al., 2001) |
| AV0436 | Δndh::FRT Δnuo::km | (Husain et al., 2008) |
| AV0438 | Δndh::FRT Δnuo::FRT | This study |
| AV0474 | ΔphoP::FRT | (Bourret et al., 2008) |
| AV0462 | ΔphoQ::km | This study |
| AV0475 | ΔphoQ::FRT | (Bourret et al., 2008) |
| AV1560 | ΔphoQ::FRT pPhoQ | (Bourret et al., 2008) |
| AV6108 | ΔrpoS::km | This study |
| TJB0601 | ΔphoQ::FRT ΔrpoS::km | This study |
| AV07174 | ΔcorA::km | This study |
| AV07175 | ΔphoQ::FRT ΔcorA::km | This study |
| AV07234 | recA-ter::FRT pCP20 | This study |
| AV07235 | recA::lacZY | This study |
| AV07250 | ΔphoQ::FRT recA::lacZY | This study |
| AV0810 | Δndh::FRT Δnuo::FRT ΔphoQ::km | This study |
| MF1085 | sodCl::Tn10 | (De Groote et al., 1997) |
| TJB0602 | ΔphoQ::FRT sodCl::Tn10 | This study |
| AV13037 | ΔmgtA::km | This study |
| AV13038 | ΔmgtBC::km | This study |
| AV13039 | ΔmgtBC::FRT | This study |
| AV13040 | ΔmgtBC::FRT ΔmgtA::km | This study |
| TJB1301 | ΔphoQ::FRT pMgtA | This study |

### Table 1. Bacterial Strains and Plasmids.

| Name | Sequence |
|------|----------|
| pTP2223 | Plac lam bet exo tet<sup>b</sup> | (Poteete and Fenton, 1984) |
| pCP20 | bla cat c857 P<sub>e</sub>fp pSC101 oriTS | (Cheerepanov and Wackernagel, 1995) |
| pKD13 | bla FRT asp FRT PS1 PS4 oriR6K | (Datsenko and Wanner, 2000) |
| pCE36 | asp FRT lacZY<sup>+</sup> t<sub>at</sub> oriR6K | (Ellermeier et al., 2002) |
| pWSK29 | bla lacZ ori<sup>b</sup>SC101 | (Wang et al., 1991) |
| pWSK29-phoQ (pPhoQ) | bla lacZ ori<sup>b</sup>SC101 | (Bourret et al., 2008) |
| pBAD/HisA | bla ori<sup>b</sup>BR322 araC | Thermo Fisher |
| pBAD/HisA:mgtA (pMgtA) | bla ori<sup>b</sup>BR322 araC | This study |

### Table 2. Primers.

| Name       | Sequence                  |
|------------|---------------------------|
| rapA-pKD13-F | F<sup>5</sup>-CATGATTCTTTAAATGAAAGACCGCGGAATTTGATGAGAACGGAGCTGGAGCTGCTTCGAAGTT |
| rapA-pKD13-R | R<sup>5</sup>-GCCCTTCACCGGAACTCGAGACGGTGCAGCGGTCAAGCTGCTTCGAAGTT |
| corA-pKD13-F | F<sup>5</sup>-TGAACTGTCCGATATTTTTACGCATTGGGAGTCCCGGTCAGCTGGAGCTGCTTCGAAGTT |
| corA-pKD13-R | R<sup>5</sup>-TGAACTGTCCGATATTTTTACGCATTGGGAGTCCCGGTCAGCTGGAGCTGCTTCGAAGTT |
| mgtA-pKD13-F | F<sup>5</sup>-TGAGGCGCGAGGCACACCGAAGATGGTTTATTACTGTCATGTCAGACTGGACTGCTTCGAAGTT |
| mgtA-pKD13-R | R<sup>5</sup>-TGAGGCGCGAGGCACACCGAAGATGGTTTATTACTGTCATGTCAGACTGGACTGCTTCGAAGTT |
| recA-pKD13-F | F<sup>5</sup>-TGAGGCGCGAGGCACACCGAAGATGGTTTATTACTGTCATGTCAGACTGGACTGCTTCGAAGTT |
| recA-pKD13-R | R<sup>5</sup>-TGAGGCGCGAGGCACACCGAAGATGGTTTATTACTGTCATGTCAGACTGGACTGCTTCGAAGTT |
| mgtA-XhoI-F | F<sup>5</sup>-AGCAGGTACTCGAGGATGCTCGGCTTTCGTACCCGAGAAGATGGTTTATTACTGTCATGTCAGACTGGACTGCTTCGAAGTT |
| mgtA-EcoRI-R | R<sup>5</sup>-AGCAGGTACTCGAGGATGCTCGGCTTTCGTACCCGAGAAGATGGTTTATTACTGTCATGTCAGACTGGACTGCTTCGAAGTT |
NonOate. At the specified timepoints, the bacteria were pelleted by centrifugation and resuspended in 200 μL of alkaline lysis buffer (25 mM Tris, 100 mM SDS, and 128 mM NaOH). The specimens were separated in 10% SDS–PAGE gels, transferred to nitrocellulose membranes, and probed with an anti-nitrotyrosine polyclonal antibody (Upstate, Lake Placid, NY) followed by a horseradish peroxidase-conjugated, anti-rabbit IgG secondary antibody. Detection was carried out using the Enhanced Chemiluminescence Kit (GE Healthcare, Piscataway, NJ) on a Molecular Imager Fx (BioRad, Hercules, CA).

**Statistical analysis.** Data are presented as mean ± standard deviation (SD). To determine statistical significance between multiple comparisons, two-way analysis of variance (ANOVA) were performed, followed by a Bonferroni posttest. Data were considered statistically significant when P was < 0.05.

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

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