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## Manganese import protects *Salmonella enterica* serovar Typhimurium against nitrosative stress

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### Abstract

Nitric oxide (NO<sup>•</sup>) is a radical molecule produced by mammalian phagocytic cells as part of the innate immune response to bacterial pathogens. It exerts its antimicrobial activity in part by impairing the function of metalloproteins, particularly those containing iron and zinc cofactors. The pathogenic Gram-negative bacterium *Salmonella enterica* serovar Typhimurium undergoes dynamic changes in its cellular content of the four most common metal cofactors following exposure to NO<sup>•</sup> stress. Zinc, iron and magnesium all decrease in response to NO<sup>•</sup> while cellular manganese increases significantly. Manganese acquisition is driven primarily by increased expression of the *mntH* and *sitABCD* transporters following derepression of MntR and Fur. ZupT also contributes to manganese acquisition in response to nitrosative stress. *S. Typhimurium* mutants lacking manganese importers are more sensitive to NO<sup>•</sup>, indicating that manganese is important for resistance to nitrosative stress.

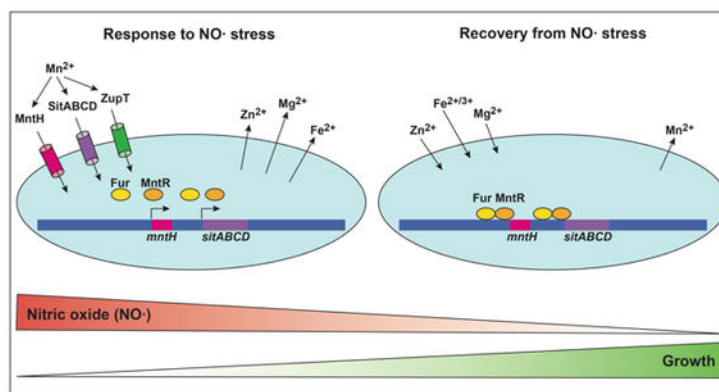
### Graphical Abstract

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Conflict of interest statement

The authors declare no conflicts of interest.



In response to nitric oxide, *Salmonella* Typhimurium acquires manganese and exports zinc, magnesium and iron. Uptake is driven by expression of transporters MntH and SitABCD as well as by ZupT.

## Introduction

Phagocytic cells of the mammalian innate immune system produce both superoxide and nitric oxide (NO $\cdot$ ) as a defense against bacterial pathogens. Mice lacking the inducible nitric oxide synthase (iNOS) are significantly more susceptible to infection than those capable of producing NO $\cdot$ <sup>1</sup>. The bacterial targets of NO $\cdot$  are less well understood than those of reactive oxygen species (ROS), therefore the mechanisms of NO $\cdot$ -mediated bacteriostasis and bacterial resistance to nitrosative stress are topics of significant biological interest.

Primary cellular targets of reactive nitrogen species (RNS) include metal centers and cysteine residues. NO $\cdot$  covalently modifies protein thiols which can disrupt redox-based signaling, inhibit enzymatic reactions, and mobilize metals, such as zinc, that rely on cysteine ligands<sup>2-5</sup>. NO $\cdot$  also interacts with iron to form dinitrosyl-iron complexes (DNICs), which can impair the activity of a variety of regulatory proteins and enzymes<sup>6-11</sup>.

A microarray to identify NO $\cdot$ -responsive genes in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) identified manganese transporters *mntH* and *sitABCD* among the most highly up-regulated genes, suggesting that manganese may play a role in cellular resistance to nitrosative stress<sup>5</sup>. Manganese exists primarily as the divalent cation Mn<sup>2+</sup> in biological systems and is the fourth most common metal cofactor after magnesium, zinc and iron<sup>12</sup>. MntH is a manganese-proton symporter in the natural resistance-associated macrophage protein (NRAMP) family<sup>13,14</sup>. While MntH function is not required under standard laboratory growth conditions in rich medium, expression is induced when cells are limited for manganese or iron as well as by oxidative stress<sup>14-17</sup>. SitABCD is an ATP-binding cassette (ABC) manganese importer that is expressed under conditions similar to those regulating MntH<sup>18,19</sup>. Manganese transport by MntH- and SitABCD-type systems promotes

survival and virulence of pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus* and *S. Typhimurium* in the face of nutritional immunity and oxidative stress<sup>13,20–25</sup>.

The requirement for manganese in response to oxidative stress is well characterized, but a role in resistance to nitrosative stress has not previously been shown in bacteria. Under conditions of oxidative stress, manganese activates the manganese-dependent superoxide dismutase SodA and catalase KatN<sup>26–28</sup>. Manganese has also been shown to prevent mismetallation by zinc and to preserve the activity of some mononuclear iron enzymes upon loss of the native iron ligand<sup>29–32</sup>. Given the potential for NO<sup>•</sup> to disrupt cellular metal homeostasis, manganese might play similar roles in preserving enzyme function and preventing zinc mismetallation under conditions of nitrosative stress. Here we show that NO<sup>•</sup> disrupts homeostasis of iron, magnesium and manganese in addition to zinc<sup>2</sup>. Manganese acquisition by MntH, SitABCD and a third transporter, ZupT, is required for the nitrosative stress response in *S. Typhimurium*.

## Materials and methods

### Growth conditions

Unless otherwise specified, *Salmonella enterica* serovar Typhimurium (ATCC 14028s) and *Escherichia coli* (TB1) were grown in Luria-Bertani (LB, Fisher) medium at 37°C with shaking at 250 rpm. Antibiotic selection was used for strain construction only at the following concentrations: 100 µg ml<sup>-1</sup> ampicillin (Amp), 50 µg ml<sup>-1</sup> kanamycin (Kan), and 20 µg ml<sup>-1</sup> chloramphenicol (Cm). The minimal medium employed in these studies was M9: 1x M9 salts (Difco), 0.1 mM CaCl<sub>2</sub>, 2 mM MgSO<sub>4</sub>, 0.4% glucose.

### Strain and plasmid construction

All strains and plasmids are listed in Supplementary Table 1, and all primers are listed in Supplementary Table 2. *S. Typhimurium* ATCC 14028s (JK237) was used as the wild-type strain and the genetic background for all *S. Typhimurium* mutant strains. Deletion mutant strains were constructed using the λ-RED mediated recombination method and, where applicable, pCP20 was used to remove antibiotic resistance cassettes by Flp-FRT recombination<sup>33</sup>. The *mntH1::kan* and *sit100::MudCm* mutations were published previously<sup>13,34</sup>. P22HT105/int bacteriophage was used for mutant strain construction by phage transduction<sup>35</sup>. All mutant strains were verified by PCR.

*E. coli* (TB1) was used as the host strain for all cloning. Plasmids pEF97 and pEF98 were generated by amplifying the upstream promoter region plus coding sequence for *mntH* using primers EFP307 and EFP308 and *sitABCD* using primers EFP309 and EFP310.

Amplification products were digested with BamHI and HindIII for *mntH* and KpnI and HindIII for *sitABCD*. Digested fragments were ligated into pRB3–273C digested with the same enzyme pair in reverse orientation to the multiple cloning site<sup>36</sup>. Constructs were confirmed by sequencing using JKP227 and JKP244. Additional internal primers EFP321 and EFP322 were used to fully sequence the insert for pEF98. Plasmids pEF97, pEF98 and pJK671 were electroporated into JK831 to create strains EF674, EF675 and EF676<sup>37</sup>.

### Gene expression analysis

Primer sequences for assessing transcript levels of *mntH*, *sitA* and *zupT* are listed in Supplementary Table 2. *S. Typhimurium* was grown overnight in LB, diluted 1:1000 into 60 mL fresh LB and grown to an optical density at 600 nm ( $OD_{600}$ ) ~1. The culture was then subdivided into 5 duplicate pairs of 5 mL cultures in 18×150 mm glass tubes. One set was treated with 2 mM diethylamine NONOate (DEANO) and one set left untreated. Cultures were returned to shaking at 37°C. At various times post-treatment (0, 5, 15, 30, 45, 60 min), 1.5 mL of treated and untreated culture were pelleted by centrifugation and resuspended in 800  $\mu$ L Trizol for RNA isolation. RNA and cDNA were prepared as described previously<sup>38</sup>. Quantitative PCR (qPCR) was carried out using SYBR Green on a BioRad CFX96 real-time system with *rpoD* as the internal control. The *rpoD* primer sequences have been published previously<sup>39</sup>. Statistical significance was determined by one-sample t-test compared to a hypothetical mean of 1 using GraphPad Prism.

### Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

All ICP-MS analyses were conducted using strain variants that were also flagellar mutants (JK377, EF622, EF623, EF624, EF650, EF651, EF653) to increase pelleting efficiency. *S. Typhimurium* overnight cultures were subcultured 1:1000 into 100 mL fresh LB and grown to  $OD_{600}$  ~1. Prior to treatment, the culture was subdivided into 10 individual 5 mL aliquots in 18×150 mm glass tubes. Five aliquots were treated with 2 mM DEANO and 5 remained untreated. Cultures were returned to shaking at 37°C. At 0, 5, 15, 30, 45 and 60 min post-treatment, 4.5 mL of treated and untreated culture were pelleted by centrifugation then washed twice with ultrapure water. Pellets were resuspended in analytical grade nitric acid, boiled, then diluted 1:10 with ultrapure water for final analysis. Samples were analyzed on an Agilent 8900x QQQ ICP-MS. CFU were also enumerated at each time point so that total metal concentrations for each sample could be normalized according to cell number. Statistical significance was determined by one sample t-test compared to a hypothetical mean of 100 in GraphPad Prism.

### Nitric oxide sensitivity assays and CFU enumeration

Wild-type *S. Typhimurium* (JK237) and mutant strains lacking manganese efflux pumps (JK601, JK603, JK605, JK715, JK719, JK720 and JK831) were grown overnight in LB medium then normalized to  $OD_{600}$  1 and diluted 1:1000 into a final volume of 300  $\mu$ L in a flat-bottom 96-well non-treated tissue culture microtiter plate (Midwest Scientific). Cultures were treated with final concentrations of 5 mM SperNO (LB), 1 mM SperNO (M9) or 1.5 mM SperNO (M9). Cultures were grown aerobically with shaking at 567 cpm (3 mm) in a Biotek Synergy HTX multi-mode 96-well plate reader at 37°C. Growth was monitored by recording  $OD_{600}$  every 15 min for 40 hr. Statistical significance was determined by comparing the time required to reach 50% maximum  $OD_{600}$  by unpaired two-tailed t-test. Complementation of the JK831 growth defect was assessed as described above using JK895 (wild-type *S. Typhimurium* containing empty plasmid) and strains EF674, EF675 and EF676. Manganese supplementation was carried out as above in M9 medium supplemented with 0.1  $\mu$ M  $MnSO_4$ . For enumeration of CFU during lag phase, overnight cultures were diluted 1:1000 in 3 mL fresh LB with 5 mM SperNO in a 18×150 mm glass culture tube and

grown at 37°C with shaking at 250 rpm. Samples (20 µL) were taken at the time of inoculation and every hour thereafter until growth resumed, diluted in phosphate-buffered saline (PBS) and plated onto LB agar in triplicate. Plates were grown overnight at 37°C before counting. Statistical significance was determined by unpaired two-tailed t-test.

## Results

### Nitrosative stress alters cellular metal abundances.

To obtain a comprehensive understanding of how metal flux changes in response to nitrosative stress, *S. Typhimurium* cultures were grown in LB, treated with 2 mM diethylamine NONOate (DEANO) and monitored using inductively coupled plasma mass spectrometry (ICP-MS). DEANO is a fast-release NO<sup>•</sup> donor that allows the response to, and subsequent recovery from, nitrosative stress to be observed over the course of 60 min<sup>2</sup>. Between 5 and 15 min post-treatment, cellular iron and magnesium levels decreased in response to NO<sup>•</sup> before recovering to near-baseline levels by 60 min post-treatment (Figure 1 A&B). These patterns are similar to what had previously been observed for zinc (Figure 1C)<sup>2</sup>. Levels of calcium, a metal that binds carboxylate ligands like magnesium though with a greater variety of bond angles and a smaller hydration radius, did not vary significantly in response to NO<sup>•</sup> (Supplementary Figure 1). In contrast to the response for other metals, manganese levels rose following treatment with NO<sup>•</sup>, peaked at 30 min, then decreased back to near untreated levels by 60 min (Figure 1D).

### The expression of manganese transporters *mntH* and *sitABCD* is upregulated in response to nitrosative stress via MntR and Fur derepression.

Previous microarray data showed that *mntH* and *sitABCD* are expressed in response to nitrosative stress, but the regulatory mechanism was not determined<sup>5</sup>. The rise and subsequent fall of cellular manganese observed by ICP-MS suggested that transporter expression is also likely to exhibit a dynamic response to NO<sup>•</sup> stress. *S. Typhimurium* cultures were treated with 2 mM DEANO, sampled for RNA isolation over the course of 60 min, and then transporter gene expression was determined by qPCR. Increased expression of *mntH* (pink) and *sitA* (purple) was observed within 5 min of 2 mM DEANO treatment (Figure 2A). Expression peaked at 15 min post-treatment before exhibiting dramatic repression by 30 min post-treatment. This repression corresponds with the peak in manganese level (Figure 1C). The 15–30 min post-treatment interval is the same period during which *S. Typhimurium* has previously been shown to have detoxified the majority of the NO<sup>•</sup> released from DEANO<sup>2</sup>.

Two different regulators have been implicated in control of *mntH* and *sitABCD* expression: the iron-binding repressor Fur and the manganese-binding repressor MntR<sup>18,15,16</sup>. OxyR, an activator that responds to oxidative stress, has also been shown to regulate *mntH* expression<sup>15</sup>. The *sitABCD* promoter of *S. Typhimurium* does not contain an OxyR binding site and thus does not respond to OxyR regulation<sup>18</sup>. A deletion mutant was constructed for each regulator to determine its contribution to manganese transporter expression following nitrosative stress. Deletion of the *mntR* and *fur* repressors leads to derepression of *mntH* and *sitA* in the absence of NO<sup>•</sup> (Supplementary Figure 2). To examine expression specifically

resulting from NO<sup>-</sup>-mediated regulation, rather than repressor deletion, data are shown as fold-change compared to expression in untreated cells of the same genetic background, thus normalizing for basal derepression. Expression in the *mntR* strain (Figure 2B) followed a similar pattern to that in the wild-type strain (Figure 2A) but, the magnitude of NO<sup>-</sup>-mediated induction was lower for both *mntH* and *sitA*. Repression between 30 and 60 min was not as dramatic in the *mntR* strain compared to wild-type. In the *fur* strain (Figure 2C), a modest increase in expression (~2–4 fold) was observed 5 min post-treatment but by 15 min expression returned to the same level as in the untreated culture, suggesting that repression by MntR was relieved in response to NO<sup>-</sup> but then quickly restored. No additional activation of *mntH* expression was observed in response to NO<sup>-</sup> in the *mntR fur* strain (Figure 2D), demonstrating that OxyR does not regulate *mntH* expression in response to NO<sup>-</sup>.

### ZupT contributes to dynamic manganese transport following NO<sup>-</sup> stress.

Since *mntH* and *sitA* exhibited a transcriptional response to NO<sup>-</sup>, mutants specifically lacking each of these transport systems were investigated for their ability to acquire manganese in response to nitrosative stress. Neither an *mntH* mutant (Figure 3A, pink) nor a *sitA* mutant (Figure 3B, violet) varied significantly from wild-type (blue) with regard to manganese acquisition in response to NO<sup>-</sup>. A double *mntH sitA* mutant (Figure 3C, red) appeared to acquire less manganese than the wild-type strain, but the difference failed to achieve statistical significance. This result suggested that an additional transporter must contribute to the observed phenotype. The sequence encoding *zupT*, a transporter with broad cation specificity that had previously been implicated in manganese transport as well as zinc transport, was therefore deleted in combination with *mntH* and *sitA*<sup>37,40</sup>. Both an *mntH zupT* mutant (Figure 3D, green) and a *sitA zupT* mutant (Figure 3E, purple) were delayed for manganese acquisition compared to wild-type, reaching peak levels 45 min post-treatment, but the maximum amounts of manganese acquired by these mutants were not significantly different from wild-type. Only an *mntH sitA zupT* mutant (Figure 3F, orange) completely failed to acquire manganese in response to NO<sup>-</sup>, thus confirming a role for NO<sup>-</sup>-responsive acquisition of manganese via ZupT. Actual values are shown in Supplementary Table 3. Expression of *zupT* does not change in response to NO<sup>-</sup> nor is it expressed at greater levels in an *mntH sitA* mutant, thus regulation of manganese acquisition in this genetic background must occur via a different mechanism (Supplementary Figure 3).

### Manganese transport mutants are more sensitive to nitrosative stress.

To determine whether manganese is required for the nitrosative stress response, *S. Typhimurium* mutants were grown in the presence of the sustained-release NO<sup>-</sup> donor spermine NONOate (SperNO). The *mntH* and *sitA* mutants that achieved wild-type levels of manganese acquisition were not impaired for growth in response to NO<sup>-</sup>, nor was a *zupT* mutant (Supplementary Figure 4). In LB medium, which contains manganese<sup>41</sup>, only the *mntH sitA zupT* mutant that was impaired for additional manganese acquisition displayed a delayed exit from lag phase (Figure 4A, orange dashed line) compared to wild-type (blue dashed line). Plasmid-based expression of any single transporter (MntH, SitABCD or ZupT) from its native promoter was sufficient to complement the recovery defect (Supplementary Figure 5). In minimal medium (M9) that does not include transition metals, both the *mntH*

*sitA zupT* strain (Figure 4B, orange dashed line) and the *mntH sitA* strain (Figure 4B, red dashed line) displayed increased sensitivity to 1 mM SperNO compared to wild-type (Figure 4B, blue dashed line). When the concentration of SperNO was increased to 1.5 mM (Figure 4C), all four mutants displayed increased sensitivity compared to wild-type (blue dashed line). The *mntH zupT* strain (green dashed line) displayed the smallest stress phenotype followed by the *sitA zupT* strain (purple dashed line) and the *mntH sitA* strain (red dashed line). Supplementation with 0.1  $\mu$ M MnSO<sub>4</sub> was sufficient to complement the recovery defects of the *mntH sitA*, *sitA zupT* and *mntH zupT* strains but not that of the *mntH sitA zupT* triple mutant (Supplementary Figure 6).

### **Manganese transport mutants recover more slowly from nitrosative stress but do not experience NO-mediated death or undergo selection for resistance.**

To understand the reason for the difference in lag-phase exit time of the *mntH sitA zupT* mutant, cells were grown in LB with 5 mM SperNO then samples were obtained for CFU enumeration and compared to initial CFU (Figure 5A). During the prolonged lag phase, the number of viable CFU remained consistent between wild-type (blue bars) and *mntH sitA zupT* (orange bars) demonstrating that inability to acquire manganese does not result in death of the transport mutants. To determine whether outgrowth is due to selection for mutants that are less NO<sup>•</sup>-sensitive, cells were treated with NO<sup>•</sup> in a standard LB assay for 40 hrs, subcultured, and subjected to subsequent challenge. The subcultured cells were no more resistant than *S. Typhimurium* strains that had not previously experienced NO<sup>•</sup> stress when grown in either rich (Figure 5B) or minimal (Figure 5C) medium. Taken together, these data show that manganese enhances the rate of recovery rather than promoting cell survival. Outgrowth represents recovery of the starting population, not growth of a small population containing secondary mutations that confer enhanced resistance.

## **Discussion**

Nitric oxide is a key chemical defense of the mammalian innate immune response, but its cellular targets and mechanisms of antimicrobial activity are incompletely characterized. NO<sup>•</sup> has previously been shown to mobilize zinc from metalloproteins by nitrosylating cysteine residues and zinc efflux was shown to be an important part of the response to nitrosative stress in *S. Typhimurium*<sup>2,4</sup>. The effect of NO<sup>•</sup> on homeostasis of other biologically relevant metals, however, was not determined. Here we show that total cellular iron and magnesium levels also decrease in response to nitrosative stress, while manganese levels increase (Figure 1). Changes in metal availability and relative abundance have the potential to alter the function of proteins that depend on a specific metal cofactor. To promote correct metallation *in vivo*, bacterial cells must hold metals with more favorable free energies to lower concentrations<sup>42</sup>. When free metal concentrations increase intracellularly, efflux is necessary to avoid metal intoxication and iron-catalyzed Fenton chemistry<sup>2,30,43,44</sup>.

The role that efflux of magnesium and iron plays in resistance to nitrosative stress has not previously been characterized and the transporters responsible for efflux of these metals are not known. Prior work has shown that magnesium supplementation is required to protect

*phoQ*, *mgtA* and *mgtBC* *S. Typhimurium* that are deficient for magnesium import from cell death caused specifically by nitroxidative species<sup>53</sup>. Metal flux in response to NO<sup>•</sup> has not been determined in these mutant backgrounds and may differ from that of wild-type observed here. It is also possible that since magnesium levels initially decrease in response to NO<sup>•</sup> but then recover to pre-stress levels, both efflux and acquisition are required during different phases of the stress response. The iron-citrate efflux transporter IceT is induced in response to NO<sup>•</sup>, and a mutant was found to be more sensitive to nitrosative stress<sup>44</sup>. IceT is therefore one possible mediator of the observed iron efflux that can be investigated further but may not be the only iron transport system involved. In general, the biological conditions and transport proteins that promote efflux of iron and magnesium are poorly understood.

Nitric oxide is known to disrupt iron binding but direct interaction of NO<sup>•</sup> with the carboxylate ligands preferred by magnesium or with magnesium ions has not been demonstrated. Nitration of tyrosine, which is found in some magnesium binding sites, can occur under aerobic conditions<sup>45,46</sup>. Metalloproteins, however, are not the only source of intracellular magnesium. A significant percentage of cellular magnesium associates with ribosomal RNAs to reinforce ribosome structure, and cellular magnesium levels are correlated with ribosome number<sup>47,48</sup>. Ribosomal proteins were found to be targets of *S*-nitrosylation in *S. Typhimurium*, which may disrupt ribosome structure<sup>2</sup>. Nucleoside triphosphates within cells exist primarily bound to magnesium and chemical disruption of ATP production results in magnesium efflux from some eukaryotic cell types<sup>49</sup>. Since nitrosative stress has been shown to reduce nucleoside triphosphate levels in *S. Typhimurium*, a similar mechanism may drive the magnesium efflux observed here<sup>50</sup>. Under standard growth conditions, *S. Typhimurium* has been reported to maintain free magnesium at a concentration of approximately 1 mM<sup>51,52</sup>. Our data show that cellular iron and magnesium levels decrease in response to nitrosative stress but determining the intracellular sources of these displaced ions and whether their efflux is required for resistance to nitrosative stress is beyond the scope of this work.

While the need to activate MnSOD in response to oxidative stress is well characterized, a demand for manganese under conditions of nitrosative stress has not been explored<sup>17,22,27</sup>. Manganese is acquired in response to NO<sup>•</sup>, driven primarily by increased expression of SitABCD and MntH, which are typically repressed by both Fur and MntR (Supplementary Figure 2, Figure 2). NO<sup>•</sup> has previously been shown to relieve repression by Fur through nitrosylation of iron, resulting in a structure that no longer binds DNA efficiently<sup>8</sup>. The mechanism by which NO<sup>•</sup> is also able to relieve repression by MntR, a manganese-binding regulator lacking cysteines, is not currently known. A third transporter, ZupT, is required for acquisition of manganese in the absence of MntH and SitABCD, despite not exhibiting a change in expression (Supplementary Figure 3). Work in *E. coli* has suggested that expression of *zupT* is constitutive<sup>40,54</sup>. A non-transcriptional mechanism of regulating manganese transport by ZupT or concurrent regulation of one or more manganese efflux transporters, such as a homologue of MntP, may therefore contribute to the *mntH sitA* phenotype<sup>55–57</sup>.

Manganese is involved in the adaptive response of *S. Typhimurium* to nitrosative stress, as evidenced by the prolonged lag phases of transporter mutants impaired for manganese

acquisition (Figure 4). In rich medium, only an *mntH sitA zupT* mutant displays increased sensitivity to NO<sup>•</sup>, but in minimal medium a clear hierarchy of phenotypes is evident. This suggests that, at least *in vitro*, SitABCD, which also exhibits the greatest expression increase, is the primary Mn transporter, with MntH playing a secondary role. ZupT appears to be the least effective transporter of the three under manganese-limited conditions, but offers some protection against the effects of nitrosative stress.

Manganese acquisition is required to speed recovery from nitrosative stress but has no impact on survival (Figure 5A) or response to subsequent NO<sup>•</sup> challenge (Figures 5B&5C). While elucidating the exact mechanisms by which manganese protects cells from NO<sup>•</sup> will require further investigation, there are several possibilities. Zinc is of particular concern during nitrosative stress, since zinc is mobilized by NO<sup>•</sup> and is highly stable as a metal cofactor under non-stress conditions<sup>2,58,59</sup>. Manganese may be required to buffer against increased free zinc and protect against mismetallation. Manganese is capable of substituting structurally and enzymatically for magnesium and iron, which decrease in response to nitrosative stress. Unlike iron, manganese does not catalyze Fenton chemistry *in vivo* and could help to maintain limited function of certain iron-dependent enzymes if they are targets of NO<sup>•</sup> or are indirectly affected by iron efflux<sup>30,60–62</sup>. Exchanging magnesium cofactors for manganese has the potential to temporarily restructure carbon flux, directing resources away from precursor pools and toward energy-generating pathways<sup>62</sup>. It is not possible to conclude from the presented data whether the observed shift in magnesium to manganese ratio would be sufficient to drive cofactor exchange in *S. Typhimurium*. However, data from other bacterial species show that even brief disruptions of the cellular ratio of magnesium to manganese can shift the ion content and function of some proteins<sup>63,64</sup>. Glycolysis has been shown to be important for *S. Typhimurium* pathogenesis, and in *Staphylococcus aureus*, nitrosative stress drives the pathogen toward glucose consumption<sup>65,66</sup>. Glycolysis is known to be disrupted by manganese limitation, and thus acquisition in response to nitrosative stress may be required to mediate this shift<sup>67</sup>. Finally, ribonucleotide reductase activity is required for DNA synthesis. While eukaryotes only have one form of this enzyme, equivalent to the iron-binding NrdAB complex, *S. Typhimurium* has several forms<sup>68</sup>. Both the eukaryotic ribonucleotide reductase complex and NrdA of *S. Typhimurium* have been shown to be modified by nitrosative species<sup>2,69,70</sup>. The genes comprising the alternative manganese-binding version of the enzyme complex (*nrdEFHI*) are upregulated in *S. Typhimurium* in response to NO<sup>•</sup><sup>5</sup>. Thus, manganese acquisition may be required to sustain ribonucleotide reductase activity following nitrosative stress.

While we have shown that manganese acquisition is required for the response of *S. Typhimurium* to NO<sup>•</sup> *in vitro*, the role of manganese in the response to NO<sup>•</sup> during infection of a mammalian host is not presently known. Given the importance of manganese for both oxidative and nitrosative stress responses, and the temporal separation of these responses *in vivo*, it may prove challenging to distinguish the specific roles of manganese in response to the individual mediators<sup>71,72</sup>. In mice lacking a functional NADPH oxidase, *mntH sitA zupT S. Typhimurium* still exhibits a growth defect compared to wild-type and also displays a defect relative to a *sodA* mutant, suggesting that manganese acquisition is important *in vivo* for reasons in addition to oxidative stress resistance<sup>22</sup>. Determining the

role of manganese in resisting the effects of nitrosative stress during infection will be an important avenue of future investigation.

Oxidative stress resistance remains the primary function of manganese in bacterial systems, but here we have shown that manganese also plays an important role in resistance to nitrosative stress. The fluxes in manganese, magnesium, iron and zinc driven by NO<sup>•</sup> reveal a global remodeling of intracellular metal contents and suggest that a comprehensive understanding of the bacterial response to nitrosative stress will require the consideration of how metals change, not just as isolated species, but in relation to one another. Identifying the transporters and regulators involved as well as the protein targets affected by these metal shifts will reveal additional novel aspects of the antimicrobial effects of NO<sup>•</sup>.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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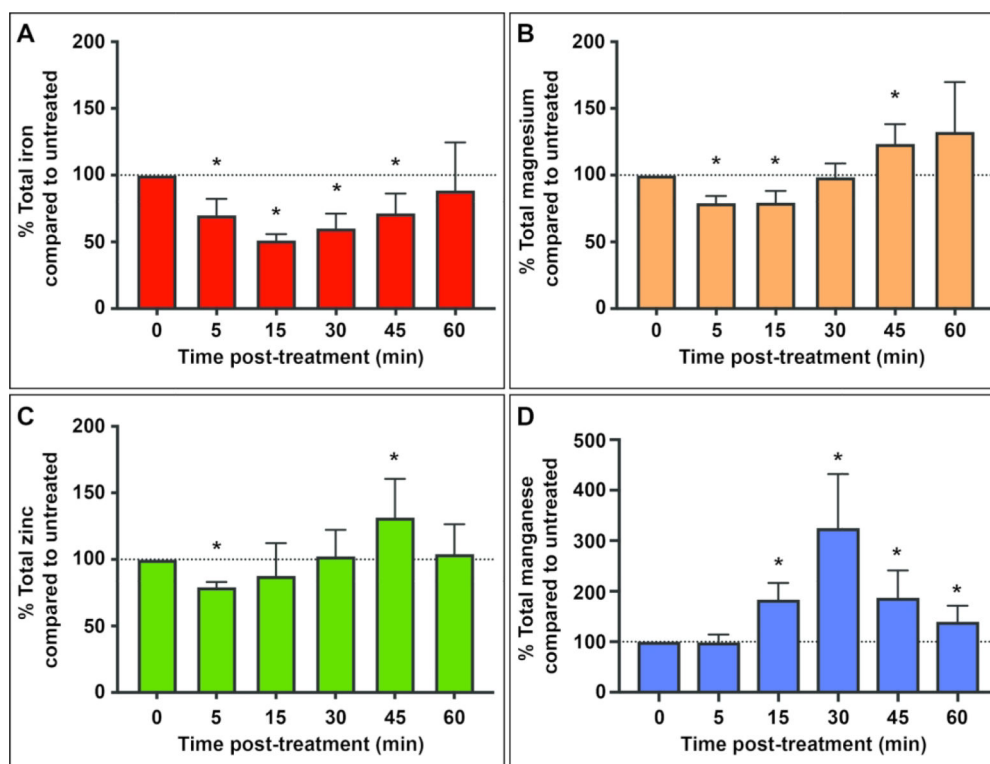
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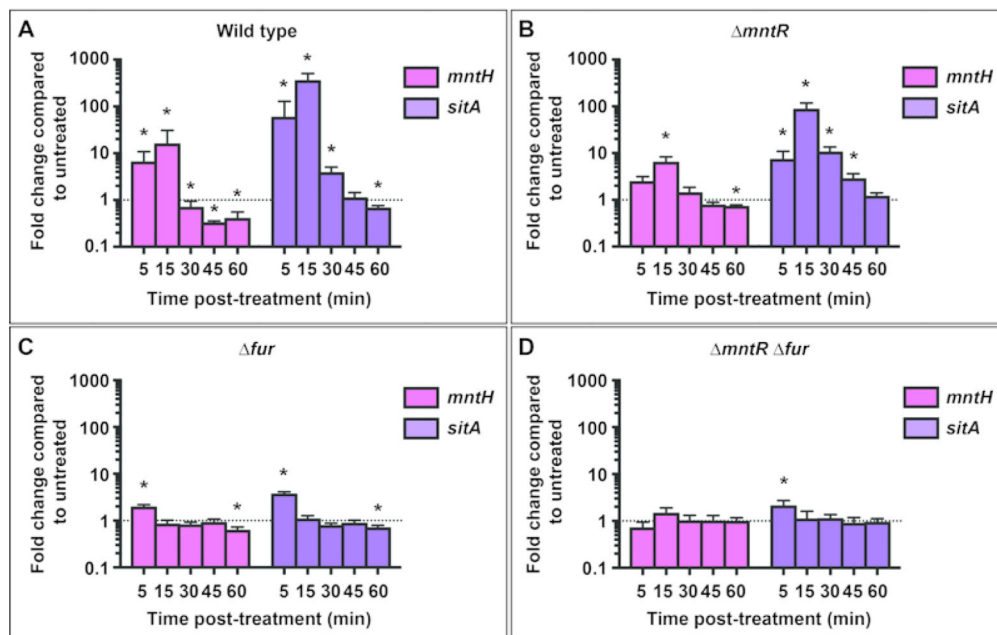
### Significance to Metallomics

Manganese is a cofactor for bacterial enzymes involved in metabolism and oxidative stress resistance. Mammalian immune cells also produce nitric oxide, which we show leads to efflux of iron, magnesium and zinc and acquisition of manganese by *Salmonella* Typhimurium. Manganese promotes recovery from nitrosative stress, possibly by preventing mismetallation by more toxic metals or activating alternative versions of enzymes. Since control of intracellular metal concentrations is required to ensure correct metal binding and protein function, our data suggest that future studies should consider global changes to metal homeostasis and examine expanded roles for manganese in bacterial pathogenesis.



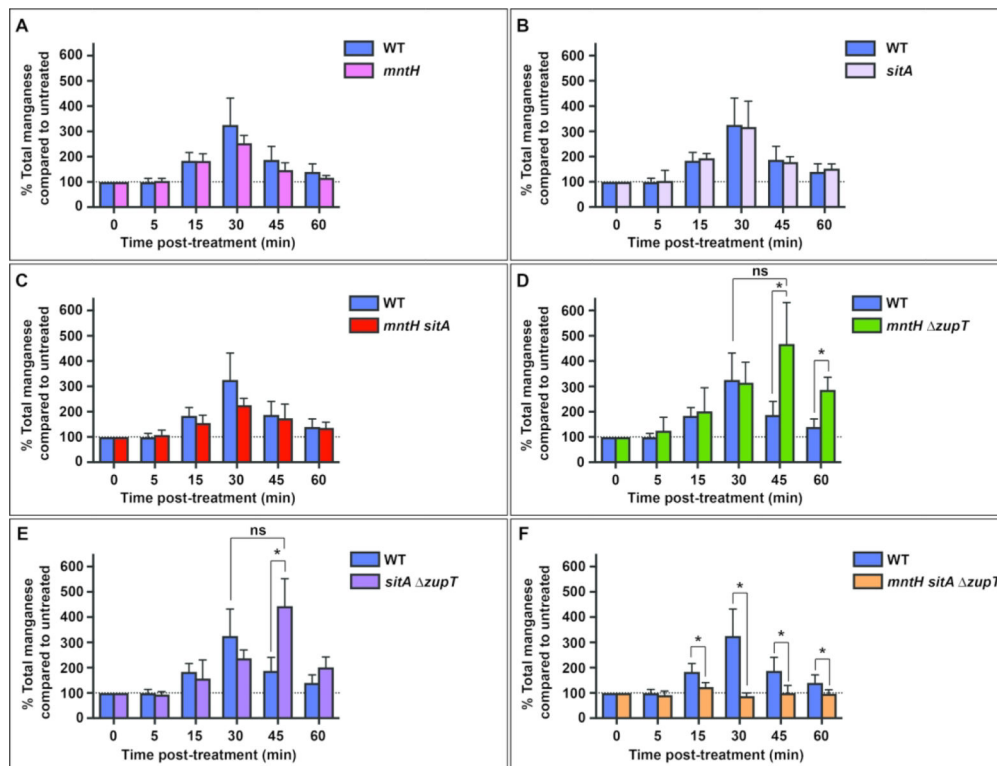
**Figure 1. Total cellular iron and magnesium decrease in response to NO<sup>•</sup> while intracellular manganese increases.**

Total cellular iron (A), magnesium (B), zinc (C) and manganese (D) contents were determined by inductively-coupled plasma mass spectrometry (ICP-MS) following addition of 2 mM diethylamine NONOate (DEANO) to OD<sub>600</sub> ≈ 1 *S. Typhimurium* cultures and compared to that of untreated cells. (A) Total cellular iron initially decreased beginning 5 min post-treatment before increasing again starting at 30 min. By 60 min post-treatment, iron levels were no longer significantly different from pre-treatment levels. (B) Magnesium levels also decreased at 5 and 15 min post-treatment before increasing and briefly exceeding pre-treatment levels at 45 min post-treatment. (C) Cellular zinc levels analyzed and published previously are reproduced here for comparison.<sup>2</sup> Zinc levels decreased at 5 min post-treatment then recovered to pre-treatment levels after briefly exceeding them at 45 min post-treatment. (D) Cellular manganese levels rose beginning at 15 min post-treatment, peaked at 30 min and then proceed to decrease and approach pre-treatment levels by 60 min. Statistical significance (\*) compared to pre-treatment metal level was determined by one sample t-test to a hypothetical mean of 100. For iron at 5, 15 and 30 min post-treatment,  $p < 0.001$ . At 45 min,  $p = 0.0021$ . For magnesium, at 5 min post-treatment,  $p < 0.001$ , at 15 min,  $p = 0.006$ , and at 45 min,  $p = 0.0213$ . For manganese at 15 min post-treatment,  $p < 0.001$ , at 30 min,  $p = 0.0014$ , at 45 min,  $p = 0.0047$  and at 60 min post-treatment,  $p = 0.0146$ . Error bars represent standard deviation.



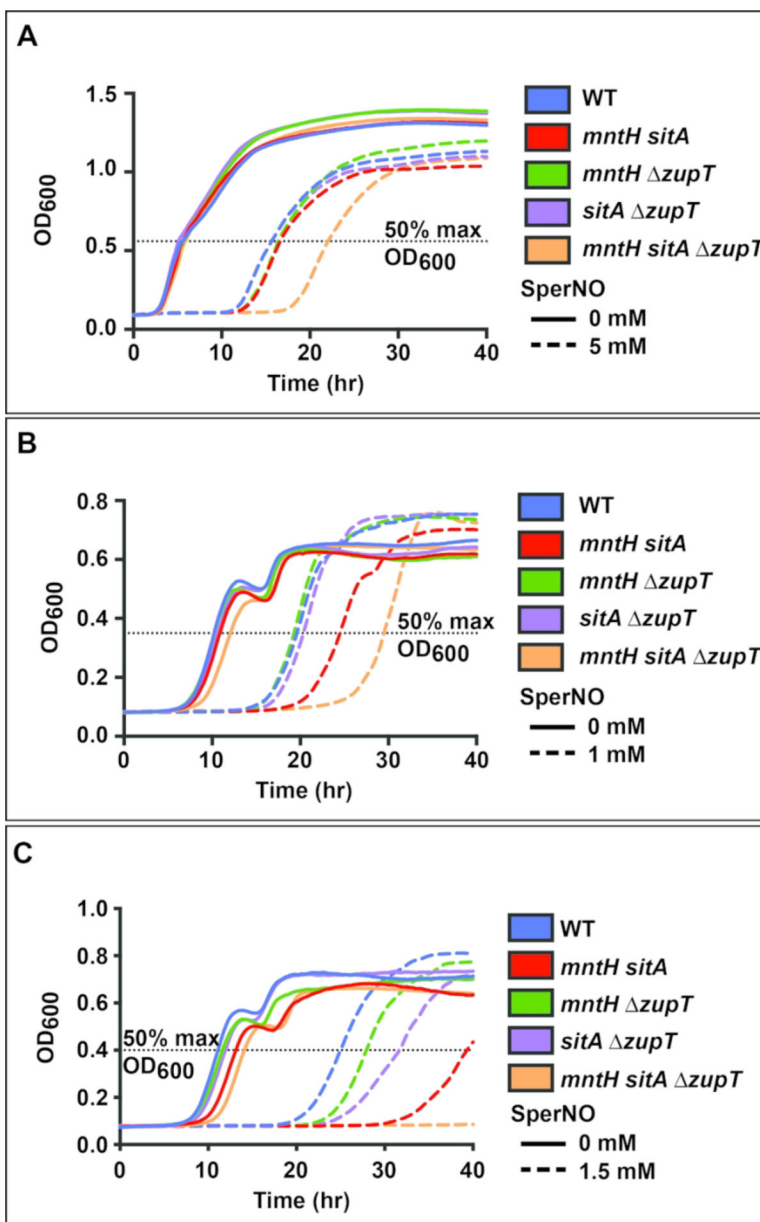
**Figure 2. Expression of *mntH* and *sitA* increases in response to NO $\cdot$  and is primarily regulated by Fur.**

Total RNA was isolated from untreated OD<sub>600</sub>  $\approx$  1 *S. Typhimurium* cultures and cultures treated with 2 mM DEANO. Expression of *mntH* and *sitA* was determined by qPCR. Data are presented as positive mean fold-change compared to untreated cells of the same genetic background. Error bars represent standard deviation. Statistical significance (\*) was determined by a one-sample t-test to a hypothetical mean of 1, indicating no difference in expression between treated and untreated cultures. (A) Expression of *mntH* (pink) and *sitA* (purple) increased at 5 and 15 min post-treatment before rapidly decreasing to levels below those of untreated cultures. For *mntH* expression at 5 min,  $p=0.0114$ , at 15 min,  $p=0.0463$ , at 30 min,  $p=0.0499$ , and at 45 and 60 min,  $p<0.001$ . For *sitA* expression at 5 min,  $p=0.0449$ , at 15 min,  $p<0.001$ , at 30 min,  $p=0.0041$  and at 60 min,  $p<0.001$ . (B) In the absence of *mntR*, the overall patterns of NO $\cdot$  induced expression for *mntH* and *sitA* were similar to wild-type though the magnitude of expression changes was smaller. Expression of *mntH* showed significant upregulation at 15 min post-treatment ( $p=0.0331$ ) and downregulation at 60 min ( $p=0.006$ ). Expression of *sitA* was significantly increased at 5 min ( $p=0.013$ ), 15 min ( $p=0.0342$ ), 30 min ( $p=0.0263$ ) and 45 min ( $p=0.0456$ ) post-treatment. (C) In the absence of *fur*, a modest increase in expression was observed in response to NO $\cdot$  5 min post-treatment for both *mntH* ( $p=0.012$ ) and *sitA* ( $p=0.004$ ) before returning to baseline levels. A small decrease in expression was observed for both genes 60 min post-treatment (*mntH*  $p=0.0221$ , *sitA*  $p=0.0236$ ). (D) In the absence of both *mntR* and *fur*, there was no further change in expression of *mntH* in response to NO $\cdot$ . Expression of *sitA* increased approximately 2-fold at 5 min post-treatment ( $p=0.0311$ ) but was no different than untreated cultures at all other times.



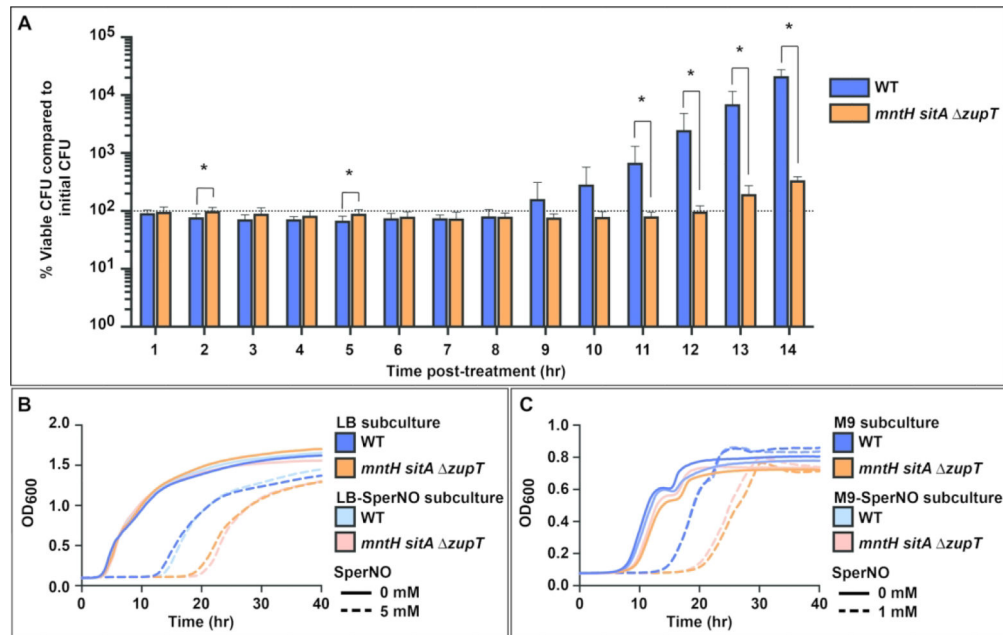
**Figure 3. Manganese acquisition by *S. Typhimurium* in response to NO<sup>•</sup> depends on ZupT as well as MntH and SitABC.**

*S. Typhimurium* cultures were treated with 2 mM DEANO at OD<sub>600</sub> ≈ 1, and total cellular manganese was monitored by ICP-MS. Data are the mean % total manganese compared to untreated culture for each mutant strain shown adjacent to the wild-type data. Error bars represent standard deviation. (A&B) Single mutants in *mntH* (pink, A) and *sitA* (grey, B) were not altered for manganese acquisition compared to wild-type (blue). (C) An *mntH sitA* mutant (red) did not acquire significantly lower levels of manganese than wild-type. (D&E) An *mntH zupT* mutant (green) and a *sitA zupT* mutant (purple) each acquired manganese in response to NO<sup>•</sup>, but the response was delayed compared to wild-type, peaking at 45 min post-treatment before falling. The total amount of manganese acquired did not differ significantly from wild-type (ns). (F) An *mntH sitA zupT* mutant (orange) did not acquire manganese in response to NO<sup>•</sup>. Statistically significant differences between mutant and wild-type (\*) were determined by unpaired two-tailed t-test. For *mntH zupT* at 45 min, p=0.0025 and at 60 min, p<0.001. For *sitA zupT* at 45 min, p<0.001. For *mntH sitA zupT* at 15 min, p=0.0084, at 30 min, p<0.001, at 45 min, p=0.0078 and at 60 min, p=0.0186.



**Figure 4. Manganese transport mutants are more sensitive to nitrosative stress.**

Growth of wild-type and mutant *S. Typhimurium* was monitored in rich medium (A) or minimal medium (B&C) following treatment with SperNO (dashed lines). (A) In LB medium with 5 mM SperNO, only the *mntH sitA zupT* strain (orange dashed line,  $p < 0.001$ ) showed a delayed exit from lag phase and increased time to reach 50% maximum optical density compared to wild-type (blue dashed line). (B) In M9 glucose medium with 1 mM SperNO, both an *mntH sitA* mutant (red dashed line) and the *mntH sitA zupT* mutant (orange dashed line) showed delayed growth ( $p < 0.001$ ). (C) In M9 glucose with 1.5 mM SperNO, the delays were more pronounced with all mutants showing delayed growth relative to wild-type (*mntH sitA*  $p < 0.001$ , *mntH zupT*  $p = 0.002$ , *sitA zupT*  $p < 0.001$ , *mntH sitA zupT* not determined). Statistical significance between wild-type and mutant growth was determined using time to reach 50% maximum OD<sub>600</sub> by unpaired two-tailed t-test.



**Figure 5. Viable CFU and sensitivity to NO $\cdot$  stress do not change following NO $\cdot$ -challenge.**

(A) Viable CFU, shown as a percentage of initial CFU, remained consistent between wild-type (blue) and *mntH sitA ΔzupT* (orange) *S. Typhimurium* during the prolonged lag phase resulting from NO $\cdot$  stress. There was no significant difference in viable CFU between the two strains except at 2 hr and 5 hr, when the mutant displayed a small but statistically significant increased percentage of viable CFU ( $p=0.011$  and  $p=0.013$ , respectively). Following recovery from the stress, normal growth kinetics resumed, and CFU began to increase with wild-type showing significantly greater viable CFU than *mntH sitA ΔzupT* from 11–14 hr ( $p=0.038$ ,  $p=0.032$ ,  $p=0.025$ ,  $p=0.004$ ). Statistical significance was determined by unpaired two-tailed  $t$ -test. (B & C) Wild-type and *mntH sitA ΔzupT* *S. Typhimurium* from NO $\cdot$  sensitivity assays that had been grown either in LB or LB with 5 mM SperNO were subcultured and used as the starting inocula for subsequent sensitivity assays. The population of *mntH sitA ΔzupT* cells arising from the initial NO $\cdot$  challenge (pale orange dashed lines) was not more resistant to NO $\cdot$  than *mntH sitA ΔzupT* cells that had not previously been exposed to NO $\cdot$  (dark orange dashed lines) in either LB (B) or M9 (C) medium.