Zinc-dependent substrate-level phosphorylation powers *Salmonella* growth under nitrosative stress of the innate host response

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Abstract

The metabolic processes that enable the replication of intracellular *Salmonella* under nitrosative stress conditions engendered in the innate response of macrophages are poorly understood. A screen of *Salmonella* transposon mutants identified the ABC-type high-affinity zinc uptake system ZnuABC as a critical determinant of the adaptation of *Salmonella* to the nitrosative stress generated by the enzymatic activity of inducible nitric oxide (NO) synthase of mononuclear phagocytic cells. NO limits the virulence of a znuB mutant in an acute murine model of salmonellosis. The ZnuABC transporter is crucial for the glycolytic function of fructose bisphosphate aldolase, thereby fueling growth of *Salmonella* during nitrosative stress produced in the innate response of macrophages. Our investigations demonstrate that glycolysis mediates resistance of *Salmonella* to the antimicrobial activity of NO produced in an acute model of infection. The ATP synthesized by substrate-level phosphorylation at the payoff phase of glycolysis and acetate fermentation powers the replication of *Salmonella* experiencing high levels of nitrosative stress. In contrast, despite its high potential for ATP synthesis, oxidative phosphorylation is a major target of inhibition by NO and contributes little to the antinitrosative defenses of intracellular *Salmonella*. Our investigations have uncovered a previously unsuspected conjunction between zinc homeostasis, glucose metabolism and cellular energetics in the adaptation of intracellular *Salmonella* to the reactive nitrogen species synthesized in the innate host response.

Author summary

Microbial pathogens are exposed to multiple antimicrobial defenses during their associations with host cells. Nitric oxide generated in the innate response exerts widespread...
antimicrobial activity against a variety of pathogenic microorganisms. Nitric oxide has high affinity for metal groups of terminal cytochromes of the respiratory chain, and thus nitrosative stress exerts extreme deleterious actions against the cellular energetics that rely on oxidative phosphorylation. Intracellular *Salmonella* have resolved this dilemma by satisfying a significant portion of their energetic demands via substrate level phosphorylation in the payoff phase of glycolysis and acetate fermentation. A high affinity zinc uptake system promotes antinitrosative defense of intracellular *Salmonella* by in great part supporting the enzymatic activity of an essential enzyme in the preparatory phase of glycolysis. Our research provides novel insights into the metabolic and energetic adaptations that allow a bacterial pathogen to thrive in the midst of the innate host response of vertebrate cells.

**Introduction**

Many of the more than 2,500 serovars of *Salmonella enterica* cause gastrointestinal or disseminated infections in millions of people and livestock every year [1, 2]. Reactive nitrogen species synthesized abiotically in the gastric lumen and the extreme acidity of the stomach constitute a formidable barrier to most microorganisms. However, *Salmonella* and other enteropathogens can endure these innate host defenses [3]. In the gastrointestinal tract, *Salmonella* competes for nutrients and space with members of the resident microbiota and, aided by the cytoskeletal remodeling induced by effectors of the *Salmonella* pathogenicity island-1 (SPI-1) type-III secretion system, forces its way into enterocytes and M cells of Peyer’s patches. The *Salmonella* SPI-1 effector SopB activates the transcription of Nos2-encoded inducible nitric oxide synthase (iNOS) long after invasion [4, 5]. Transcription of Nos2 is independently activated in mononuclear phagocytic cells in response to lipopolysaccharide, fimbriae or porins imbedded in *Salmonella*’s cell envelope [6]. The iNOS flavohemoprotein synthesizes nitric oxide (NO) from the guanidino group of L-arginine and molecular oxygen (O$_2$) [6–8]. The diatomic gas NO combines with O$_2$, superoxide anion, iron and low-molecular weight thiols, generating a plethora of reactive nitrogen species that are endowed with vigorous antimicrobial activity [6].

By modifying thiol groups in redox active cysteines, the Feo of iron-sulfur clusters, and ferrous or ferric ions in heme cofactors, NO and its oxidative and nitrosative congeners exert cytostasis against *Salmonella* and various other microbial pathogens [6]. Reactive nitrogen species inhibit quinol oxidases, aconitase, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and dihydroxy acid dehydratase, thereby restricting oxidative phosphorylation, the tricarboxylic acid (TCA) cycle, and the biosynthesis of methionine, lysine, leucine, isoleucine and valine [9–11]. Nitrosative stress is often accompanied by demetallation and loss of protein function [6, 12, 13]. The released iron, manganese or zinc ions can further disrupt cellular functions by mismetallating metabolic enzymes [14]. The liberation of ferrous iron can also cause genotoxicity via the Fenton-catalyzed synthesis of hydroxyl and ferryl radicals [15]. Despite the many molecular targets poisoned by NO and its congeners, the antimicrobial activity of reactive nitrogen species varies widely against diverse intracellular and extracellular bacterial pathogens. Whereas *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, and *Bur- kholderia spp.* are readily killed by NO [16–20], *Staphylococcus aureus* continuously replicates in the presence of nitrosative stress levels that are inhibitory to other microorganisms [21].

*S. enterica* shows intermediate phenotypes. Chemically-generated NO is bacteriostatic against this facultative intracellular enteropathogen [22], but intracellular *Salmonella* grow remarkably well in the presence of high fluxes of NO synthesized enzymatically by iNOS in the
The flavohemoglobin Hmp, cytochrome bd, and low-molecular weight thiols are the main effectors of the antinitrosative toolbox of Salmonella [6, 9, 21, 24, 25]. In addition to these detoxification systems, the Salmonella pathogenicity island-2 type III secretion system helps Salmonella evade contact with iNOS-containing vacuoles [26]. Little is known about the metabolic adaptations that protect intracellular Salmonella against nitrosative stress produced by the host. Herein, we have exploited an unbiased Tn-seq approach to identify hitherto unknown antinitrosative defenses of Salmonella. Our investigations indicate that the high-affinity zinc transporter ZnuABC enables ATP synthesis via substrate-level phosphorylation in glycolysis and acetate fermentation, thereby sustaining Salmonella growth during the nitrosative stress that is generated in the innate host response of macrophages.

Results

Diversification of carbon sources minimizes the anti-Salmonella activity of NO

NO exerts potent bacteriostasis against Salmonella in culture media, but this diatomic radical is well tolerated by intracellular Salmonella. In contrast to specialized bacterial pathogens that have undergone genomic and metabolic reduction [27], nontyphoidal Salmonella such as serovar Typhimurium can catalyze numerous carbon sources during their association with mammalian hosts [28]. Given the pressure NO exerts on central metabolism [9, 10, 12, 29], we investigated whether the availability of diverse carbon sources influences the tolerance of Salmonella to NO. The addition of the NO donor spermine NONOate induced a similarly prolonged lag phase in Salmonella grown in glucose or casamino acids (Fig 1A). The combination of casamino acids and glucose prevented most of the bacteriostasis associated with NO treatment (Fig 1A, S1 Fig). Similar trends were noted when Salmonella were challenged with the NO donors S-nitrosoglutathione (GSNO) or diethylenetriamine-NONOate (DETA NONOate) (S1 Fig). These findings indicate that Salmonella recovers rapidly from NO-induced cytotoxicity as long as it can gain access to diverse carbon sources. As suggested earlier [10, 11, 29], lysine, methionine and branch chain amino acids may allow Salmonella to circumvent NO-induced amino acid functional auxotrophies associated with the inactivation of lipoamide-dependent lipoamide dehydrogenase and dihydroxy acid dehydratase. However, the mechanisms by which glucose strengthens the antinitrosative defenses of Salmonella have not been determined yet.

To gain insights into the metabolic pathways by which glucose and casamino acids power the antinitrosative defenses of Salmonella, we examined the growth of mutants in glycolysis, acetate fermentation and TCA in glucose or casamino acids media (Fig 1B). Electron transport chain mutants were also tested. The inability of Δpgi, ΔpfkAB, ΔgltA, ΔacnAB and ΔicdA mutants to replicate in glucose indicate that glycolysis and the oxidative branch of the TCA cycle support Salmonella growth in this hexose (Fig 1C and 1D). A ΔpfkAB Salmonella strain lacking both isoforms of the glycolytic enzyme phosphofructokinase grew in glycerol (S1 Fig), demonstrating that this mutant can grow in carbon entering glycolysis below fructose-1,6-bisphosphate. ΔatpB and ΔackA Δpta deletion mutants thrived in glucose, suggesting that ATP formed via oxidative phosphorylation or acetate fermentation is largely dispensable for growth of S. Typhimurium in glucose (Fig 1E). Aside from the minor growth defect of ΔasdAB Salmonella, glycolytic and TCA cycle mutants grew robustly in casamino acids (Fig 1C and 1D). Casamino acids, a mixture of small peptides and amino acids, enter central metabolism at different steps in glycolysis and the TCA cycle (Fig 1B), likely explaining why single glycolytic or TCA cycle mutants flourished in this carbon source. Salmonella, however, required
Fig 1. Effects of carbon sources on resistance of Salmonella to NO. (A) Growth of Salmonella treated (+ sNO) or untreated (ctrl) with 750 μM of spermine NONOate in MOPS minimal media supplemented with either glucose (GLC), casamino acids (CAA), or both carbon sources (GLC + CAA) (N = 4, mean ± S.E.M.). (B) Depiction of genes in glycolysis and tricarboxylic acid cycle (TCA) targeted for mutation. Sites of entry of GLC and CAA into glycolysis and TCA are shown. Steps of ATP synthesis by substrate-level phosphorylation and fbaAB genes encoding fructose bisphosphate aldolase are also indicated. Growth of Salmonella harboring mutations in TCA (C) or glycolysis (D) after 20 h of growth in MOPS minimal media supplemented with GLC or CAA (N = 3, mean ± S.E.M.). (E) Growth of wild-type (WT), ΔatpB, and ΔackA Δpta Salmonella in MOPS supplemented with GLC or CAA (N = 4, mean ± S.E.M.). (F) Anaerobic growth of Salmonella in MOPS media supplemented with 50 mM NaNO₃ (N = 4, mean ± S.E.M.). *, p < 0.05; ***, p < 0.001.

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oxidative phosphorylation and acetate fermentation for optimal growth on casamino acids (Fig 1E). Moreover, casamino acids supported Salmonella growth as long as the terminal electron acceptors O₂ or NO₃⁻ were available. Conversely, glucose fueled Salmonella growth in the absence of O₂ or NO₃⁻ (Fig 1F). Cumulatively, this research indicates that glucose and casamino acids energize Salmonella growth by engaging substrate-level and oxidative phosphorylation, respectively. Our data also indicate that the metabolic diversification associated with glycolysis, acetate fermentation, TCA cycle and the electron transport chain yields a population of Salmonella that is highly immune to NO.

**Importance of metabolism in resistance of Salmonella to nitrosative stress**

To gain further insights into the mechanisms by which nutritional diversity promotes antinitrosative defenses in Salmonella, we chose an unbiased transposon-based approach. A library of barcoded transposon mutants was separately challenged with the NO donors spermine NONOate, GSNO, or DETA NONOate in MOPS minimal media supplemented with glucose, casamino acids, or a combination of glucose and casamino acids. When the results were corrected to the media alone and the false discovery rate was set to < 10%, spermine NONOate, GSNO, and DETA NONOate exerted selectable advantages or disadvantages to 1132, 1729, and 299 genes, respectively. Differentially selected loci (S1 Table) were sorted into Venn diagrams (Fig 2A, 2C and 2E), and the annotated genes were converted into Go-terms. Panther Pathway analysis indicated that NO exerts the greatest negative and positive selection on loci related to metabolic processes (Fig 2B, 2D and 2F).

There were more common positively or negatively selected genes within each NO donor than within each medium (S2 Table and S3 Table). The fast releasing NO donor spermine NONOate selected against mutants in genes related to glycolysis (pgk, pfkA, gpmA), Tat-mediated secretion (tatABC), cell division (zipA, ftsNL) or proteostasis (hflKCX, hslVU, clpA), whereas mutations in SPI-1 (invAFH, pipB2, sipA) generated a growth advantage. As expected, mutations in the dipeptide uptake system (dppABCDFG), which transports S-nitrosoglutathione cysteine across the cytoplasmic membrane [22], were positively selected in GSNO-treated Salmonella. The transnitrosating agent GSNO also enriched for mutations in purine (purFH) and cysteine (cysCFEGQ) biosynthesis, but selected against mutants in molybdopterin biosynthesis (moaACDE, moeAB). The slow NO donor DETA NONOate imposed a weaker selective pressure than spermine NONOate or GSNO. Mutations in the high-affinity, ABC-type, Zn²⁺ transporter (znuABC) and biotin biosynthesis (bioABCDFH) were negatively selected by DETA NONOate.

Genes were also grouped by medium (S3 Table). The NO donors tested selected for or against 11 (e.g., znuABC, thiCF, nadC) or 126 genes in Salmonella grown in glucose or casamino acids, respectively. Many of the positively selected transposon mutants in casamino acid-containing medium were deficient in glyoxylate shunt (aceABK), or biosynthesis of amino acids (argABCEGI, thrBC), thiamine (thiCDFGH), and lipopolysaccharide (rfABGIJKLPZ, rfbABCDKMN, cfc, arrAT, basRS, pmrDFJLM), suggesting that expression of these genes imposes a considerable metabolic burden when diverse nutrient sources are available to Salmonella undergoing nitrosative stress. As expected, NO negatively selected against mutations in hmpA and gshAB encoding the known antinitrosative defenses flavohemoglobin and glutathione synthase, respectively (S4 Table) [24, 25]. In addition, mutants in efflux and acquisition of Zn²⁺ (zntA, znuC, respectively), and modification of tRNAs (cmoB) were at a disadvantage when exposed to NO in casamino acids.

Overall, there were very few mutations that were consistently deleterious in most experimental conditions tested. Mutations in genes encoding the high-affinity, ABC-type, Zn²⁺
uptake system ZnuABC were the strongest exception. ZnuA is the zinc-binding, periplasmic cassette; ZnuB comprises the membrane-spanning permease; and ZnuC is the cytosolic ATPase that fuels uptake of this divalent cation [30]. Genetically, \( \Delta znuA \) is divergently transcribed from the \( znuCB \) operon [31]. Henceforth, this locus will be referred to as \( znuABC \).

Although transposon interruptions of either \( znuA, znuB, \) or \( znuC \) were under negative selection in most of the screen conditions tested, the disadvantage of the \( znuA, znuB \) and \( znuC \) mutants was most evident in glucose (Fig 2G, S5 Table), suggesting that \( Zn^{2+} \) uptake is particularly important in the antinitrosative defenses of glycolytic bacteria. However, not all components of zinc uptake contributed equally to the antinitrosative defenses of \( Salmonella \), because NO did not exert significant pressures on \( zntB, zitB, \) or \( zupT \) genes encoding low-affinity \( Zn^{2+} \) transporters (S6 Table). NO did not seem to exert negative selection against \( sodCI \) or \( sodCII \) mutants deficient in copper-zinc superoxide dismutases (Cu-Zn SODs), which have been involved in resistance to phagocyte NADPH oxidase and iNOS [32].

**The \( znuABC \)-encoded \( Zn^{2+} \) uptake system protects \( Salmonella \) against the nitrosative stress engendered in the innate host response**

We evaluated in more detail the role that the high-affinity \( Zn^{2+} \) uptake system ZnuABC plays in resistance to NO generated chemically *in vitro* or enzymatically in the innate response of macrophages and mice. Compared to wild-type controls, \( \Delta znuB \) \( Salmonella \) required longer times to enter exponential growth in all media examined (Fig 3A, S2 Fig). The \( \Delta znuB \) mutant also had longer doubling times in glucose than wild-type controls (S2 Fig), further reinforcing the idea that zinc plays an essential role in glycolysis. The addition of 5 mM \( ZnCl_2 \) restored normal growth to \( \Delta znuB \) \( Salmonella \), whereas the NO donor DETA NONOate exacerbated the growth defects of \( \Delta znuB \) \( Salmonella \) in all carbon sources tested (Fig 3A, S2 Fig). Expression of a \( znuB \) allele in trans restored growth of \( \Delta znuB \) \( Salmonella \) in EG minimal media to wild-type levels and it prevented the hypersusceptibility of this mutant to nitrosative stress (S2 Fig). Isogenic \( \Delta znuA \) and \( \Delta znuC \) \( Salmonella \) mutants also displayed hypersusceptibility to DETA NONOate (S2 Fig), indicating that mutations in any of the subunits that encode the high-affinity ZnuABC zinc uptake system predispose \( Salmonella \) to nitrosative stress. In contrast, \( \Delta zur \) \( Salmonella \) was as resistant to DETA NONOate as wild-type controls (S2 Fig), perhaps reflecting derepression of \( znuABC \) transcription [30, 31]. Collectively, these investigations suggest that glycolysis is particularly reliant on \( Zn^{2+} \) and that zinc-starved \( Salmonella \) are particularly sensitive to the bacteriostatic actions of NO.

We examined whether the antinitrosative defenses associated with the ZnuABC system protect \( Salmonella \) against NO engendered in the innate response of mononuclear phagocytic cells. Compared to wild-type controls, \( \Delta znuB \) \( Salmonella \) replicated poorly in NO-producing J774 cells (Fig 3B, S2 Fig). Ectopic expression of the \( znuB \) gene reversed the growth defect of \( \Delta znuB \) \( Salmonella \) (S2 Fig). Moreover, the addition of the iNOS inhibitor aminoguanidine substantially reduced NO production (S2 Fig), and greatly promoted intracellular growth of \( \Delta znuB \) \( Salmonella \) (Fig 3B). The iNOS inhibitor L-NIL also promoted intracellular growth of
ΔznuB Salmonella in J774 cells (S2 Fig). Neither aminoguanidine nor L-NIL affected growth of Salmonella in vitro (S2 Fig), suggesting that the improved growth of ΔznuB Salmonella in J774 cells treated with aminoguanidine or L-NIL cannot be explained by direct effects of these drugs on the bacteria but rather on the inhibition of host iNOS enzymatic activity. Accordingly, ΔznuB Salmonella were also more susceptible than wild-type controls to the antimicrobial activity derived from NO congeners synthesized in the innate response of periodate-elicited macrophages (Fig 3C, S2 Fig). We also examined whether the ZnuABC system is a key component of the antinitrosative arsenal of Salmonella in a model of acute infection. Wild-type Salmonella killed C57BL/6 and iNOS−/− mice 9 and 7.5 days post infection, respectively (Fig 3D). Consistent with previous investigations [33], ΔznuB Salmonella was attenuated in C57BL/6 mice (p < 0.0001). Interestingly, ΔznuB Salmonella became virulent in iNOS−/− mice, indicating that this Zn2+ acquisition system is an integral aspect of the antinitrosative defenses of Salmonella. iNOS−/− mice infected with ΔznuB Salmonella live on average 4 days longer (p < 0.001) than those infected with wild-type controls, suggesting that zinc acquisition also participates in Salmonella virulence in ways that are independent of antinitrosative defense.
The Zn\textsuperscript{2+} uptake system ZnuABC is needed for maximal fructose bisphosphate aldolase activity

In the preparative phase of glycolysis, fructose bisphosphate aldolase splits fructose-1,6-bisphosphate into dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate. As is the case for \textit{E. coli}, the \textit{Salmonella} genome encodes for two isoforms of fructose-bisphosphate aldolase. The constitutively expressed class II fructose bisphosphate aldolase (encoded by \textit{fbaA}) is zinc-dependent, whereas the expression of the zinc-independent class I isoform \textit{FbaB} is induced in response to gluconeogenic substrates. Hence, in \textit{E. coli} grown in glucose, 95–100% of the total fructose bisphosphate aldolase activity is contributed by the zinc-dependent class II fructose bisphosphate aldolase [34]. We noticed that \textit{ΔznuB Salmonella} harbored about two thirds of the fructose bisphosphate aldolase enzymatic activity of wild-type controls (Fig 4A). Fructose bisphosphate activity was inhibited by the Zn\textsuperscript{2+}-chelator N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (Fig 4A). The addition of 5 μM ZnCl\textsubscript{2} to the EG minimal media eliminated the differences in fructose bisphosphate aldolase activity between wild-

![Fructose bisphosphate aldolase content](https://doi.org/10.1371/journal.ppat.1007388.g004)
type and ΔznuB Salmonella (Fig 4B). Together, these findings suggest that most differences in fructose bisphosphate aldolase content between wild-type and ΔznuB Salmonella are due to poor zinc uptake. NO inhibited (p < 0.05) by 10% the enzymatic activity of fructose bisphosphate aldolase in wild-type Salmonella (Fig 4C). NO appears to inhibit fructose bisphosphate aldolase to a greater extent in ΔznuB Salmonella (p < 0.01) than wild-type controls (p < 0.05).

We wondered if a 33% drop in fructose bisphosphate aldolase activity has a significant impact in the intracellular growth defect of ΔznuB Salmonella. To test this idea, ΔznuB Salmonella were complemented with the fbaA or fbaB genes, encoding zinc-dependent or -independent fructose bisphosphate aldolase isoforms, respectively (Fig 4D). Although intracellular growth was dramatically improved (p < 0.001), expression of fbaA gene did not fully restore growth of ΔznuB Salmonella to wild-type levels (p < 0.001 when ΔznuB pFBAA is compared to wild-type Salmonella). However, expression of the fbaB gene encoding the zinc-independent fructose bisphosphate aldolase isoform fully restored the intracellular growth of ΔznuB Salmonella. Together, these findings strongly indicate that the decreased enzymatic activity of fructose bisphosphate aldolase is a major contributor to the poor intracellular growth exhibited by ΔznuB Salmonella.

Zinc serves as cofactor for multiple proteins, including Cu-Zn SODs that detoxify superoxide anion in the periplasm. By limiting peroxynitrite formation from superoxide and NO, Cu-Zn SODs contribute to the antinitrosative defenses of Salmonella [32]. To assess if ΔznuB Salmonella has defects in detoxifying superoxide anion formed in the plasma membrane from the adventitious reduction of oxygen by NADH dehydrogenases [35], we monitored the formation of nitrotyrosine residues as a proxy of peroxynitrite. Wild-type and ΔznuB Salmonella harbored similar levels of nitrotyrosine after treatment with 500 μM spermine NONOate (S3 Fig). As predicted by the critical role NADH dehydrogenases play in the production of endogenous superoxide in Salmonella, a Δnuo Δndh mutant showed a dramatic decrease in nitrotyrosine formation in response to spermine NONOate. Together, these findings suggest that ΔznuB Salmonella has normal SodC function, perhaps because, as it has been suggested earlier [36], metallation of apo-SodC is likely to occur in the periplasm.

**Glycolysis contributes to the antinitrosative defenses of Salmonella**

Given the role the ZnuABC uptake system plays in glycolysis and antinitrosative defense, we tested the contribution of glycolysis in the resistance of Salmonella to NO. The gene fba, encoding the zinc-dependent, fructose bisphosphate aldolase is an essential gene in *E. coli*. The lack of transposons in the gene indicate that it is also essential in Salmonella. Therefore, we tested the phenotypes of a glycolytic mutant deficient in the pfkAB-encoded phosphofructokinase, and found that ΔpfkAB Salmonella suffered a prolonged lag phase when challenged with DETA NONOate (Fig 5A, S4 Fig). Consistent with previous reports [37, 38], ΔpfkAB Salmonella failed to replicate within macrophages (Fig 5B). Blockage of NO synthesis with the iNOS inhibitor N6-(1-iminoethyl)-L-lysine (L-NIL) partially rescued the intracellular growth defect of ΔpfkAB Salmonella in J774 macrophage cells (Fig 5B, S4 Fig). The intracellular replication defect and hypersusceptibility of ΔpfkAB Salmonella to DETA NONOate could be complemented by expression of pfkA or pfkB genes in trans (S4 Fig). The ΔpfkAB Salmonella strain was extremely attenuated in C57BL/6 mice but became virulent in iNOS−/− mice (Fig 5C), strongly suggesting that glycolysis helps *Salmonella* to overcome the cytotoxicity of NO synthesized in the innate host response. As noted with ΔznuB Salmonella, iNOS−/− mice succumbed more slowly to ΔpfkAB Salmonella infection than controls infected with wild-type Salmonella (p < 0.01), suggesting that glycolysis supports *Salmonella* virulence in NO-dependent and -independent ways.
In addition to glycolysis, oxidative phosphorylation can be a sizable source of ATP. Therefore, we also tested whether oxidative phosphorylation contributes to the antinitrosative defenses of *Salmonella*. We noted that a *Salmonella* strain deficient in the *atpB*-encoded subunit of ATP synthase recovered from nitrosative stress as efficiently as wild-type controls (Fig 5D). Furthermore, wild-type and Δ*atpB* *Salmonella* replicated to similar densities within NO-producing J774 macrophages (Fig 5E, S4 Fig). Given its excellent growth in J774 cells, we were surprised to find that Δ*atpB* *Salmonella* were not only severely attenuated in C57BL/6 mice but remained avirulent in iNOS-deficient mice. Similar results were obtained with a Δ*nuo* Δ*ndh* strain lacking both isoforms of the NADH dehydrogenase of the electron transport chain. Thus, the ATP synthase seems to be dispensable for antinitrosative defenses of intracellular *Salmonella*, but it is critical for *Salmonella* pathogenesis.

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Substrate-level phosphorylation sustains growth of intracellular Salmonella undergoing nitrosative stress

Because substrate-level phosphorylation in glycolysis can be a sizable source of ATP, we examined the degree to which glucose utilization helps Salmonella maintain the ATP pool during nitrosative stress. Thin layer chromatography and independent firefly luciferase determinations indicated that spermine NONOate reduces the ATP pool in Salmonella growing in glucose or casamino acids (reductions of 90 vs. 99%, respectively) (Fig 6A and 6B). Salmonella growing in both glucose and casamino acids retained about 60% of the ATP pool after NO treatment (Fig 6A and 6B), indicating that access to diverse carbon sources that feed into glycolysis and the electron transport chain protects most of the ATP pool from the toxic actions of NO. The ATP pools were also measured in glycolytically-deficient ΔackA and oxidative phosphorylation-deficient ΔatpB Salmonella. Because of its extreme growth defect in glucose, the ΔpykAF mutant was tested in LB broth. Wild-type, ΔatpB, ΔpykAF and ΔznuB Salmonella suffered about 3-, 5-, 8-, and 21-fold reductions, respectively, in the ATP pool upon NO challenge (Fig 6C and 6D, S5 Fig). Cumulatively, these findings suggest that zinc-dependent glycolysis is more effective than oxidative phosphorylation at preserving cellular energetics during periods of nitrosative stress.

The pgk-encoded phosphoglycerate kinase and pykAF-encoded pyruvate kinases generate ATP by substrate-level phosphorylation in the payoff phase of glycolysis. In addition, an ATP molecule is synthesized by the fermentation of acetyl-CoA to acetate by the ackA-encoded acetate kinase. Because pgk is an essential gene in Salmonella, we directed our attention to the pykAF and pta-ackA pathways. In increasing order, spermine NONOate depleted ATP from wild-type, ΔackA Δpta, ΔpykAF and ΔpykAF ΔackA Δpta Salmonella (Fig 6E), suggesting that glycolysis contributes most of the ATP synthesized by substrate-level phosphorylation in NO-treated Salmonella but that acetate fermentation is also an important source of ATP. Spermine NONOate inflicted more severe bacteriostasis to a ΔpykAF ΔackA Δpta mutant than ΔpykAF or ΔackA Δpta isogenic strains (Fig 6F), and NO produced in the innate response of J774 cells stunted the growth of ΔpykAF ΔackA Δpta Salmonella more severely than that of ΔpykAF or ΔackA Δpta controls (Fig 6G). The growth defects of ΔpykAF ΔackA Δpta Salmonella could be complemented by expression of the ackA pta operon in trans (S5 Fig). ΔpykAF ΔackA Δpta Salmonella were also more susceptible to the antimicrobial activity of iNOS expressed in the innate response of primary macrophages (Fig 6H, S5 Fig). Moreover, in contrast to the ΔpykAF or ΔackA Δpta parent strains, the ΔpykAF ΔackA Δpta mutant was attenuated in C57BL/6 mice (p < 0.05 as compared to wild-type Salmonella). Interestingly, ΔpykAF ΔackA Δpta Salmonella became as virulent as wild-type bacteria in iNOS−/− mice (p = 0.5) (Fig 6I). Collectively, these investigations demonstrate that ATP synthesized by substrate-level phosphorylation in both glycolysis and acetate fermentation protects Salmonella against the cytotoxicity of NO produced in the innate response.

Discussion

Zn2+, the second most abundant metal cofactor, provides structural, regulatory, antioxidant, and catalytic properties to diverse metalloproteins [39, 40]. Given its critical importance in bacterial cell physiology, mammalian hosts actively limit the bioavailability of Zn2+ to bacterial pathogens, thereby contributing to what is now known as nutritional immunity [41]. As a countermeasure, bacterial pathogens use high affinity Zn2+ transporters, such as ZnuABC or the Gram-positive AdcABC orthologue, to compete with the host for zinc [41]. Interestingly, Salmonella utilizes ZnuABC to compete for zinc with indigenous microbiota of the gut, and exploits this high-affinity uptake system to gain advantages in systemic sites and macrophages.
Fig 6. Effects of carbon source on the ATP pool of Salmonella undergoing nitrosative stress. (A) ATP was visualized after TLC analysis of $^{32}$P-labeled Salmonella grown in MOPS minimal media supplemented with either glucose (GLC) and/or casamino acids (CAA). When indicated, the cultures were treated with 750 μM spermine NONOate (sNO) for 5 min. Images are representative of experiments performed on at least two independent days. (B, C-E) The ATP pools were independently estimated with firefly luciferase. The bacteria were grown in EG (C and E) or LB broth (D). Select bacteria were treated with 750 μM sNO for 5 min (N = 6, mean ± S.E.M.). *, **, *** p < 0.05, 0.01, 0.001, respectively, as determined by one-way or two-way ANOVA. (F) Growth of the indicated Salmonella strains after treatment with 1 mM DETA NONOate (dNO) in EG media. (G) Intracellular growth of Salmonella in J774 cells 20 h after infection. Some of the specimens were treated with 500 μM L-NIL. (H) Intracellular survival of Salmonella in periodate-elicited macrophages from C57BL/6 or iNOS$^{-/-}$ mice (N = 4–5, mean ± S.D.). ***, p < 0.001 as determined by two-way ANOVA. (I) Survival of C57BL/6 or iNOS$^{-/-}$ mice after i.p. inoculation of 100 CFU of the indicated Salmonella strains. N = 9–15; */ p < 0.05, WT vs. ΔpykAF ΔackA Δpta in C57BL/6 mice as determined by logrank analysis; all other comparisons to WT were not statistically significant.

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The mechanisms underlying poor growth of ΔznuB Salmonella within macrophages have not been identified yet. Herein, we show that ZnuABC potentiates Salmonella pathogenesis by in part antagonizing the nitrosative stress generated in the innate response of macrophages and mice. Despite its widespread utilization in metabolism and multiple regulatory processes, the scarcity of zinc in ΔznuB Salmonella is particularly detrimental to zinc-dependent fructose bisphosphate aldolase in glycolysis as suggested by the complementation of the intracellular growth defects of ΔznuB Salmonella with the fbaB gene encoding zinc-independent fructose bisphosphate aldolase. Thus, not only does high affinity zinc uptake defend microbes against the metabolic stress associated with either nutritional immunity or competition with microbiota, but it also arms bacteria with the glycolytic flexibility needed to overcome the deficiencies in energetics that are triggered by the inhibition of cytochromes by NO of the innate response of professional phagocytes.

The widespread utilization of zinc in multiple metabolic pathways may explain why ΔznuB Salmonella exhibits growth delays in all carbon sources tested. Our investigations have demonstrated that the ZnuABC uptake system plays a particularly salient role in the antinitrosative defenses of Salmonella, by perhaps promoting DksA-mediated regulation of transcription and the enzymatic activity of RecBCD DNA repair proteins [13, 40, 43]. Salmonella infection results in elevated levels of free zinc in macrophages, which is then readily available for the needs of the pathogen, but also undermines production of reactive oxygen and nitrogen species [44]. Despite the widespread utilization of zinc in the cell, a sizable component of the antinitrosative defenses associated with zinc uptake appears to be dependent on glycolysis. We noticed that ΔznuB Salmonella is particularly sensitive to the antimicrobial actions of NO when grown in glucose. The high demand for zinc in glycolysis likely reflects usage of zinc by class II fructose bisphosphate aldolase. Poor glycolytic activity might predispose ΔznuABC mutants to NO, an idea that is independently supported by the attenuation of ΔpfrKAB Salmonella in mice expressing a functional iNOS hemoprotein. Glycolysis allows intracellular Salmonella to use the glucose available in macrophages [28], satisfying the requirements for carbon. In addition, the attenuation of ΔpykAF ΔackA Δpta Salmonella indicates that glycolysis protects against nitrosative stress by promoting ATP synthesis in substrate-level phosphorylation (Fig 7). Glycolysis could also boost antinitrosative defenses by balancing redox away from the electron transport chain, as has been demonstrated in S. aureus [45].

ATP generated by oxidative phosphorylation contributes very little to the antinitrosative defenses of Salmonella (Fig 7). Cytochrome bd and cytochrome bo are among the most sensitive targets of NO [46]. Nitrosylation of terminal cytochromes of the electron transport chain has a devastating effect on the ATP synthesized by oxidative phosphorylation [47]. Additionally, aconitate, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase are all sensitive to NO [10, 48], thereby not only limiting the ATP synthesized in the TCA cycle but also preventing the generation of NADH reducing power that fuels oxidative phosphorylation. Although Salmonella does not seem to strongly rely on oxidative phosphorylation to overcome nitrosative stress, ΔatpB and Δnuo Δndh mutants lacking ATP synthase and NADH dehydrogenases, respectively, are highly attenuated. These phenotypes may be explained by the fact that the electron transport chain is critical for maintaining redox balance, uptaking and effluxing substrates across the cytoplasmic membrane, and driving protein folding in the cell envelope [49]. The ATP synthase working in reverse can also be an important aspect of the proton motive force [50].

As shown by others in Borrelia burgdorferi [12], fructose bisphosphate aldolase was found to be inactivated in Salmonella after NO treatment. However, fructose-bisphosphate aldolase enzymatic activity appears to be more resistant to NO toxicity than terminal cytochromes of the electron transport chain [9, 46, 51]. The high sensitivity of the electron transport chain and TCA cycle to nitrosative stress provides a reasonable explanation for the capital importance of...
substrate-level phosphorylation in maintaining cellular energetics of *Salmonella* undergoing nitrosative stress. The excellent recovery of *Salmonella* from nitrosative stress in the presence of both glucose and casamino acids could be partially explained by the retention of much of the ATP pool. Amino acids may also promote the recovery of *Salmonella* from NO by relieving the functional auxotrophies for branch chain amino acids, lysine and methionine that follow damage of dihydroxy-acid dehydratase and lipoamide-dependent lipoamide dehydrogenase by reactive nitrogen species [10, 11].

In summary, the ability of *Salmonella* to simultaneously exploit different carbon sources during its intracellular life-cycle [28] and diversification of metabolic pathways used to synthesize ATP may underlie the remarkable resistance of *Salmonella* to NO generated by the innate response of macrophages [7, 23]. Our investigations demonstrate that zinc allows for metabolic flexibility during nitrosative stress by allowing for proper fructose bisphosphate aldolase activity and glycolysis.

**Methods**

**Ethics statement**

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

Bacterial strains

Salmonella enterica serovar Typhimurium strain ATCC 14028s and its mutant derivatives were used in these studies (S7 Table). In-frame deletion mutations were constructed using the λ Red recombinase system as originally described [52–54]. Briefly, the kan gene from pKD4 or pKD13, or the cat gene from pKD3, were PCR amplified using primers with 40–45 bases of overhang on the 5’ end that were homologous to the 40–45 bases following the ATG and the 40–45 bases preceding the stop codon of the gene of interest that was to be deleted. These linear DNA segments were gel purified, and electroporated into Salmonella expressing the λ Red recombinase from the plasmid pKD46 or pTP233. Transformants were selected on either LB (Luria Bertoni) kanamycin (50 μg/mL) or LB chloramphenicol (20 μg/mL) plates. Mutations were verified by PCR and were then phage (P22) transduced into 14028s wild-type Salmonella. Transduced colonies were selected on antibiotic plates, were confirmed by PCR, and were then cured of phage contamination. Primers used to generate and check these mutations are listed in S7 Table.

Construction of the Tn-seq Salmonella library

Construction of the barcoded Tn-seq library in S. enterica sv Typhimurium 14028s has been described in detail elsewhere [55]. In brief, EZ-Tn5 <KAN-2> (http://www.lucigen.com) was modified to introduce an N18 barcode adjacent an Illumina Read 1 sequence. A library of over 230,000 different insertion mutants was constructed by mixing transposase and barcoded construct and subsequent electroporation into electrocompetent bacterial cells. The barcode associated with each unique Tn5 insertion position was determined by Illumina sequencing of PCR-amplified flanking regions, as described [55].

Growth conditions

Strains and mutants were maintained in LB broth in the presence of 50 μg/mL kanamycin or 20 μg/mL chloramphenicol, as needed. When indicated, Salmonella were grown in MOPS minimal media (MOPS) [56], supplemented with either 0.4% D-glucose, 0.4% casamino acids, or 0.4% D-glucose and 0.4% casamino acids. Salmonella were also grown in E salts media [57] supplemented with either 0.4% D-glucose (EG), 0.1% casamino acids (ECA), or 0.4% D-glucose and 0.1% casamino acids (EGCA). When indicated, Salmonella were grown in N9 media (100 mM Tris-HCl, pH 7.6, 5 mM KCl, 7.5 mM (NH4)2SO4, 1 mM KH2PO4, 38 mM glycerol, and 0.1% casamino acids). All cultures were grown aerobically in incubators shaking vigorously at 37˚C. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Hampton, NH).

NO recovery assays

Salmonella grown in MOPS minimal media to an OD600 of 0.1–0.2 were challenged with either 750 μM spermine NONOate, 5 mM GSNO or 2.5 or 5 mM DETA NONOate (Cayman Chemical Company, Ann Arbor, MI). Growth was recorded by measuring the OD600 of a 200 μL sample every 30 or 60 mins in a 96-well plate. The cultures’ doubling times of the initial OD600 were calculated by exponential regression. Alternatively, 200 μL of overnight culture diluted 1:1000 into fresh media were seeded into honeycomb microplates and treated with either the polyamine diethylenetriamine (DETA) or DETA NONOate, and OD600 was recorded every 15
min for up to 40 h in a Bioscreen C plate reader (Growthcurves USA, Piscataway, NJ). Doubling times were calculated according to the equation \( DT = \ln(2)/r \), where \( r \) is the growth rate as calculated by regression analysis. The time at which the cultures reached half of their maximum growth (\( T = \frac{1}{2} \text{OD}_{600}\text{Max} \)), a method to calculate the growth delay [10], was calculated by exponential regression.

**Screen conditions**

Three different NO donors with different chemical properties were selected for our screen. Spermine NONOate spontaneously releases 2 molecules of NO per parent molecule with a half-life of 39 minutes at pH 7.4, 37˚C. The NO donor DETA NONOate decays with a half-life of 20 h at pH 7.4, 37˚C, producing a long and sustained flux of NO for the duration of the experiment [58]. GSNO can homolytically release NO, or heterolytically transfer an NO\(^+\)-like species to redox active thiol groups in cysteine residues. The transposon library was challenged with these NO donors in early exponential phase in MOPS minimal media supplemented with either glucose, casamino acids, or glucose and casamino acids. The resulting populations were compared to the library grown in media alone. More specifically, the *Salmonella* Tn5 library was grown for 20 h in LB broth at 37˚C with vigorous shaking. An aliquot of the culture was stocked in 10% glycerol at -80˚C as the input, and another aliquot was diluted 1:100 into 10 mL of MOPS minimal media supplemented with glucose, casamino acids, or glucose and casamino acids. The cultures were grown for 2.5–4 h to an approximate OD\(_{600}\) of 0.2. The cultures were split into 2 mL aliquots and were either left untreated or were challenged with either 750 \( \mu \)M spermine NONOate, 5 mM GSNO, or 5 mM DETA NONOate. After 20 h of culture, the resulting populations were stocked in 10% glycerol at -80˚C. The procedure was performed in biological triplicates on 3 different days. We analyzed two of those biological replicates by deep-sequencing.

Sequencing and analysis of the barcoded input and output Tn libraries was performed as described in detail in de Moraes *et al* [55]. Briefly, bacterial pellets representing approximately 5 \( \times \) 10\(^7\) bacteria were washed three times in water followed by proteinase K digestion, enzyme inactivation, and nested PCR to amplify the N\(_{18}\) barcode region and add sample- and experiment-specific N\(_{8}\) indices. Different samples, with different indexes, were pooled and QIAquick purified (Qiagen, Hilden, Germany), followed by Illumina sequencing with standard primers. The first 18 bases in each sequencing read, which represented the unique N\(_{18}\) tag for each Tn5 mutant, were extracted, and the abundance of these 18-mers was calculated using custom perl scripts.

**Statistical analysis of the *Salmonella* Tn-seq library screen**

Detailed methods of how the location and sequence of N\(_{18}\) barcode tag flanked by conserved priming sites for each Tn5 insertion mutant were identified are reported elsewhere [55]. Abundances of all mutants within each annotated feature were summed up which resulted into a data matrix where rows were annotated features and columns were samples. This data matrix was used to generate log\(_2\) ratios as compared to either input library or library passaged in control media (without stress inducing agents) using edgeR [59].

**Fructose bisphosphate aldolase assay**

Fructose-1,6-bisphosphate aldolase activity was assayed as previously described [12]. Briefly, wild-type and \( \Delta \text{znuB} \) *Salmonella* were grown in EG minimal media to an OD\(_{600}\) of 0.4–1.0. Where indicated, the medium was supplemented with 5 \( \mu \)M ZnCl\(_2\). Some of the cultures were treated with 750 \( \mu \)M spermine NONOate for 5 min. Cell pellets were stored at -80˚C until
assayed. The pellets were resuspended in 500 μL of 100 mM Tris, pH 7.2 and the bacterial cells were lysed by sonication on ice. The specimens were centrifuged at 13,000 RPM for 15 min at 4˚C. Lysates were diluted to approximately 5 mg/mL of protein as measured by A$_{280}$ readings. Some of the specimens were treated with 100 μM of the zinc chelator N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) at room temperature for 30 min. Aliquots of 100 μL of lysate were added to 700 μL of 100 mM Tris, pH 7.2, 100 μL of 4 mM NADH in 100 mM Tris, pH 7.2, 100 μL of 58 mM fructose-1,6-bisphosphate trisodium salt in water, 2.5 U of type X triosephosphate isomerase from rabbit tissue and 1.5 U of type I 3-phosphoglycerate dehydrogenase, from rabbit muscle (Sigma-Aldrich, St. Louis, MO). Specimens were mixed well and four 200 μL samples were pipetted into a 96-well plate. The consumption of NADH was measured by recording the A$_{340}$ every 20 sec for 10 min. The ΔA$_{340}$/min was generated by linear regression and technical replicates were averaged and normalized to the protein in the sample. All activities were normalized to untreated samples gathered from wild-type *Salmonella*.

### Intracellular replication

J774A1 murine macrophage-like cells (ATCC TIB67) were maintained at 37˚C, 5% CO$_2$ in RPMI$^+$ media (RPMI media supplemented with 2 mM L-glutamate, 1 mM sodium pyruvate, 15 mM HEPES buffer, 10% fetal bovine serum, and 100 U/mL Penicillin-Streptomycin). Twenty hours prior to infection, J774 cells were plated at 10$^5$ cells per well in a 96-well plate in 100 μL of media. *Salmonella* cultures were grown for 20 h at 37˚C with vigorous shaking. Overnight bacterial cultures were diluted to approximately 2x10$^6$ CFU/mL in macrophage media without Pen/Strep. J774 cells were washed with prewarmed RPMI$^+$ media, and were challenged with *Salmonella* at an MOI of 2. The plates were centrifuged at 4000 RPM for 1 min at room temperature, and then incubated at 37˚C, 5% CO$_2$. After 25 min, the culture media was removed and fresh RPMI$^+$ media containing 50 μg/mL of gentamicin was added to the macrophages at 37˚C and, after 1h, the media was replaced with media containing 10 μg/mL gentamicin. One and seventeen h later (T = 2 and 18 h of infection), the media was removed and the macrophages were lysed with 0.25% (w/v) deoxycholic acid prepared in PBS. The bacterial burdens were quantified after 10-fold serial dilutions on LB agar plates. Fold-replication was calculated by normalizing the CFU/mL at T = 18 h to the CFU/mL at T = 2 h. When applicable, nitrite in the culture supernatants was assayed with the Griess reaction as previously described [60].

### Survival in primary macrophages

The intracellular survival of wild-type and mutant *Salmonella* was tested in periodate-elicited macrophages as described [61]. Macrophages from C57BL/6 and iNOS$^{-/-}$ [62] mice were infected at MOI of 2, and survival was determined after 16–20 h of challenge.

### Murine infections

These following studies were approved by the Institutional Animal Care and Use Committee at the University of Colorado—Denver Anschutz Medical Campus. Eight to ten week-old C57BL/6 and congenic iNOS$^{-/-}$ mice were infected i.p. with 100–200 CFU of the indicated *Salmonella* strains. Mouse survival was monitored over time.

### Thin layer chromatography estimations of ATP pools

Wild-type *Salmonella* were grown for 20 h in MOPS supplemented with 2 mM HK$_2$PO$_4$ and either glucose, casamino acids, or glucose and casamino acids. The overnight cultures were
diluted 1:100 into MOPS supplemented with the same carbon source and 0.4 mM HK$_2$PO$_4$. The bacteria were grown to an OD$_{600}$ of 0.2 and then labeled with 10 μCi of $^{32}$P orthophosphate in 1 mL aliquots. After approximately 2.5 generations, at an OD$_{600}$ of 0.5, cultures were either left untreated or were challenged for 5 min with 750 μM spermine NONOate. One mL cultures were mixed with 0.4 mL of ice-cold 50% formic acid and samples were put on ice for at least 15 min prior to centrifugation for 5 min at 13,000 RPM. 10 μL of lysates were spotted along the bottom of polyethyleneimine-cellulose TLC plates (EMD Millipore, Darmstadt, Germany). The TLC plates were air-dried and then placed into a chromatography chamber containing either 1.25 or 0.9 M KH$_2$PO$_4$, pH 3.4. The solvent system was allowed to migrate 15–19 cm up the 20 cm TLC plate. The plates were air-dried, placed inside plastic wrap, and placed on a phosphorscreen overnight. The following day the screens were scanned with a phosphorimager. Images were cropped and brightness and contrast were adjusted in PhotoShop 11.0.

**Firefly luciferase estimations of ATP pools**

Intracellular pools of ATP were calculated with the luciferase-based ATP Determination Kit (Molecular Probes, Eugene, OR) as instructed by the manufacturer with a few minor adjustments. Briefly, wild-type *Salmonella* grown to an OD$_{600}$ of 0.2–0.5 were either left untreated or challenged with 750 μM spermine NONOate for 5 min. Samples (0.5 mL culture) were thoroughly mixed with 0.6 mL freshly prepared, ice-cold 380 mM formic acid and 17 mM EDTA, and small samples were taken to quantify bacterial density. Formic acid-EDTA samples were saved at -80˚C until assayed. Specimens were centrifuged for 1 min at 13,000 RPM, and the supernatants were diluted 25-fold into 100 mM TES buffer, pH 7.4 to neutralize the formic acid. Ten μL of samples or solutions with known concentrations of ATP were mixed with 90 μL of reaction master mix (10 mL; 8.9 mL water, 500 μL of 20X buffer, 500 μL of 10 mM D-luciferin, 100 μL of 100 mM DTT, and 2.5 μL of 5 mg/mL firefly luciferase) in white 96-well plates, and luminescence was recorded in a Lmax 1.1L machine (Molecular Devices, San Jose, CA), and the data analyzed using the SOFTmax Pro software. Concentrations of ATP in the culture were generated using linear regression of ATP standards. Intracellular ATP concentrations of the original samples were calculated using the CFU/mL counts assuming a cell volume of 1 fL.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5.0b Software. One-way and two-way ANOVA, t-tests and logrank tests were used. Results were determined to be significant when $p < 0.05$.

**Supporting information**

S1 Table. All genes.
(XLSX)

S2 Table. Genes for Venn diagram.
(XLSX)

S3 Table. Shared genes.
(XLSX)

S4 Table. NO-related genes.
(XLSX)
S5 Table. Zinc-related genes.
(XLSX)

S6 Table. Bacterial strains.
(DOCX)

S7 Table. Primers.
(DOCX)

S1 Fig. Effect of carbon source on the recovery of Salmonella from S-nitrosylglutathione (GSNO) and diethylenetriamine NONOate (dNO). (A) The time (min) required for Salmonella to double the initial culture density was calculated for bacteria growing in MOPS minimal media supplemented with either glucose (GLC), casamino acids (CAA), or glucose and casamino acids (GLC + CAA) with or without 750 μM spermine NONOate challenge (N = 4, mean ± S.E.M.). * , ** , *** , p < 0.05, 0.01, 0.001, respectively, as determined by one-way ANOVA. Salmonella grown in MOPS minimal media supplemented with either GLC, CAA, or GLU + CAA were either untreated (ctrl) or challenged with 5 mM of either GSNO (B) or dNO (C). Bacterial growth was estimated by following OD_{600} measurements every hour. (D) Growth of wild-type (WT) and ΔpfkAB Salmonella in EG minimal media or MOPS minimal media supplemented with glycerol (N = 12, mean).
(DOCX)

S2 Fig. Recovery of Salmonella deficient in Zn^{2+} metabolism from nitrosative stress. Delay in growth (A) and rate of growth (B) of wild-type (WT) and ΔznuB Salmonella in EG, ECA, and EGCA minimal media. Select samples were supplemented with 5 μM ZnCl_{2} or challenged with 1 mM diethylenetriamine (DETA) or DETA NONOate (dNO) (N = 5 or 10, mean ± S.E.M.). (C) Growth of ΔznuB Salmonella complemented with a wild-type znuB gene in EG media. Selected samples were treated with 1 mM dNO. (D) Growth of WT and Δzur, ΔznuA, ΔznuB, and ΔznuC Salmonella in EGCA minimal media challenged with either 5 mM DETA or 5 mM dNO (N = 10, mean). Growth rates (E) and delays (F) were calculated by exponential regression (N = 10, mean ± S.E.M.). (G, H) Replication of Salmonella after 16–20 h of culture in J774 cells. Selected samples were treated with 500 μM L-NIL. (I, K) The concentration of nitrite in the supernatants of Salmonella-infected macrophages was estimated by the Griess reaction (N = 16, mean ± S.E.M.). * , ** , *** , p < 0.05, 0.01, 0.001, respectively, as determined by two-way ANOVA or t-test. (J) Effect of AG or L-NIL on the growth of Salmonella in EG media.
(DOCX)

S3 Fig. Nitrotyrosine formation in NO-treated Salmonella. Salmonella were grown in EG minimal media to OD_{600} of 0.4 at 37˚C with shaking. The bacteria were lysed by sonication and the specimens were tested for the presence of nitrotyrosine residues by Western blotting as described [1]. Where indicated (+), the bacteria were treated with 500 μM spermine NONOate for 30 min prior to sonication. The blot is representative of 2 independent samples. * , proteins nonspecific labeled by the anti-nitrotyrosine antibodies. Arrows indicate proteins bearing nitrotyrosine residues.
(DOCX)

S4 Fig. Growth and recovery of glycolysis and ATP synthesizing mutants. Growth delay (A) and rate (B) of wild-type (WT) and ΔpfkAB Salmonella in LB broth challenged with 5 mM DETA NONOate were calculated by exponential regression (N = 5, mean ± S.E.M.). (C) NO production from J774 cells infected with Salmonella was estimated by the Griess reaction (N = 4 or 8, mean ± S.E.M.). Replication of ΔpfkAB Salmonella complemented with pfkA or pfkB genes in J774 cells (D) or EG media +/- 1 mM dNO (E). (F) NO production from J774...
cells infected with WT, ΔatpB, and ΔackA Δpta Salmonella was estimated by the Griess reaction (N = 4 or 8, mean ± S.E.M.). *, **, ***; p < 0.05, 0.01, 0.001, respectively, as determined by two-way ANOVA.

(DOCX)

S5 Fig. ATP pools in ΔatpB Salmonella. (A) ATP pools in WT, ΔatpB, and ΔackA Δpta Salmonella was estimated with firefly luciferase and normalized to culture density (N = 6, mean ± S.E.M.). Selected cultures were treated with 750 μM spermine NONOate (sNO). (B) Effect of 750 μM spermine NONOate (sNO) on the growth of Salmonella. The ΔpykAF ΔackA Δpta mutant was complemented with the low copy number plasmid pWSK29 harboring the ackA pta operon (pACKPTA). (C) Intracellular growth of the indicated Salmonella strains after 16h of culture in J774 cells. (D) Production of nitrite by Salmonella-infected, periodate-elicited macrophages was estimated spectrophotometrically by the Griess reaction.

(DOCX)

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References

1. Ao TT, Feasey NA, Gordon MA, Keddy KH, Angulo FJ, Crump JA. Global burden of invasive nontyphoidal Salmonella disease, 2010(1). Emerg Infect Dis. 2015; 21(6). https://doi.org/10.3201/eid2106.140999 PMID: 25860298; PubMed Central PMCID: PMCPM4451910.

2. Wieelhoetter AK, Beltran-Alcrudo D, Kock R, Mor SM. Global trends in infectious diseases at the wildlife-livestock interface. Proc Natl Acad Sci U S A. 2015; 112(31):9662–7. https://doi.org/10.1073/pnas.1422741112 PMID: 26195733; PubMed Central PMCID: PMCPMC4534210.

3. Alvarez-Ordonez A, Prieto M, Bernardo A, Hill C, Lopez M. The Acid Tolerance Response of Salmonella spp.: An adaptive strategy to survive in stressful environments prevailing in foods and the host. Food Research International. 2012; 45(2):482–92. https://doi.org/10.1016/j.foodres.2011.04.002 WOS:000302032200003.

4. Que FX, Wu SY, Huang R. Salmonella Pathogenicity Island 1(SPI-1) at Work. Current Microbiology. 2013; 66(6):582–7. https://doi.org/10.1007/s00284-013-0307-8 WOS:000317893000009. PMID: 23370732

5. Bueno SM, Wozniak A, Leiva ED, Riquelme SA, Carreno LJ, Hardt WD, et al. Salmonella pathogenicity island 1 differentially modulates bacterial entry to dendritic and non-phagocytic cells. Immunology. 2010; 130(2):273–87. https://doi.org/10.1111/j.1365-2567.2009.03233.x WOS:000277411900013. PMID: 20201987
6. Henard CA, Vazquez-Torres A. Nitric oxide and *Salmonella* pathogenesis. Front Microbiol. 2011; 2:84. Epub 2011/08/13. https://doi.org/10.3389/fmicb.2011.00084 PMID: 21833325; PubMed Central PMCID: PMCPMC3153045.

7. Mastroeni P, Vazquez-Torres A, Fang FC, Xu Y, Khan S, Hormaeche CE, et al. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. J Exp Med. 2000; 192:237–48. Epub 2000/07/19. PMID: 10899910; PubMed Central PMCID: PMCPMC193252.

8. Aktan F. iNOS-mediated nitric oxide production and its regulation. Life Sci. 2004; 75:639–53. https://doi.org/10.1016/j.lfs.2003.10.042 PMID: 15172174.

9. Husain M, Bouret TJ, McCollister BD, Jones-Carson J, Laughlin J, Vazquez-Torres A. Nitric oxide evokes an adaptive response to oxidative stress by arresting respiration. J Biol Chem. 2008; 283:7682–9. Epub 2008/01/17. M708845200 [pii] https://doi.org/10.1074/jbc.M708845200 PMID: 18198179.

10. Richardson AR, Payne EC, Younger N, Karlinsey JE, Thomas VC, Becker LA, et al. Multiple targets of nitric oxide in the tricarboxylic acid cycle of *Salmonella enterica* serovar typhimurium. Cell Host Microbe. 2011; 10:33–43. Epub 2011/07/20. https://doi.org/10.1016/j.chom.2011.06.004 PMID: 21767810; PubMed Central PMCID: PMCPMC3142370.

11. Fitzsimmons LF, Liu L, Kim JS, Jones-Carson J, Vazquez-Torres A. *Salmonella* Reprograms Nucleotide Metabolism in Its Adaptation to Nitrosative Stress. MBio. 2018; 9. https://doi.org/10.1128/mBio.00211-18 PMID: 29468278; PubMed Central PMCID: PMCPMC5682828.

12. Bourret TJ, Boylan JA, Lawrence KA, Gherardini FC. Nitrosative damage to free and zinc-bound cysteine thiols underlies nitric oxide toxicity in wild-type *Borrelia burgdorferi*. Mol Microbiol. 2011; 81:259–73. https://doi.org/10.1111/j.1365-2958.2011.07681.x PMID: 21564333; PubMed Central PMCID: PMCPMC3147059.

13. Henard CA, Tapscott T, Crawford MA, Husain M, Doulias PT, Porwollik S, et al. The 4-cysteine zinc-finger motif of the RNA polymerase regulator DksA serves as a thiol switch for sensing oxidative and nitrosative stress. Mol Microbiol. 2014; 91:790–804. Epub 2013/12/21. https://doi.org/10.1111/mmi.12498 PMID: 24354846.

14. Imlay JA. The mismetallation of enzymes during oxidative stress. J Biol Chem. 2014; 289:28121–8. https://doi.org/10.1074/jbc.R114.588814 PMID: 25160623; PubMed Central PMCID: PMCPMC4192467.

15. Imlay JA, Chin SM, Linn S. Toxic DNA damage by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro*. Science. 1988; 240:640–2. PMID: 2834621.

16. Jones-Carson J, Laughlin JR, Stewart AL, Voskul MI, Vazquez-Torres A. Nitric oxide-dependent killing of aerobic, anaerobic and persistent *Burkholderia pseudomallei*. Nitric Oxide. 2012; 27:25–31. https://doi.org/10.1016/j.niox.2012.04.001 PMID: 22252123; PubMed Central PMCID: PMCPMC3517295.

17. Workman AD, Kohanski MA, Kennedy DW, Palmer JN, Adappa ND, et al. Relative susceptibility of airway organisms to antimicrobial effects of nitric oxide. Int Forum Allergy Rhinol. 2017. https://doi.org/10.1002/alr.21966 PMID: 28544570.

18. Jones-Carson J, Laughlin J, Hamad MA, Stewart AL, Voskul MI, Vazquez-Torres A. Inactivation of [Fe-S] metalloproteins mediates nitric oxide-dependent killing of *Burkholderia mallei*. PLoS One. 2008; 3: e1976. https://doi.org/10.1371/journal.pone.0001976 PMID: 1898646; PubMed Central PMCID: PMCPMC2276317.

19. Hattrick EM, Shin JH, Paul HS, Schenfisch MH. Anti-biofilm efficacy of nitric oxide-releasing silica nanoparticles. Biomaterials. 2009; 30:2782–9. https://doi.org/10.1016/j.biomaterials.2008.09.052 PMID: 19233464; PubMed Central PMCID: PMCPMC2692680.

20. Long R, Light B, Talbot JA. Mycobacteriocidal action of exogenous nitric oxide. Antimicrob Agents Chemother. 1999; 43:403–5. PMID: 9925545; PubMed Central PMCID: PMCPMC89090.

21. Richardson AR, Dunnan PM, Fang FC. The nitrosative stress response of *Staphylococcus aureus* is required for resistance to innate immunity. Molecular Microbiology. 2006; 61:927–39. https://doi.org/10.1111/j.1365-2958.2006.09290.x WOS:000239336100008 PMID: 16859493.

22. De Groot MA, Granger D, Xu Y, Campbell G, Prince R, Fang FC. Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. Proc Natl Acad Sci U S A. 1995; 92:6399–403. PMID: 7604033.

23. Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC. Antimicrobial actions of the NAPDH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. Journal of Experimental Medicine. 2000; 192(2):227–36. https://doi.org/10.1084/jem.192.2.227 WOS:000088261100009 PMID: 10899909.
24. Song M, Husain M, Jones-Carson J, Liu L, Henard C, Vázquez-Torres A. Low-molecular-weight thiol-dependent antioxidant and antinitrosative defences in *Salmonella* pathogenesis. Molecular microbiology. 2013; 87:609–22. https://doi.org/10.1111/mmi.12119 PMID: 23217033

25. Bang IS, Liu L, Vázquez-Torres A, Crouch ML, Stamler JS, Fang FC. Maintenance of nitric oxide and redox homeostasis by the *Salmonella* flavohemoglobin hmp. J Biol Chem. 2006; 281:28039–47. https://doi.org/10.1074/jbc.M605174200 PMID: 16873371

26. Eriksson S, Bjorkman J, Borg S, Syk A, Pettersson S, Andersson DI, et al. *Salmonella typhimurium* mutants that downregulate phagocyte nitric oxide production. Cellular Microbiology. 2000; 2:239–50. https://doi.org/10.1046/j.1462-5822.2000.00051.x WOS:000087726000006. PMID: 11207580

27. Georgiades K. Genomics of epidemic pathogens. Clin Microbiol Infect. 2012; 18(3):213–7. https://doi.org/10.1111/odi.12119 PMID: 22369153

28. Steeb B, Claudi B, Burton NA, Tienz P, Schmidt A, Farhan H, et al. Parallel exploitation of diverse host nutrients enhances *Salmonella* virulence. PLoS Pathog. 2013; 9:e1003301. https://doi.org/10.1371/journal.ppat.1003301 PMID: 23633950; PubMed Central PMCID: PMCPMC3636032.

29. Hyduke DR, Jarboe LR, Tran LM, Chou KJ, Liao JC. Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in *Escherichia coli*. Proc Natl Acad Sci U S A. 2007; 104:8484–9. Epub 2007/05/15. 0610888104 [pii] https://doi.org/10.1073/pnas.0610888104 PMID: 17494765; Pubmed Central PMCID: PMC1895976.

30. Patzer SI, Hantke K. The zinc-responsive regulator Zur and its control of the znu gene cluster encoding the ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol Microbiol. 1998; 28:119–20. PMID: 9680209.

31. De Groote MA, Ochsner UA, Shiloh MU, Nathan C, McCord JM, Dinauer MC, et al. Periplasmic superoxide dismutase protects Escherichia coli. Proc Natl Acad Sci U S A. 1997; 94:13997–4001. PMID: 9391141.

32. Ammendola S, Pasquali P, Pistoia C, Petrucci P, Petrarca P, Rotilio G, et al. High-affinity Zn2+ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of *Salmonella enterica*. Infect Immun. 2007; 75:5867–76. https://doi.org/10.1128/IAI.00559-07 PMID: 17923515; Pubmed Central PMCID: PMC1895976.

33. Zgiby SM, Thomson GJ, Qamar S, Berry A. Exploring substrate binding and discrimination in fructose1,6-bisphosphate and tagatose 1,6-bisphosphate aldolases. Eur J Biochem. 2000; 267:1858–68. Epub 2000/03/11. PMID: 10712619.

34. Bourret TJ, Liu L, Shaw JA, Husain M, Vazquez-Torres A. Magnesium homeostasis protects *Salmonella* against nitrooxidative stress. Sci Rep. 2017; 7:15083. https://doi.org/10.1038/s41598-017-15445-y PMID: 29118452; PubMed Central PMCID: PMCPMC5678156.

35. Gort AS, Ferber DM, Imlay JA. The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. Mol Microbiol. 1999; 32:179–91. PMID: 10268171.

36. Paterson GK, Cone DB, Peters SE, Maskell DJ. Redundancy in the requirement for the glycolytic enzymes phosphofructokinase (Pfk) 1 and 2 in the in vivo fitness of *Salmonella enterica serovar Typhimurium*. Microbial Pathogenesis. 2009; 46:261–5. https://doi.org/10.1016/j.micpath.2009.01.008 WOS:000266150600004. PMID: 19486643.

37. Bowden SD, Rowley G, Hinton JC, Thompson A. Glucose and glycolysis are required for the successful infection of macrophages and mice by *Salmonella enterica serovar Typhimurium*. Infect Immun. 2009; 77:3117–26. https://doi.org/10.1128/IAI.00093-09 PMID: 19380470; PubMed Central PMCID: PMCPMC2708584.

38. Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM. Metal ions in biological catalysis: from enzyme databases to general principles. J Biol Inorg Chem. 2008; 13:1205–18. https://doi.org/10.1007/s00775-008-0404-5 PMID: 18604568.

39. Crawford MA, Tapsott T, Fitzsimmons LF, Liu L, Reyes AM, Libby SJ, et al. Redox-Active Sensing by Bacterial DisksA Transcription Factors Is Determined by Cysteine and Zinc Content. MBio. 2016; 7: e02161–15. https://doi.org/10.1128/mBio.02161-15 PMID: 27094355; Pubmed Central PMCID: PMCPMC4850274.

40. Palmer LD, Skaar EP. Transition Metals and Virulence in Bacteria. Annual Review of Genetics, Vol 50. 2016; 50:67–81. https://doi.org/10.1146/annurev-genet-120215-035146 WOS:000389584900004. PMID: 27617971.

41. Liu JZ, Jellbauer S, Poe AJ, Ton V, Pesciaroli M, Kehl-Fie TE, et al. Zinc sequestration by the neutrophil protein calprotectin enhances *Salmonella* growth in the inflamed gut. Cell Host Microbe. 2012; 11:227–39. https://doi.org/10.1016/j.chom.2012.01.017 PMID: 22423963; Pubmed Central PMCID: PMCPMC3308348.
53. Porwollik S, Santiviago CA, Cheng P, Long F, Desai P, Fredlund J, et al. Defined single-gene and multi-gene deletion mutant collections in Salmonella enterica sv Typhimurium. PLoS Pathog. 2014; 9:e99820. https://doi.org/10.1371/journal.ppat.1000477 PMID: 25007190; PubMed Central PMCID: PMCPMC4089911.

54. Santiviago CA, Reynolds MM, Porwollik S, Choi SH, Long F, Andrews-Polymenis HL, et al. Analysis of pools of targeted Salmonella deletion mutants identifies novel genes affecting fitness during competitive infection in mice. PLoS Pathog. 2009; 5:e1000477. Epub 2009/07/07. https://doi.org/10.1371/journal.ppat.1000477 PMID: 19578432; PubMed Central PMCID: PMCPMC2698986.

55. de Moraes MH, Desai P, Porwollik S, Canals R, Perez DR, Chu W, et al. Salmonella Persistence in Tomatoes Requires a Distinct Set of Metabolic Functions Identified by Transposon Insertion Sequencing. Appl Environ Microbiol. 2017; 83. Epub 2017/01/01. https://doi.org/10.1128/AEM.03028-16 PMID: 28039131; PubMed Central PMCID: PMCPMC511394.

56. Neidhardt FC, Bloch PL, Smith DF. Culture medium for enterobacteria. J Bacteriol. 1974; 119:736–47. PMID: 4604283; PubMed Central PMCID: PMCPMC245675.

57. Crawford MA, Henard CA, Tappcott T, Porwollik S, McClennand M, Vazquez-Torres A. DksA-Dependent Transcriptional Regulation in Salmonella Experiencing Nitrosative Stress. Front Microbiol. 2016; 7:444. https://doi.org/10.3389/fmicb.2016.00444 PMID: 27065993; PubMed Central PMCID: PMCPMC4815678.

58. Henard C, Vázquez-Torres A. DksA-dependent resistance of Salmonella enterica serovar Typhimurium against the antimicrobial activity of inducible nitric oxide synthase. Infection and immunity. 2012; 80:1373–80. https://doi.org/10.1128/IAI.06316-11 PMID: 22311927.

59. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res. 2012; 40:4288–97. Epub 2012/01/31. https://doi.org/10.1093/nar/gks402 PMID: 22287627; PubMed Central PMCID: PMCPMC3379882.

60. Vazquez-Torres A, Stevanin T, Jones-Carson J, Castor M, Read RC, Fang FC. Analysis of nitric oxide-dependent antimicrobial actions in macrophages and mice. Methode Enzymol. 2008; 437:521–38. https://doi.org/10.1016/S0076-8929(07)37026-2 PMID: 18433645; PubMed Central PMCID: PMCPMC2704381.

61. McCollister BD, Bourret TJ, Gill R, Jones-Carson J, Vazquez-Torres A. Repression of SPI2 transcription by nitric oxide-producing, IFNγ-activated macrophages promotes maturation of Salmonella phagosomes. J Exp Med. 2005; 202:625–35. https://doi.org/10.1084/jem.20050246 PMID: 16129704.
62. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, et al. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell. 1995; 81:641–50. PMID: 7538909.