Ferric Uptake Regulator-Dependent Antinitrosative Defenses in *Salmonella enterica* Serovar Typhimurium Pathogenesis

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Herein we report an important role for the ferric uptake regulator (Fur) in the resistance of *Salmonella enterica* serovar Typhimurium to the reactive nitrogen species produced by inducible nitric oxide (NO) synthase in an NRAMP1[r] murine model of acute systemic infection. The expression of *fur* protected *Salmonella* grown under normoxic and hypoxic conditions against the bacteriostatic activity of NO. The hypersusceptibility of *fur*-deficient *Salmonella* to the cytotoxic actions of NO coincides with a marked repression of respiratory activity and the reduced ability of the bacteria to detoxify NO. A *fur* mutant *Salmonella* strain contained reduced levels of the terminal quinol oxidases of the electron transport chain. Addition of the heme precursor δ-aminolevulinic acid restored the cytochrome content, respiratory activity, NO consumption, and wild-type growth in bacteria undergoing nitrosative stress. The innate antinitrosative defenses regulated by Fur added to the adaptive response associated with the NO-detoxifying activity of the flavohemoprotein Hmp. Our investigations indicate that, in addition to playing a critical role in iron homeostasis, Fur is an important antinitrosative determinant of *Salmonella* pathogenesis.

almonellosis encompasses a spectrum of clinical diseases ranging from typhoid fever, a serious condition that kills about 600,000 people annually (http://www.who.int/inf-pr-1998/en/pr98-18.html), to nontyphoidal zoonotic infections that afflict more than 1.4 million Americans a year (http://www.who.int/mediacentre/factsheets/fs139/en/). The parasitic relationship between *Salmonella* and host mononuclear phagocytes is one of the hallmarks of both typhoidal and nontyphoidal salmonellosis (1). Within macrophages, *Salmonella* is sequentially exposed to the antimicrobial activity of the NADPH oxidase and inducible nitric oxide (NO) synthase (iNOS) flavohemoproteins (2). Following the initial oxidative burst emanating from the NADPH oxidase (2), reactive nitrogen species derived from iNOS-expressing human (3–5) and murine (2, 6) macrophages exert sustained bacteriostatic activity at later stages of the intracellular *Salmonella* infection.

The coordinated actions of low-molecular-weight thiols and superoxide dismutases detoxify a variety of reactive nitrogen species produced from the reaction of NO with either oxygen (O_2) or superoxide anion (7–9). These innate detoxification systems are strengthened upon the induction of Hmp, a flavohemoprotein that denitrosylates NO to nitrate (NO_3^-) (10), thereby protecting *Salmonella* against NO congeners produced by human and murine macrophages (3, 11, 12). Overall, the enzymatic activity of Hmp appears to be the most important antinitrosative defense in *Salmonella* pathogenesis (11). *Salmonella* can also diminish the extent of nitrosative stress endured in macrophages through the expression of the *Salmonella* pathogenicity island 2 type III secretion system that excludes iNOS-containing vesicles from phagosomes (13). Despite these protective mechanisms, reactive nitrogen species must damage *Salmonella* biomolecules, as demonstrated by the fact that DNA damage repair systems contribute to the antinitrosative arsenal of this enteric pathogen (14). Many of these antinitrosative defenses are carefully orchestrated at the transcriptional level. The proteins NsrR and NorR are NO-dedicated sensors that regulate genes encoding antinitrosative ac-

tivity in aerobic and anaerobic bacteria (15, 16), whereas OxyR, SoxR, FNR, DksA, and Fur, which primarily serve as sensors of reactive oxygen species, O_2, nutritional starvation, and iron, contribute important aspects of the antinitrosative arsenal of enteric bacteria (17–21).

Traditionally, the ferric uptake regulator (Fur) has been described to be a major determinant in the control of iron homeostasis. Binding of Fe^{2+} Fur to −35 and −10 promoter regions represses transcription by interfering with the RNA polymerase, whereas binding to upstream regions activates transcription (22). In addition to serving as a cofactor that modulates Fur activity in response to intracytoplasmic metal concentrations, the iron cofactor is a sensor of nitrosative stress, as indicated by the fact that iron-nitrosyl Fur complexes derepress gene transcription (18). The ability of Fur to sense nitrosative stress appears to be important for the antinitrosative defenses of *Bacillus subtilis*, *Staphylococcus aureus*, *Helicobacter pylori*, and *Escherichia coli* (23–26). Because Fur has recently been shown to play a critical role in *Salmonella* virulence (27), we tested whether Fur regulates antinitrosative defenses in *Salmonella* pathogenesis.

MATERIALS AND METHODS

Bacterial strains. *Salmonella enterica* serovar Typhimurium strain ATCC 14028s was used as the wild type and as a background for the construction

TABLE 1 Bacterial strains

S. enterica serovar Typhimurium strain	Relevant characteristic	Source or reference
14028s	Wild type	ATCC
AV13110	Δ*hmp*::km	6
AV0468	Δ*hmp*::FRT[a]	6
KLM001	Δ*fur*::bla	28
AV10274	Δ*fur*::bla Δ*hmp*::km	This study

[a] FRT, FLP recombination target.

of mutants (Table 1). The *hmp*, *hmp*::km, and *fur*::bla mutants used in the course of our investigations have been described earlier (6, 28). The mutations were confirmed by PCR analysis. A Δ*hmp*::km mutation was moved from strain AV13110 into *S.* Typhimurium strain KLM001 by P22-mediated transduction to generate strain AV10274. Pseudolysogens were eliminated by streaking on Evans blue uranine agar plates.

Cytochrome spectroscopy. Wild-type and *fur* mutant *Salmonella* strains grown overnight in LB broth were subcultured in fresh LB broth to an optical density at 600 nm (OD_{600}) of 0.5. Where indicated, some of the cultures were supplemented with 100 μM δ-aminolevulinic acid (δ-ALA). Inner membranes were prepared as described by Husain et al. (29). Briefly, bacterial pellets were resuspended in 10 mM EDTA, 100 mM Tris HCl buffer, pH 8.5. The bacteria were lysed by passing the cell suspension through a French press cell disruptor (Thermo Electron Corporation, Milford, MA) 3 times at 18,000 lb/in^2 at a flow rate of 5 ml/min. Cell debris was removed after centrifugation at 10,000 × g for 20 min. The supernatants were then centrifuged at 200,000 × g for 1 h, and the pellets were solubilized in 75 mM K_2HPO_4, 150 mM KCl, 5 mM EDTA, and 60 mM *N*-dodecyl-*N*,*N* dimethyl-3-ammonio-1-propane sulfonate buffer, pH 6.4. Supernatants containing inner membranes were collected, and the protein concentration was assayed using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL). The protein concentration was adjusted to 1.5 mg/ml in 75 mM K_2HPO_4, 150 mM KCl, 5 mM EDTA, 10 mM ascorbate, and 60 mM *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate buffer, pH 6.4. Spectra were collected by absorbance spectroscopy with a Hewlett-Packard diode array spectrophotometer (model 8452A).

O_2 and NO measurements. Overnight *Salmonella* cultures diluted 1:100 in EG medium (1.66 mM $MgSO_4$, 9.5 mM citric acid monohydrate, 57 mM K_2HPO_4, 16.7 mM $NaNH_3PO_4$, 0.4% [wt/vol] glucose) were grown to an OD_{600} of 0.5 in a shaker incubator at 37°C. The cultures were diluted to an OD_{600} of 0.2 with EG medium and equilibrated in a shaker incubator at 37°C for 3 min before transfer into an air-sealed, multiport measurement chamber equipped with ISO-OXY-2 O_2 and ISO-NOP NO probes. The evolution of O_2 and NO in the cultures was recorded with an Apollo 4000 free radical analyzer (World Precision Instruments, Inc., Sarasota, FL). Selected cultures were supplemented with 100 μM δ-ALA. The fast inhibition of respiratory activity in response to NO was studied by adding 5 μM proli NONOate [1-(hydroxy-NNO-azoxy)-L-proline] (half-life [$t_{1/2}$] = 1.8 s at 37°C) to the bacterial cultures when the concentration of O_2 in the chamber was ~130 μM. To assess the ability of the bacteria to adapt to NO, O_2 consumption was also studied in cultures treated for 10 min with 50 μM spermine NONOate ($t_{1/2}$ = 39 min at 37°C). The rates of oxygen consumption supported by bacteria at a cell density of an OD_{600} of 1 are expressed as μM/s. The concentration of NO in the cultures is expressed as μM.

Bacterial growth kinetics. The growth kinetics of wild-type and mutant *Salmonella* were studied using a Bioscreen C growth analyzer (Labsystems, Helsinki, Finland). *Salmonella* strains grown overnight in LB broth were diluted to 2 × 10^6 CFU/ml with fresh LB broth. The cultures were treated with 2.5 to 7.5 mM diethylentriamine (DETA) polyamine control or the NO donor DETA NONOate. Two hundred microliters of the bacterial suspension was dispensed into a Bioscreen 100-well microtiter plate. For analysis of growth under a normoxic environment, the

microtiter plates were incubated at 37°C for 30 h with continuous shaking. The OD_{600} of the cultures was recorded every 15 min. To study the effect of δ-ALA on normoxic growth, some of the cultures diluted 1:100 in LB broth were supplemented with 100 μM δ-ALA until they reached an OD_{600} of 0.5. The cultures were then diluted to 2 × 10^6 CFU/ml with fresh LB broth, and the bacterial growth was recorded under normoxic conditions as described above. To measure growth under hypoxic conditions, the bacterial cultures in the microtiter plates were overlaid with 50 μl of mineral oil. The cultures were incubated at 37°C for 30 h without shaking, and the bacterial density at 600 nm was recorded every 15 min.

Bacterial virulence in mice. Eight- to 10-week-old NRAMP1^r C3H/ HeN mice were bred at the animal facility of the University of Colorado School of Medicine according to the University of Colorado at Denver, Anschutz Medical Campus, Institutional Animal Care and Use Committee guidelines. NRAMP1^r C3H/HeN mice were challenged intraperitoneally with 3 × 10^3 CFU/mouse of wild-type or isogenic *fur* mutant *Salmonella* prepared in phosphate-buffered saline from overnight cultures grown in LB broth. To evaluate the role of Fur in the resistance of *Salmonella* to iNOS-dependent cytotoxicity, the drinking water of selected groups of C3H/HeN mice was supplemented with 500 μg/ml of the specific iNOS inhibitor N^6-(1-iminoethyl)-L-lysine, dihydrochloride (L-NIL). The survival of *Salmonella*-infected mice was recorded over time.

Statistical analysis. Data are presented as the mean ± standard error of the mean or standard deviation. The statistical significance was calculated with a two-way analysis of variance, followed by a Bonferroni posttest. The statistical significance of the difference in O_2 consumption between the control and spermine NONOate-treated cells was calculated using a paired Student's *t* test. Differences in mouse survival after *Salmonella* infection were determined by a log-rank Martel-Cox test. Data were considered statistically significant when the *P* value was <0.05.

RESULTS

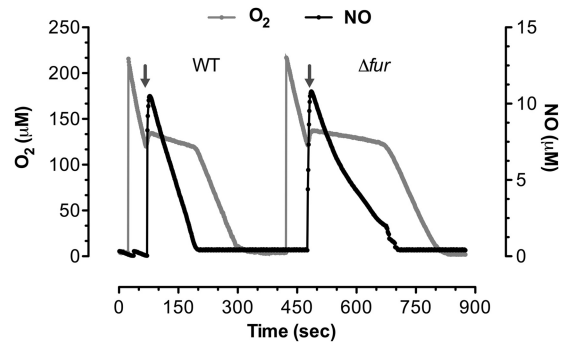**Fur contributes to the antinitrosative defenses of *Salmonella*.** The cytoplasm of *E. coli* exposed to NO harbors nitrosyl-iron Fur complexes; and *fur* mutants of *E. coli*, *H. pylori*, *S. aureus*, and *B. subtilis* are hypersusceptible to the cytotoxicity of this diatomic radical. We therefore tested whether Fur contributes to the antinitrosative armamentarium of *Salmonella*. Wild-type and *fur* mutant *Salmonella* strains grown overnight to stationary phase in LB broth were diluted 1:100 in fresh LB broth. The cultures were treated with 7.5 mM either DETA NONOate or the DETA polyamine base. The *fur* mutant grew similarly to the isogenic wild-type controls during the first 6 h of culture in LB broth supplemented with DETA but exhibited slightly lower growth rates once the bacterial cells entered late log phase (Fig. 1A). The overall doubling times of wild-type and *fur* mutant bacteria growing in LB broth were 1.1 and 1.7 h, respectively. The addition of 7.5 mM DETA NONOate, which generates about 7.5 μM NO for the duration of the experiment (30), inhibited the growth of wild-type bacteria. The *fur* mutant was even more sensitive to the bacteriostatic activity of DETA NONOate, showing an extended lag period compared to wild-type controls. The doubling time of wild-type *Salmonella* after the initial bacteriostasis imposed upon NO treatment was similar to that of the untreated controls (1.2 versus 1.1 h, respectively). In comparison to the untreated controls, NO significantly (*P* < 0.001) slowed the doubling time (i.e., 3.9 h) of *fur* mutant *Salmonella*. Cumulatively, these data indicate that *Salmonella fur* mutant strains are hypersusceptible to NO during both the lag and log phases of growth. We also tested the anti-*Salmonella* activity of NO under O_2-limiting conditions (Fig. 1B). To attain a hypoxic environment, 50 μl of sterile mineral oil was overlaid on top of the bacterial cultures. The addition of 7.5 mM DETA NONOate was highly inhibitory for both wild-type and *fur*
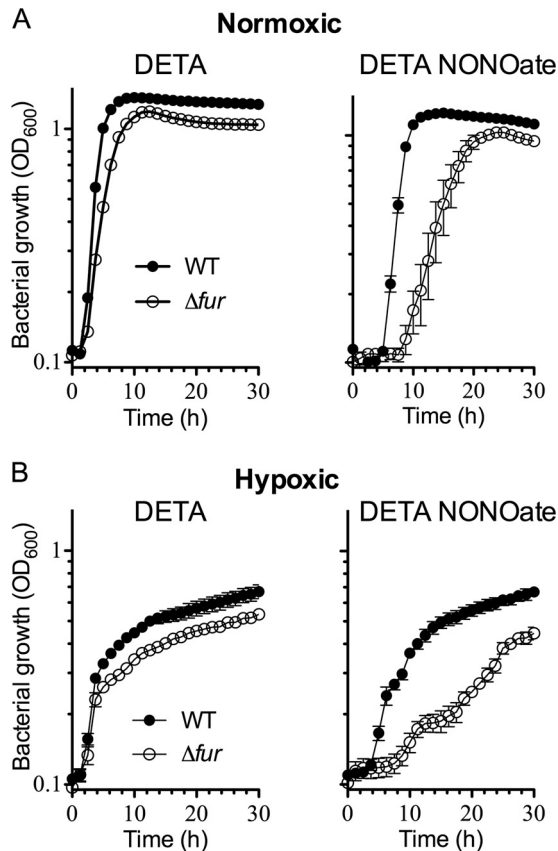
FIG 1 Effect of NO on bacterial growth. (A) Growth of wild-type (WT) *Salmonella* and its isogenic *fur* mutant in LB broth supplemented with either 7.5 mM DETA or DETA NONOate in a 100-well microtiter plate with shaking (normoxic). (B) Bacterial growth was also monitored in standing cultures overlaid with mineral oil after treatment with 2.5 mM DETA or DETA NONOate (hypoxic). The data are representative of those from experiments performed on three independent days with five replicates each day.

FIG 2 Consumption of O_2 by *fur* mutant *Salmonella* undergoing nitrosative stress. The consumption of O_2 was recorded in *Salmonella* grown to an OD_{600} of 0.5 in EG medium. The bacteria were diluted to an OD_{600} of 0.2 in an air-sealed chamber. Bacterial cultures were treated with 5 μM proli NONOate (arrows) at a time when the concentration of O_2 in the medium was about 130 μM. The evolution of NO in the cultures was also recorded. The data are representative of those from three independent experiments performed on three separate days.

mutant bacteria grown under hypoxic conditions. Therefore, we tried lower concentrations of DETA NONOate. It was found that *fur* mutant bacteria grown under hypoxic conditions were hypersusceptible to 2.5 mM DETA NONOate compared to wild-type *Salmonella*. Together, these data indicate that Fur contributes to the antinitrosative defenses of *Salmonella*.

Fur protects the respiratory activity of *Salmonella* undergoing nitrosative stress. NO nitrosylates a variety of biomolecules, including [4Fe-4S] clusters, redox-active cysteines, organic radicals, and quinol oxidases of the electron transport chain. NO reacts with metal groups in the cytochromes of terminal oxidases of the electron transport chain with k_{on} (association constant) rates of 1.25×10^7 to 2.3×10^8 M^{-1} s^{-1} (31), making these biomolecules one of the preferred targets of NO. We therefore tested the respiratory activity of *fur* mutant bacteria undergoing nitrosative stress (Fig. 2). Under the experimental conditions of our investigations, log-phase wild-type and *fur* mutant *Salmonella* bacteria respired at a rate of about 3.5 μM O_2/s/OD_{600} unit. Proli NONOate, which at 37°C and pH 7.4 releases NO with a half-life of 1.8 s, allowed us to deliver a bolus of ~10 μM NO. We then followed the evolution of NO in the cultures over time. The addition of 5 μM proli NONOate inhibited the respiration of wild-type *Salmonella*. After 2 min, wild-type *Salmonella* resumed respiration at rates

similar to the ones sustained before exposure to NO. The reestablishment of respiratory activity coincided with the detoxification of NO from the bacterial cultures. In comparison, the respiratory activity of *fur* mutant *Salmonella* was inhibited for about 3 min after NO treatment. The longer period of inhibition of respiration in proli NONOate-treated, *fur* mutant bacteria coincided with a prolonged presence of NO in the cultures ($P < 0.05$).

Hmp-independent, Fur-dependent antinitrosative defenses. The flavohemoprotein Hmp catalyzes the denitrosylation of NO to NO_3^-, thereby serving as a primary defense mechanism against the nitrosative stress generated by iNOS (11). The transcription of *hmp* is derepressed in a *fur* mutant grown under anaerobic conditions (32), raising the possibility that the sustained inhibition of respiratory activity endured by *fur*-deficient *Salmonella* undergoing nitrosative stress is independent of Hmp function. To test this hypothesis, we constructed a double *hmp fur* mutant strain. Wild-type *Salmonella* grew in LB broth with kinetics similar to those for the *hmp*, *fur*, and *fur hmp* mutants (Fig. 3A). The addition of 7.5 mM DETA NONOate completely inhibited the growth of the *hmp* mutant for the 30 h tested. Therefore, 1 mM DETA NONOate was used instead. The *hmp* mutant was significantly more susceptible than the *fur*-deficient strain to the bacteriostasis of 1 mM DETA NONOate (Fig. 3B), indicating that the flavohemoprotein Hmp contributes to the antinitrosative defenses of *Salmonella* more than Fur. Interestingly, the *fur hmp* double mutant was even more susceptible than single *fur* or *hmp* mutant strains, supporting our hypothesis that Hmp and Fur independently contribute to the antinitrosative defenses of *Salmonella*.

Hmp protects the respiratory activity of terminal cytochromes of the electron transport chain (33). Therefore, the contribution of Fur and Hmp to *Salmonella*'s antinitrosative defenses was independently tested by monitoring the respiratory activity of log-phase bacteria after spermine NONOate treatment. The bacteria were exposed to 50 μM spermine or spermine NONOate. This donor was chosen because its 39-min half-life facilitates the long-term release of NO, thereby allowing the expression of inducible antinitrosative determinants. After 10 min of treatment, the bacterial cultures were transferred to an air-sealed, multiport measurement chamber and the evolution of O_2 in the cultures was
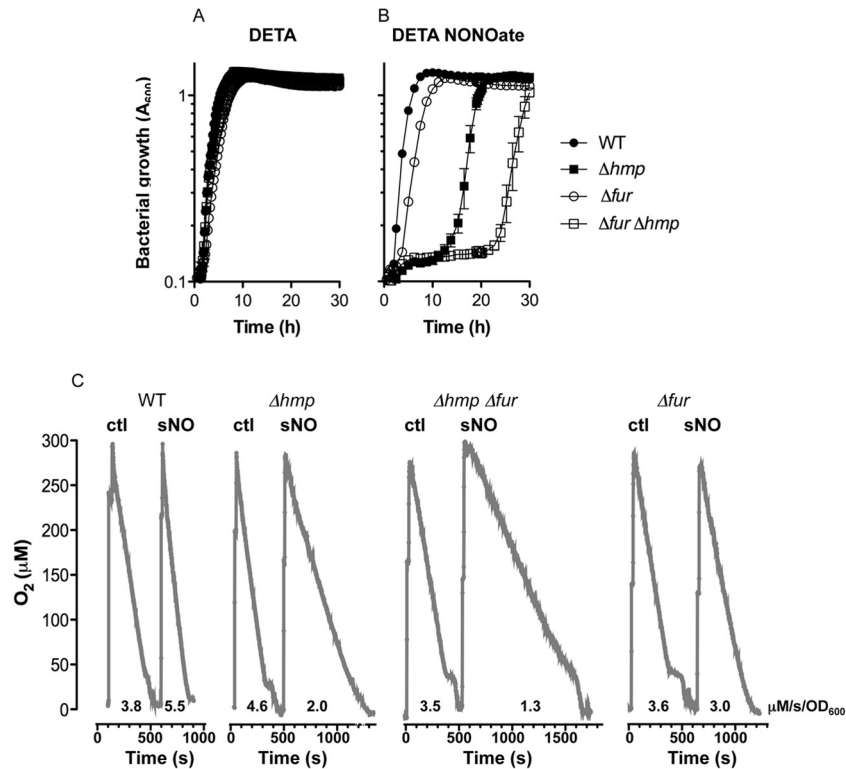
FIG 3 Contribution of Hmp and Fur to the antinitrosative defenses of *Salmonella*. Growth of stationary-phase *Salmonella* diluted to 2×10^6 CFU/ml in LB medium over time. The bacterial cultures were treated with either 1 mM DETA (A) and DETA NONOate (B). (C) O_2 consumption in log-phase *Salmonella* grown in EG medium was monitored 10 min after the addition of 50 μM the spermine control (ctl) or 50 μM the NO donor spermine NONOate (sNO). The data are representative of those from three independent experiments performed on three separate days. The O_2 consumption rates supported by bacterial cells at a cell density of OD_{600} of 1 are expressed as μM/s.

measured using an Apollo 4000 free radical analyzer. The respiratory activity of wild-type bacteria was not inhibited ($P > 0.05$) after treatment with 50 μM spermine NONOate (i.e., 3.8 versus 5.5 μM O_2/s/OD_{600} unit for spermine and spermine NONOate, respectively). In contrast, the respiratory activity of log-phase *hmp*-deficient *Salmonella* was still inhibited 10 min after treatment with spermine NONOate (i.e., 4.6 versus 2.0 μM O_2/s/OD_{600} unit for spermine and spermine NONOate, respectively). The respiration of the *fur* mutant was not affected ($P > 0.05$) 10 min after the addition of spermine NONOate (3.6 versus 3.0 μM O_2/s/OD_{600} unit for spermine or spermine NONOate, respectively), suggesting that *fur* mutant *Salmonella* can adapt to prolonged exposure to NO. Remarkably, a lack of both *fur* and *hmp* further reduced the respiratory activity of *Salmonella* treated with 50 μM spermine NONOate (i.e., 1.3 μM O_2/s/OD_{600} unit). Cumulatively, these findings indicate that Hmp- and Fur-regulated targets can independently protect respiration against the inhibitory actions of the diatomic radical NO.

Cytochrome content in Fur-deficient *Salmonella* and susceptibility to nitrosative stress. UV-visible spectroscopic analysis of cytoplasmic membranes revealed that Fur-deficient *Salmonella* strains have decreased absorbance peaks at the 430-nm Soret and 560-nm visible regions of the spectrum (Fig. 4A), indicating that Fur regulates the expression of cytochromes in *Salmonella*. The addition of δ-ALA, which is an intermediate of heme biosynthesis, restored the 430- and 560-nm absorption peaks in *fur* mutant *Salmonella*. In agreement with the spectroscopy data, membranes

isolated from *fur* mutant bacteria grown in LB broth containing 100 μM δ-ALA yielded a pellet with a brown stain that is consistent with an increase in cytochrome content (Fig. 4B). We tested whether the addition of δ-ALA could enhance the resistance of *fur* mutant bacteria to the antimicrobial activity of 2 mM DETA NONOate. The bacteria grew in the presence of 100 μM δ-ALA; however, the addition of 2 mM DETA NONOate to LB broth containing δ-ALA completely inhibited the growth of both wild-type and *fur* mutant bacteria, which might reflect the toxicity associated with high levels of heme (34). Therefore, to test the effect of δ-ALA on the susceptibility of *Salmonella* to DETA NONOate, the bacteria were grown to an OD_{600} of 0.5 in LB broth supplemented with 100 μM δ-ALA. The cells were then washed and exposed to 2 mM DETA or DETA NONOate in fresh LB broth in the absence of δ-ALA. Log-phase wild-type *Salmonella* bacteria were highly resistant to the bacteriostatic activity of 2 mM DETA NONOate (Fig. 4C and D). In contrast, *fur* mutant bacteria exposed to 2 mM DETA NONOate were susceptible to NO. The *fur* mutant bacteria that had been grown in the presence of 100 μM δ-ALA were as resistant to NO as the wild-type controls during the first 2 h of challenge but became susceptible thereafter. The transient protective effects may be explained by the dwindling δ-ALA concentrations as *fur* mutant bacteria grew in fresh LB broth. The addition of 5 μM proli NONOate more profoundly inhibited the consumption of O_2 in *fur* mutant *Salmonella* than in the wild-type controls (Fig. 4E). Wild-type *Salmonella* growth in EG medium supplemented with 100 μM δ-ALA detoxified NO more efficiently
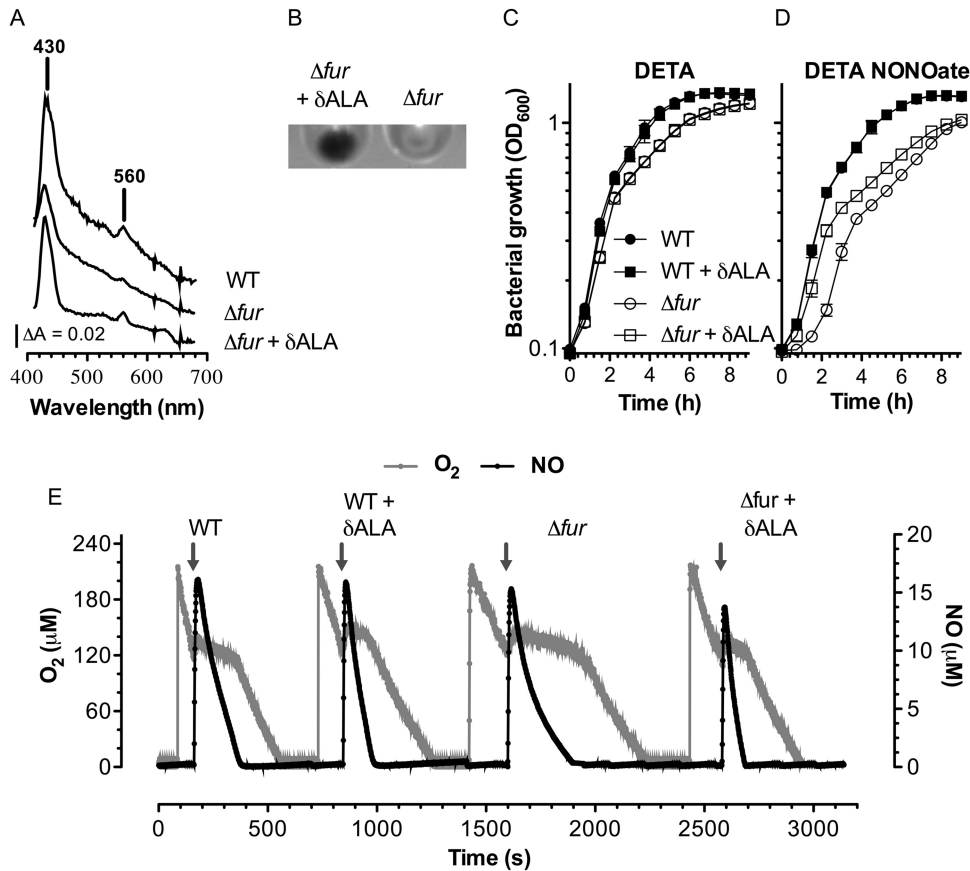
FIG 4 Effect of δ-ALA supplementation on the cytochrome content, growth, and O$_2$ consumption. (A) UV/visible difference absorbance (ΔA) spectra were recorded in *Salmonella* grown in LB broth with or without 100 μM δ-ALA. The absorption band at 560 and the Soret peak at 430 nm correspond to the fully reduced cytochrome *b*. (B) Inner membrane preparations from *fur* mutant *Salmonella* grown with or without δ-ALA. (C, D) Effect of 100 μM δ-ALA on the growth of log-phase wild-type and *fur* mutant *Salmonella* in the presence of 2 mM DETA (C) or DETA NONOate (D). Where indicated, the *Salmonella* strains for which the results are presented in panels C and D were grown to log phase in LB broth supplemented with 100 μM δ-ALA. The cells were then washed and exposed to NO in fresh LB broth. (E) Effect of 100 μM δ-ALA on the respiratory activity of *fur* mutant *Salmonella* grown to log phase in EG medium. Selected cultures were treated with 5 μM proli NONOate (arrows) when the concentration of O$_2$ in the cultures reached about 130 μM.

than control growth in EG medium, an activity that coincided with a shortened inhibition of respiration. Remarkably, the robust repression of respiratory activity noted in NO-treated *fur*-deficient *Salmonella* was prevented by growing the bacteria in EG medium containing 100 μM δ-ALA. Together, these findings suggest that the reduced cytochrome content of *fur* mutant bacteria decreases antinitrosative defenses.

Fur contributes to *Salmonella* virulence by protecting against NO-dependent host defenses. The results of the experiments described above demonstrate the importance of Fur in the antinitrosative arsenal of *Salmonella*. Next, we tested the possibility that Fur-dependent antinitrosative defenses may contribute to *Salmonella* virulence in a murine model of acute infection. Because the antinitrosative defenses of *Salmonella* have consistently been better appreciated in mice expressing the wild-type NRAMP1^r allele (7, 11, 35), we used C3H/HeN mice in these investigations. C3H/HeN mice were inoculated intraperitoneally with about 3,000 CFU of either wild-type or *fur* mutant *Salmonella*. The *fur* mutant was found to be profoundly attenuated in this acute model of *Salmonella* infection (Fig. 5A), an observation that is consistent with our previously published data (27). Using this murine model of salmonellosis, we tested whether Fur antagonizes iNOS-dependent anti-*Salmonella* host defenses. This idea
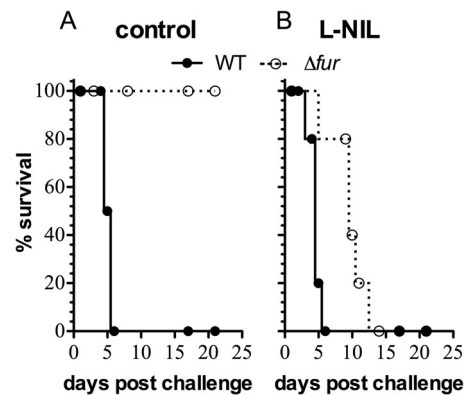
FIG 5 Effect of iNOS inhibition on the virulence of *fur* mutant *Salmonella*. (A) The survival of C3H/HeN mice infected intraperitoneally with wild-type or *fur* mutant *Salmonella* strains was studied over time. (B) Some of the animals were treated with 500 μg/ml of the iNOS-inhibitor L-NIL in drinking water. The data are from two independent experiments (*n* = 5 to 11 mice per group).
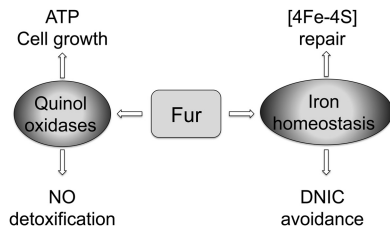
FIG 6 Model for Fur-mediated, innate, antinitrosative defenses. Fur-mediated regulation of terminal quinol oxidases of the electron transport chain can boost the innate antinitrosative defenses of *Salmonella* by (i) generating the ATP that is necessary for bacterial growth and (ii) directly detoxifying NO. The critical role played by Fur in iron homeostasis may also add to the defense mechanisms against nitrosative stress. For example, Fur-regulated expression of the *sufABCDSE* system could help repair [4Fe-4S] clusters damaged by NO and its oxidative congeners. Moreover, the Fur-dependent control of the labile iron pool could limit the formation of cytotoxic dinitrosyl-iron complexes (DNIC).

was examined by adding 500 μg/ml of the iNOS-specific inhibitor L-NIL to the drinking water of C3H/HeN mice. As previously noted (35), the addition of L-NIL to drinking water did not increase the virulence of wild-type *Salmonella*. In contrast, the addition of this iNOS competitive inhibitor to the drinking water of C3H/HeN mice significantly ($P < 0.05$) increased the virulence of *fur*-deficient *Salmonella* (Fig. 5B). These findings indicate that Fur-regulated antinitrosative defenses contribute to the virulence potential of *Salmonella*. It should be noted, however, that *fur* mutant *Salmonella* was slightly more attenuated in L-NIL-treated mice than wild-type isogenic bacteria, indicating that some aspects of the Fur-mediated *Salmonella* virulence cannot be accounted for by the contribution of this regulator to antinitrosative defenses.

DISCUSSION

Fur has been shown to be important in bacterial pathogenesis. The Fur metalloprotein could coordinate virulence through the regulation of metabolic processes, and more specifically, in the case of enteropathogenic bacteria, it could enhance pathogenicity by regulating the acid tolerance response that increases bacterial fitness in the low pH of the stomach (36). Our investigations have identified regulation of antinitrosative defenses as a novel mechanism by which Fur contributes to *Salmonella* pathogenesis in an acute model of infection.

Our investigations indicate that Fur helps maintain normal cytochrome content in *Salmonella*. The regulation of cytochrome expression could mediate *Salmonella* virulence by at least two non-mutually exclusive mechanisms (Fig. 6). First, the proton-translocating activity of terminal cytochromes of the electron transport chain is essential for the production of ATP during aerobic metabolism. Low cytochrome levels in *fur* mutant bacteria could affect *Salmonella* virulence because production of ATP is correlated with bacterial cell growth. In this context, cytochromes were recently found to add to the pathogenicity of *S. aureus* (37). Copper and iron cofactors in quinol cytochrome oxidases are some of the preferred targets of the cytotoxicity of NO (38). Therefore, the extended inhibition of respiration experienced by *fur* mutant bacteria undergoing nitrosative stress could have a particularly devastating effect on ATP synthesis, cell growth, and *Salmonella* pathogenesis. Second, lower concentrations of the cytochrome *bd* oxidase, which is known to detoxify NO (31), might

have contributed to the slow detoxification of this diatomic radical in *fur* mutant *Salmonella*. The lingering concentrations of NO noted in cultures of *fur* mutant *Salmonella* could exert lasting cytotoxicity to critical enzymes, such as ribonucleotide reductase and dihydroxy acid dehydratase (38). Low concentrations of cytochrome *bd* oxidase could also explain why the doubling time of *fur* mutant *Salmonella* undergoing nitrosative stress is less than the one recorded for wild-type controls.

The reduced cytochrome content in *fur* mutant *Salmonella* is surprising since the *cyoABCDE* and *cydAB* genes encoding cytochrome *bo* and *bd* oxidases, respectively, are overexpressed in the absence of this regulatory metalloprotein (27). These observations suggest that the decreased cytochrome content of *fur*-deficient *Salmonella* must be explained by defects at steps of their biosynthesis other than the transcriptional regulation of the *cyoABCDE* and *cydAB* operons. The addition of δ-ALA to the culture medium not only increased the cytochrome content of *fur* mutant bacteria to wild-type levels but also enhanced NO detoxification. These findings indicate that Fur must directly or indirectly control a step in the biosynthesis of δ-ALA. This model is consistent with the fact that the supplementation of culture medium with δ-ALA reestablishes wild-type expression levels of the hemoproteins hydroperoxidase I and II in *fur* mutant bacteria (39). In *Salmonella* and *E. coli*, the *hemA*-encoded δ-aminolevulinic acid synthase generates δ-ALA from glutamate by the C5 pathway (40). Analysis of a *lacZY* transcriptional fusion revealed similar expression of *hemA* in wild-type and *fur* mutant controls (M. Song and A. Vázquez-Torres, unpublished data). Further investigations are needed in order to address how Fur regulates the biosynthesis of heme proteins.

Our investigations suggest that Fur regulates an innate antinitrosative detoxification system. However, *fur* mutant *Salmonella* strains respire and consume NO after prolonged exposure to NO, indicating that they are capable of mounting a delayed protective adaptive response against NO. The marked susceptibility of *fur hmp* mutant bacteria to nitrosative stress indicates that the flavohemoprotein Hmp mediates the adaptive antinitrosative defenses seen in *fur* mutant bacteria. Our investigations are consistent with a model in which Fur controls an innate mechanism of detoxification of NO (data herein), whereas the induction of the flavohemoprotein Hmp in response to the nitrosylation of the NsrR repressor provides adaptive, long-term, and amplified NO resistance (41). Terminal cytochromes of the electron transport chain are logical suspects for the Fur-regulated innate NO detoxification noted in our investigations, as suggested by previous studies in *E. coli* (31). Our observations also indicate that Fur- and Hmp-mediated antinitrosative defenses mostly operate independently. In support of this notion, a *fur hmp* double mutant not only was significantly more susceptible to the bacteriostatic actions of NO, but it also detoxified NO with apparently slower kinetics than *fur* and *hmp* single mutant isogenic *Salmonella* strains. Collectively, our investigations indicate that both innate and adaptive antinitrosative defenses are necessary for the growth of *Salmonella* in the presence of reactive nitrogen species generated by the inducible NO synthase.

In addition to the regulation of cytochrome expression, Fur could enhance the antinitrosative defenses of *Salmonella* by other mechanisms as well (Fig. 6). For example, Fur is a positive regulator of the *sufABCDSE* operon that encodes an enzymatic pathway for the repair of oxidized [4Fe-4S] clusters (42). As mentioned earlier, [4Fe-4S] clusters in dehydratases are some of the

preferred targets of NO and some of its oxidative products, such as peroxynitrite (38, 43). Low levels of expression of SufABCDSE in *fur* mutant bacteria could have devastating effects on the repair of dehydratases, such as aconitase, the *nuo*-encoded NADH dehydrogenase, glutamate synthetase, and dihydroxy acid dehydratase, which are responsible for key aspects of central metabolism. In addition, deregulated iron homeostasis could play a key role in the hypersusceptibility of *fur* mutant *Salmonella* to nitrosative stress. Increases in the cytoplasmic pool of labile iron in *fur* mutant bacteria could lead to the formation of highly cytotoxic dinitrosyl-iron complexes that, according to work published by Lancaster and collaborators (44), are the leading nitrosating species in the cell.

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REFERENCES

1. **Fields PI, Swanson RV, Haidaris CG, Heffron F.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. U. S. A. **83:**5189–5193. http://dx.doi.org/10.1073/pnas.83.14.5189.

2. **Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC.** 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. J. Exp. Med. **192:**227–236. http://dx.doi.org/10.1084/jem.192.2.227.

3. **Stevanin TM, Poole RK, Demoncheaux EA, Read RC.** 2002. Flavohemoglobin Hmp protects *Salmonella enterica* serovar Typhimurium from nitric oxide-related killing by human macrophages. Infect. Immun. **70:**4399–4405. http://dx.doi.org/10.1128/IAI.70.8.4399-4405.2002.

4. **Paolillo R, Carratelli CR, Rizzo A.** 2011. Effect of resveratrol and quercetin in experimental infection by *Salmonella enterica* serovar Typhimurium. Int. Immunopharmacol. **11:**149–156. http://dx.doi.org/10.1016/j.intimp.2010.10.019.

5. **Azenabor AA, Kennedy P, York J.** 2009. Free intracellular Ca^{2+} regulates bacterial lipopolysaccharide induction of iNOS in human macrophages. Immunobiology **214:**143–152. http://dx.doi.org/10.1016/j.imbio.2008.06.001.

6. **McCollister BD, Bourret TJ, Gill R, Jones-Carson J, Vazquez-Torres A.** 2005. Repression of SPI2 transcription by nitric oxide-producing, IFNγ-activated macrophages promotes maturation of *Salmonella* phagosomes. J. Exp. Med. **202:**625–635. http://dx.doi.org/10.1084/jem.20050246.

7. **Song M, Husain M, Jones-Carson J, Liu L, Henard CA, Vazquez-Torres A.** 2013. Low-molecular-weight thiol-dependent antioxidant and antinitrosative defences in *Salmonella* pathogenesis. Mol. Microbiol. **87:**609–622. http://dx.doi.org/10.1111/mmi.12119.

8. **De Groote MA, Testerman T, Xu Y, Stauffer G, Fang FC.** 1996. Homocysteine antagonism of nitric oxide-related cytostasis in *Salmonella typhimurium*. Science **272:**414–417. http://dx.doi.org/10.1126/science.272.5260.414.

9. **Fang FC, DeGroote MA, Foster JW, Baumler AJ, Ochsner U, Testerman T, Bearson S, Giard JC, Xu Y, Campbell G, Laessig T.** 1999. Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. Proc. Natl. Acad. Sci. U. S. A. **96:**7502–7507. http://dx.doi.org/10.1073/pnas.96.13.7502.

10. **Hausladen A, Gow A, Stamler JS.** 2001. Flavohemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. Proc. Natl. Acad. Sci. U. S. A. **98:**10108–10112. http://dx.doi.org/10.1073/pnas.181199698.

11. **Bang IS, Liu L, Vazquez-Torres A, Crouch ML, Stamler JS, Fang FC.** 2006. Maintenance of nitric oxide and redox homeostasis by the *Salmonella* flavohemoglobin *hmp*. J. Biol. Chem. **281:**28039–28047. http://dx.doi.org/10.1074/jbc.M605174200.

12. **Crawford MJ, Goldberg DE.** 1998. Role for the Salmonella flavohemoglobin in protection from nitric oxide. J. Biol. Chem. **273:**12543–12547. http://dx.doi.org/10.1074/jbc.273.20.12543.

13. **Chakravortty D, Hansen-Wester I, Hensel M.** 2002. *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. J. Exp. Med. **195:**1155–1166. http://dx.doi.org/10.1084/jem.20011547.

14. **Richardson AR, Soliven KC, Castor ME, Barnes PD, Libby SJ, Fang FC.** 2009. The base excision repair system of *Salmonella enterica* serovar Typhimurium counteracts DNA damage by host nitric oxide. PLoS Pathog. **5:**e1000451. http://dx.doi.org/10.1371/journal.ppat.1000451.

15. **Bodenmiller DM, Spiro S.** 2006. The *yjeB* (*nsrR*) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. J. Bacteriol. **188:**874–881. http://dx.doi.org/10.1128/JB.188.3.874-881.2006.

16. **D'Autreaux B, Tucker NP, Dixon R, Spiro S.** 2005. A non-haem iron centre in the transcription factor NorR senses nitric oxide. Nature **437:**769–772. http://dx.doi.org/10.1038/nature03953.

17. **Cruz-Ramos H, Crack J, Wu G, Hughes MN, Scott C, Thomson AJ, Green J, Poole RK.** 2002. NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. EMBO J. **21:**3235–3244. http://dx.doi.org/10.1093/emboj/cdf339.

18. **D'Autreaux B, Touati D, Bersch B, Latour JM, Michaud-Soret I.** 2002. Direct inhibition by nitric oxide of the transcriptional ferric uptake regulation protein via nitrosylation of the iron. Proc. Natl. Acad. Sci. U. S. A. **99:**16619–16624. http://dx.doi.org/10.1073/pnas.252591299.

19. **Ding H, Demple B.** 2000. Direct nitric oxide signal transduction via nitrosylation of iron-sulfur centers in the SoxR transcription activator. Proc. Natl. Acad. Sci. U. S. A. **97:**5146–5150. http://dx.doi.org/10.1073/pnas.97.10.5146.

20. **Hausladen A, Privalle CT, Keng T, DeAngelo J, Stamler JS.** 1996. Nitrosative stress: activation of the transcription factor OxyR. Cell **86:**719–729. http://dx.doi.org/10.1016/S0092-8674(00)80147-6.

21. **Henard CA, Vazquez-Torres A.** 2012. DksA-dependent resistance of *Salmonella enterica* serovar Typhimurium against the antimicrobial activity of inducible nitric oxide synthase. Infect. Immun. **80:**1373–1380. http://dx.doi.org/10.1128/IAI.06316-11.

22. **Spiro S, D'Autreaux B.** 2012. Non-heme iron sensors of reactive oxygen and nitrogen species. Antioxid. Redox Signal. **17:**1264–1276. http://dx.doi.org/10.1089/ars.2012.4533.

23. **Moore CM, Nakano MM, Wang T, Ye RW, Helmann JD.** 2004. Response of *Bacillus subtilis* to nitric oxide and the nitrosating agent sodium nitroprusside. J. Bacteriol. **186:**4655–4664. http://dx.doi.org/10.1128/JB.186.14.4655-4664.2004.

24. **Richardson AR, Dunman PM, Fang FC.** 2006. The nitrosative stress response of *Staphylococcus aureus* is required for resistance to innate immunity. Mol. Microbiol. **61:**927–939. http://dx.doi.org/10.1111/j.1365-2958.2006.05290.x.

25. **Qu W, Zhou Y, Shao C, Sun Y, Zhang Q, Chen C, Jia J.** 2009. *Helicobacter pylori* proteins response to nitric oxide stress. J. Microbiol. **47:**486–493. http://dx.doi.org/10.1007/s12275-008-0266-0.

26. **Mukhopadhyay P, Zheng M, Bedzyk LA, LaRossa RA, Storz G.** 2004. Prominent roles of the NorR and Fur regulators in the *Escherichia coli* transcriptional response to reactive nitrogen species. Proc. Natl. Acad. Sci. U. S. A. **101:**745–750. http://dx.doi.org/10.1073/pnas.0307741100.

27. **Troxell B, Sikes ML, Fink RC, Vazquez-Torres A, Jones-Carson J, Hassan HM.** 2011. Fur negatively regulates *hns* and is required for the expression of HilA and virulence in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **193:**497–505. http://dx.doi.org/10.1128/JB.00942-10.

28. **Velayudhan J, Castor M, Richardson A, Main-Hester KL, Fang FC.** 2007. The role of ferritins in the physiology of *Salmonella enterica* sv. Typhimurium: a unique role for ferritin B in iron-sulphur cluster repair and virulence. Mol. Microbiol. **63:**1495–1507. http://dx.doi.org/10.1111/j.1365-2958.2007.05600.x.

29. **Husain M, Bourret TJ, McCollister BD, Jones-Carson J, Laughlin J, Vazquez-Torres A.** 2008. Nitric oxide evokes an adaptive response to oxidative stress by arresting respiration. J. Biol. Chem. **283:**7682–7689. http://dx.doi.org/10.1074/jbc.M708845200.

30. **Henard CA, Bourret TJ, Song M, Vazquez-Torres A.** 2010. Control of redox balance by the stringent response regulatory protein promotes antioxidant defenses of *Salmonella*. J. Biol. Chem. **285:**36785–36793. http://dx.doi.org/10.1074/jbc.M110.160960.

31. **Mason MG, Shepherd M, Nicholls P, Dobbin PS, Dodsworth KS, Poole RK, Cooper CE.** 2009. Cytochrome *bd* confers nitric oxide resistance to

Escherichia coli. Nat. Chem. Biol. **5**:94–96. http://dx.doi.org/10.1038/nchembio.135.

32. **Troxell B, Fink RC, Porwollik S, McClelland M, Hassan HM.** 2011. The Fur regulon in anaerobically grown *Salmonella enterica* sv. Typhimurium: identification of new Fur targets. BMC Microbiol. **11**:236. http://dx.doi.org/10.1186/1471-2180-11-236.

33. **Stevanin TM, Ioannidis N, Mills CE, Kim SO, Hughes MN, Poole RK.** 2000. Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes *bo'* or *bd*, from nitric oxide. J. Biol. Chem. **275**:35868–35875. http://dx.doi.org/10.1074/jbc.M002471200.

34. **Anzaldi LL, Skaar EP.** 2010. Overcoming the heme paradox: heme toxicity and tolerance in bacterial pathogens. Infect. Immun. **78**:4977–4989. http://dx.doi.org/10.1128/IAI.00613-10.

35. **Husain M, Jones-Carson J, Song M, McCollister BD, Bourret TJ, Vazquez-Torres A.** 2010. Redox sensor SsrB Cys[203] enhances *Salmonella* fitness against nitric oxide generated in the host immune response to oral infection. Proc. Natl. Acad. Sci. U. S. A. **107**:14396–14401. http://dx.doi.org/10.1073/pnas.1005299107.

36. **Foster JW, Moreno M.** 1999. Inducible acid tolerance mechanisms in enteric bacteria. Novartis Found. Symp. **221**:55–69.

37. **Hammer ND, Reniere ML, Cassat JE, Zhang Y, Hirsch AO, Indriati Hood M, Skaar EP.** 2013. Two heme-dependent terminal oxidases power *Staphylococcus aureus* organ-specific colonization of the vertebrate host. mBio **4**(4):e00241–13. http://dx.doi.org/10.1128/mBio.00241-13.

38. **Henard CA, Vazquez-Torres A.** 2011. Nitric oxide and *Salmonella* pathogenesis. Front. Microbiol. **2**:84. http://dx.doi.org/10.3389/fmicb.2011.00084.

39. **Troxell B.** 2010. The role of ferric uptake regulator in regulation of metal homeostasis, metabolism, virulence, and protection against hydrogen peroxide in *Salmonella enterica* serovar Typhimurium. Ph.D. thesis. North Carolina State University, Raleigh, NC.

40. **Avissar YJ, Beale SI.** 1989. Identification of the enzymatic basis for delta-aminolevulinic acid auxotrophy in a *hemA* mutant of *Escherichia coli*. J. Bacteriol. **171**:2919–2924.

41. **Hausladen A, Gow AJ, Stamler JS.** 1998. Nitrosative stress: metabolic pathway involving the flavohemoglobin. Proc. Natl. Acad. Sci. U. S. A. **95**:14100–14105. http://dx.doi.org/10.1073/pnas.95.24.14100.

42. **Nachin L, El Hassouni M, Loiseau L, Expert D, Barras F.** 2001. SoxR-dependent response to oxidative stress and virulence of *Erwinia chrysanthemi*: the key role of SufC, an orphan ABC ATPase. Mol. Microbiol. **39**:960–972. http://dx.doi.org/10.1046/j.1365-2958.2001.02288.x.

43. **Henard CA, Vazquez-Torres A.** 2013. Regulation of Salmonella resistance to oxidative and nitrosative stress, p 425–440. *In* Vasil ML, Darwin AJ (ed), Regulation of bacterial virulence. ASM Press, Washington, DC.

44. **Bosworth CA, Toledo JC, Jr, Zmijewski JW, Li Q, Lancaster JR, Jr.** 2009. Dinitrosyliron complexes and the mechanism(s) of cellular protein nitrosothiol formation from nitric oxide. Proc. Natl. Acad. Sci. U. S. A. **106**:4671–4676. http://dx.doi.org/10.1073/pnas.0710416106.