Maintenance of Nitric Oxide and Redox Homeostasis by the Salmonella Flavohemoglobin Hmp*

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Intracellular pathogens must resist the antimicrobial actions of nitric oxide (NO) produced by host cells. To this end pathogens possess several NO–metabolizing enzymes. Here we show that the flavohemoglobin Hmp is the principal enzyme responsible for aerobic NO metabolism by Salmonella enterica serovar typhimurium. We further show that Hmp is required for Salmonella virulence in mice, in contrast to S-nitrosoglutathione reductase, flavoredbredoxin, or cytochrome c nitrite reductase. Abrogation of murine-inducible NO synthase restores virulence to hmp mutant bacteria. In the presence of nitrosative stress, Hmp-deficient Salmonella exhibits reduced NO consumption, impaired growth, increased protein S-nitrosylation, and filamentous morphology. However, under aerobic conditions in the absence of nitrosative stress, elevated hmp expression by the transcriptional repressor NsrR in response to nitrosative stress. This provides a rationale for the regulation of hmp expression by the transcriptional repressor NsrR in response to both nitrosative stress and intracellular free iron concentration. The Hmp flavohemoglobin plays a central role in the response of Salmonella to nitrosative stress but requires precise regulation to avoid the exacerbation of oxidative stress that can result if electrons are shuttled to extraneous iron.

Host phagocytic cells use the NADPH phagocyte oxidase (Phox) and inducible nitric-oxide synthase (iNOS) to generate the antimicrobial radicals superoxide anion and nitric oxide, respectively (1). Superoxide (O2) and nitric oxide (NO) in turn can be converted to other reactive oxygen species or reactive nitrogen species (RNS) such as hydrogen peroxide (H2O2), hydroxyl radical (·OH), nitrogen dioxide (NO2), peroxynitrite (ONOO−), dinitrogen trioxide (N2O3), and nitrosothiols (RSNO). Enzymes responsible for the metabolism and detoxification of reactive oxygen species, including catalases, superoxide dismutases, and peroxidases, have been extensively studied and shown to contribute to bacterial virulence in experimental infections (2–4). However, although some bacterial enzymes capable of RNS detoxification have been characterized, their importance in pathogenesis has not been directly demonstrated.

The enteric pathogens such as Salmonella enterica serovar typhimurium possess a number of enzymes with the ability to metabolize RNS. The flavohemoglobin Hmp detoxifies NO by an O2-dependent denitrosylase mechanism, producing NO3 under aerobic or microaerobic conditions or by the slower O2-independent reduction of NO to N2O (5–7). The flavoredbredoxin NorV can reduce NO to N2O under anaerobic or microaerobic conditions (8, 9) and is induced during experimental infection of macrophages (10). The GSH-dependent formaldehyde dehydrogenase AdhC has S-nitrosogluthathione (GSNO) reductase activity (11), which can limit levels of S-nitrosogluthathione formed during nitrosative stress. Last, the periplasmic cytochrome c nitrite reductase NrfA, which reduces NO3 to NH3, may also be able to directly reduce NO (12).

The biochemistry of Hmp has been extensively characterized. As one of bacterial globins, Hmp binds NO at its heme ligand. Structural analysis of flavohemoglobins has also revealed binding domains for FAD and NAD(P) in the C-terminal portion of the molecule (13). This reductase domain is believed to transfer electrons from FAD(P)H to the heme iron ligand via FAD, ultimately resulting in reduction of the liganded NO to form a heme-bound nitroxyl anion (NO−) equivalent (7, 14, 15). NO−/HNO is alternatively converted to NO3 or N2O in the presence or absence of O2, respectively.

Biochemical studies of the Escherichia coli Hmp enzyme have revealed some evidence that Hmp might exacerbate oxidative stress under selected circumstances. In the absence of NO, Hmp binds O2, NADH oxidase activity of Hmp can then generate O2− at the heme, and further dismutation or reduction could produce H2O2 (16, 17). Moreover, by consuming NADH and reducing free flavins, Hmp has the ability to reduce external electron acceptors, including ferric iron (Fe3+) (18–20).
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20. Woodmansee and Imlay (21) have demonstrated the ability of reduced flavins generated by the NADPH-dependent flavin oxidoreductase Fre to promote oxidative damage by reducing intracellular free iron. Homology between Fre and the C-terminal portion of Hmp (22) as well as the ability of Hmp to act as a ferrisiderophore reductase (18) supports a possible role of Hmp in reducing intracellular iron under physiological conditions.

To examine the importance of RNS metabolism to *Salmonella* virulence in mice, we have constructed *Salmonella* mutant strains lacking Hmp, NorV, AdhC, or NrfA. Infections of mice with these strains show that the flavohemoglobin Hmp plays the most important role of these enzymes in detoxifying host-derived RNS produced during *Salmonella* infections. We have also used site-specific mutations to demonstrate the contribution of the heme and flavin binding domains of Hmp to NO\(^{-}\) denitrosylase activity and the potentiation of oxidative stress. Finally, we have elucidated the mechanism by which hmp transcription is regulated in response to nitrosative stress and intracellular iron concentration.

**EXPERIMENTAL PROCEDURES**

**Media and Chemicals**—Luria-Bertani (LB) complex medium and minimal E medium containing 0.2% glucose were used for the routine growth of bacterial cells (23). The chemicals were purchased from Sigma-Aldrich. GSNO was synthesized from the routine growth of bacterial cells (23). The chemicals were purchased each mutation into a clean wild-type background and complementing plasmid, the nsrR gene was amplified from *S. typhimurium* chromosomal DNA using *Pfu* polymerase (Stratagene, La Jolla, CA) with primers 5’-CAGTGTGATACATTGCTGTG-3’ and 5’-AGGATCTAGAGACATTGAGGTTC-3’ and cloning into pBAD30.

**Mouse Virulence Assay**—Female 6–8-week-old C57BL/6, 129Xi/SvJ (The Jackson Laboratory, Bar Harbor, ME) and C3H/HeN (Charles River Laboratory, Wilmington, MA) mice were used for the determination of *Salmonella* virulence. C57BL/6 iNOS\(^{-/-}\) mice were bred at the University of Washington and University of Colorado Health Science Center animal facilities according to the animal care and use regulations of each institution. To inhibit iNOS expression in C3H/HeN mice, L-NIL (L-N6-1-iminoethyl-lysine; 500 \(\mu\)g ml\(^{-1}\) was administered with drinking water throughout the experiment. *Salmonella* cells were grown overnight in LB medium and diluted in phosphate-buffered saline (PBS; Difco). For acute infections, 500 colony-forming units (cfu) and 2000 cfu were administered intraperitoneally to C56BL/6 and C3H/HeN mice, respectively. For competition assays in chronically infected mice, strains were mixed at a 1:1 ratio of hmp mutant to wild type before oral administration of 2 \(\times\) 10\(^5\) cfu to 129Xi/SvJ mice. Infected mice were sacrificed at designated time intervals for isolation of mesenteric lymph nodes, which were homogenized in PBS, diluted, and plated onto selective and non-selective media for quantitation of cfu. Each virulence assay was performed a minimum of two times with 10 mice per group.

**Macrophage Killing Assay**—Peritoneal exudate cells from wild-type C57BL/6 or congenic iNOS\(^{-/-}\) mice were harvested 4 days after intraperitoneal inoculation of 1 mg ml\(^{-1}\) sodium periodate, as described previously (28). The peritoneal exudate cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gemini Bio-Products, Calabasas, CA), 1 \(\times\) 10\(^5\) human interferon-\(\gamma\) (Invitrogen) 16h before infection. Macrophages were selected by adherence to a 96-well plate and cultured for 48 h at 37°C in a 5% CO\(_2\) incubator. Selected groups of macrophages were incubated with 100 units ml\(^{-1}\) murine interferon-\(\gamma\) (Invitrogen) 16h before infection. Macrophages were challenged with *Salmonella* opsonized with 10% normal mouse serum at a multiplicity of infection of 10:1 and allowed to internalize the bacteria for 15 min. Extracellular bacteria were removed by washing with prewarmed medium containing 6 \(\mu\)g ml\(^{-1}\) gentamicin. The *Salmonella*-infected macrophages were lysed 20 h after challenge, and the surviving bacteria were enumerated on LB agar plates. Results are expressed as percent survival. Nitrite production by macrophages in response to infection was determined by the Griess reaction (29).

**Measurement of NO Consumption**—Wild-type *S. typhimurium*, hmp mutant *S. typhimurium*, and wild-type *S. typhimurium* heated to 95°C for 10 min were resuspended in buffer (A\(\text{NO}_2\) = 2) before the addition of NO\(^{-}\) (\(\approx\)3 \(\mu\)M). NO\(^{-}\) consumption was measured in 1 ml of PBS with 20 \(\mu\)M diethylenetriamine pentaacetic acid using an NO\(^{-}\) electrode (World Precision Instruments, Sarasota, FL).

**Measurement of S-Nitrosothiols**—SNO levels in bacterial cells were measured essentially as described (30). *Salmonella* grown in minimal medium were treated with 2 \(\mu\)M GSNO for 1 h before lysis by sonication. Lysates were cleared by centrifugation at 20,000 \(\times\) g for 10 min and either treated or not treated (total XNO) with HgCl\(_2\) to deplete S-nitrosothiols. Quantities of total S-nitrosothiols in the lysates were measured by photolysis-chemiluminescence (31). Standard curves were derived using S-nitrosoglutathione, and data were normalized to total protein content.

**Measurement of Growth Kinetics**—Growth kinetics were measured by determining the optical density at 600 nm (A\(\text{600nm}\)) at 37°C with agitation on a BioScreen C Microbiology Microplate reader (Growth Curves USA, Piscataway, NJ). Cells
grown overnight in LB medium were diluted in PBS to $A_{600 \text{ nm}} = 0.1$ before inoculating equal quantities into minimal E glucose (0.2%) medium containing various NO donors.

Cloning and Site-directed Mutagenesis of the S. typhimurium hmp Gene—The hmp gene was amplified from S. typhimurium 14028s chromosomal DNA using Pfu polymerase (Stratagene) with primers 5' TCTCTAGA TTTTCAATAAAGGAAGCA-3' and 5'-AACGGGCTTGCCTTACTA-3'. The purified product was digested with restriction enzyme XbaI and cloned into pBluescript SK+ (Stratagene). Identity of the cloned insert was confirmed by DNA sequencing and performing phenotypic complementation in hmp mutant bacteria before use as the template plasmid for site-directed mutagenesis according to the method of (32) with modifications. Briefly, two complementary mutagenic primers and Pfu DNA polymerase were used to amplify DNA from the template plasmid. The PCR product was treated with restriction enzyme DpnI to specifically cut methylated parental plasmid DNA sequences before purification, ligation, and transformation into Electromax DH10B competent cells (Invitrogen). Each mutant construct was confirmed by sequencing. Primers used for site-directed mutagenesis were as follows: H85A, 5'-CGGTTATACGTAACATCGG-3' (hmp), 5'-CCGTTATGTTGAACCTGTC-3' and 5'-GCCGCTGGGT TCTAATGTA-3' (iroN), and 5'-GGGACTTGTTGAACCTG-3' and 5'-TTCCAGCAGATGGTAATGGCCTTC-3' (rpoD). Each sample was independently tested three times and assayed in duplicate during each run.

RESULTS

A Mutation in hmp but Not the adhC, norV, or nrfA Genes Attenuates Salmonella Virulence in Mice—To compare the contribution of flavohemoglobin, GSNO reductase, flavoredoxin, and cytochrome c nitrite reductase to Salmonella virulence, the virulence of isogenic hmp, adhC, norV, and nrfA mutant and parental S. typhimurium 14028s wild-type bacteria was compared after intraperitoneal inoculation into mice. No significant differences in virulence were observed in genetically susceptible (Nramp1+) C57BL/6 mice (data not shown). However, in genetically resistant (Nramp1+/−) C3H/HeN mice, the hmp mutant strain exhibited almost complete loss of virulence, whereas adhC, norV, or nrfA mutants possessed virulence similar to that of wild-type bacteria (Fig. 1A). Complementation of the hmp virulence phenotype in vivo was not attempted because hmp is monocistronic, excluding the possibility that the hmp mutation exerted a polar effect. Moreover, expression of hmp from a plasmid vector was found to have a detrimental effect on cell growth (see below). Notably, treatment of mice with the iNOS inhibitor L-NIL (35) restored virulence to hmp mutant Salmonella (Fig. 1B), indicating that the flavohemoglobin Hmp promotes Salmonella virulence by detoxifying RNS generated by host cells. Moreover, in comparison with wild-type Salmonella, hmp mutants were more susceptible to killing by murine macrophages expressing iNOS than to congenic iNOS−/− macrophages (Fig. 1C). Macrophages infected with hmp mutants produced slightly higher quantities of nitrite compared with macrophages infected with wild-type Salmonella (Fig. 1D), although it cannot be distinguished whether this represents direct consumption of NO− by Hmp or lesser production of NO− in macrophages with greater organism burdens.

Mice that are highly resistant to Salmonella infection can be persistently infected with wild-type S. typhimurium in mesenteric lymph nodes (36). To determine whether Hmp is required for persistent Salmonella infection, we challenged 129Xi/SvJ mice with oral inocula containing equal quantities of wild-type and isogenic hmp mutant bacteria. At intervals after infection, mice were sacrificed and competition index was calculated by enumerating bacterial load in mesenteric lymph nodes. The hmp mutant strain was not significantly different in competitive index at day 5 (0.465 ± 0.189) but was 100-fold less abundant than wild-type bacteria after 98 days of infection (0.017 ± 0.019). These results demonstrate that the flavohemoglobin Hmp promotes Salmonella virulence during chronic infection as well as during acute lethal infection of mice.

An hmp Mutant S. typhimurium Strain Is Unable to Metabolize Nitric Oxide, Leading to S-Nitrosylation of Proteins and Cell Filamentation—The rate of NO− consumption by hmp mutant and wild-type bacteria cultured in minimal medium was measured using an NO− sensitive electrode. Wild-type bac-
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FIGURE 1. Flavohemoglobin Hmp is required for Salmonella virulence in mice and survival in macrophages. A, wild-type (WT) S. typhimurium 14028s or isogenic hmp, adhC, norV, or nirA mutant derivatives (∼2000 cfu) were inoculated intraperitoneally into 6–8-week-old C3H/HeN mice (n = 10). Infected animals were monitored with euthanasia of moribund animals. B, C3H/HeN mice were infected as in A except that the iNOS inhibitor L-NIL (500 μM) was administered in drinking water. Results in A and B are representative of two or more reproducible, independent experiments using 10 mice per strain. *, p < 0.001 by Student’s t test for mortality compared with wild type. C, peritoneal macrophages from C57BL/6 and congenic iNOS−/− mice were infected with wild-type or hmp mutant Salmonella. Viable intracellular bacteria within untreated or interferon (IFN)−γ-treated macrophages were recovered and enumerated 20 h after infection. Data are the mean ± S.D. of three independent experiments. *, p < 0.05 compared with wild type. D, nitrite production by C57BL/6 macrophages infected with wild-type or hmp mutant Salmonella was measured over a 1-h period. Data are expressed as the mean ± S.D. of three independent experiments. *, p < 0.05 compared with wild type.

Flavohemoglobin Hmp rapidly consumed NO−, whereas hmp mutants consumed NO− as slowly as heat-inactivated wild-type cells, demonstrating that Hmp is responsible for virtually all NO− decomposition in Salmonella under these aerobic experimental conditions (Fig. 2A). Accordingly, the growth of hmp mutant bacteria was severely impaired in the presence of NO−-donor compounds spermine-NONOate (37), diethylamine triamine-NONOate (37), and GSNO (24) (Fig. 2B). Furthermore, hmp mutant cells treated with GSNO accumulated approximately twice as much S-nitrosylated proteins compared with wild-type (Fig. 2C). GSNO has previously been shown to induce cell filamentation in association with the SOS response, suggesting that nitrosative stress arrests DNA replication (38). Microscopy revealed that hmp mutant bacteria exhibit filamentation after overnight culture in GSNO concentrations (500 μM) insufficient to induce filamentation of wild-type cells (Fig. 2D). Collectively, these observations indicate that Hmp is responsible for the majority of NO− detoxification in Salmonella and can promote bacterial growth during nitrosative stress.

Mutation of Either the Heme or Flavoreductase Domain of Hmp Abrogates Salmonella Resistance to Nitrosative Stress—An earlier study showed that separate expression of the E. coli Hmp heme and flavin domains in trans cannot confer resistance to nitrosative stress (39). To evaluate the importance of each domain in the activity of intact Hmp protein, site-directed mutagenesis of the Salmonella Hmp heme and flavoreductase domains was performed. Mutant and wild-type hmp genes were expressed from an episomal vector in an hmp mutant background. X-ray crystallographic analysis of the E. coli Hmp protein (13) and a comparison of amino acid sequences conserved among diverse flavohemoglobins (22) identified histidine 85 and tyrosine 206-serine 207 as conserved residues in the Salmonella Hmp heme and flavoreductase domains, respectively. The hmp mutant cells harboring wild-type hmp on a plasmid exhibited GSNO resistance equivalent to that of wild-type cells. However, alanine substitution of histidine 85 in the heme domain and tyrosine 206-serine 207 in the flavoreductase domain abrogated GSNO resistance (Fig. 3), demonstrating the requirement of both heme and flavoreductase domains for NO− metabolism by Hmp. This result is consistent with the proposed transfer of electrons from NAD(P)H to FAD to heme for NO− detoxification (7, 20).

Overexpression of Hmp in the Absence of Nitrosative Stress Increases Salmonella Susceptibility to Hydrogen Peroxide by a Flavoreductase- and Iron-dependent Mechanism—Hmp overexpression from a high copy number plasmid (pBluescript) complemented the NO− resistance of an hmp mutant strain but slowed bacterial growth under aerobic conditions in the absence of nitrosative stress (data not shown) in association with cell filamentation (Fig. 4A), indicating that increased Hmp expression might have deleterious effects. The Conserved Domain Data base (40) revealed homology between the C-terminal portion of Hmp and the flavin reductase Fre. Common
ferric iron reductase activity of Hmp and Fre (18) also suggested functional conservation. Under conditions of NADH excess, the Fre flavin reductase of *E. coli* reduces FAD to FADH$_2$, which can in turn act as a ferric iron reductant and drive the Fenton reaction (21). For example, in non-respiring cells, a fre mutation protects cells from iron-dependent oxidative damage, and fre overexpression enhances susceptibility to hydrogen peroxide (21). We, therefore, determined whether overexpression of Hmp affects *Salmonella* susceptibility to hydrogen peroxide. Log-phase cells grown in iron-rich LB media were treated with cyanide to inhibit respiration and challenged with H$_2$O$_2$. As shown in Fig. 4B, within 30 min of H$_2$O$_2$ challenge the survival of Hmp-overexpressing cells was reduced more than 10-fold compared with cells harboring a control plasmid. Treatment with the chelators deferoxamine or 2,2′-dipyridyl rescued the cells, indicating that the potentiation of oxidative stress susceptibility by Hmp overexpression is iron-dependent. Concentrations of total iron in cells with and without Hmp overexpression were not significantly different (data not shown), indicating that Hmp overexpression does not alter iron content. To examine the contribution of Hmp heme and flavoreductase domains to hydrogen peroxide susceptibility, cells

![FIGURE 2. Hmp-dependent NO–detoxification. A, NO–consumption in room air was measured using an NO–electrode in the presence of wild-type (black), hmp mutant (red), and heat-inactivated (cyan) *Salmonella* or a cell-free buffer control (purple). NO– (−3 μM) was added at times designated by arrows to wild-type cells and only once to the other specimens. Results of a representative experiment are shown. B, growth of wild-type (WT) (filled symbols) and isogenic hmp mutant (open symbols) bacteria was measured in minimal E medium (diamonds) or in the presence of 1 mM GSNO (circles), spermine-NONOate (triangles), or diethylamine triamine-NONOate (rectangles). The experiment was repeated three times with essentially identical results. C, S-nitrosothiol (SNO) accumulation was measured in wild-type and hmp mutant bacteria after 3 mM GSNO treatment for 2h. Nitrosylation was detected by photolysis-chemiluminescence. S-Nitrosothiols are represented by the mercury-displaceable component. Data represent the mean ± S.D., *p < 0.05 compared with WT control. D, morphology of wild-type and hmp mutant *Salmonella* after overnight culture in minimal medium containing 1 mM GSNO was examined by light microscopy with Nomarski optics (1000×). The photograph is representative of three independent experiments.](image)

![FIGURE 3. Both the heme and flavoreductase domains of Hmp are required for *Salmonella* resistance to nitrosative stress. Overnight cultures of wild-type (WT) *Salmonella* cells and hmp mutant cells harboring a hmp complementing plasmid (pHmp), empty vector (pSK), or site-directed mutant hmp alleles (H85A for the heme domain and Y206A-S207A for the flavoreductase domain) were diluted 1:1000 into fresh minimal E medium containing 0 μM (blue), 125 μM (red), 250 μM (yellow), 500 μM (cyan), or 1 mM (purple) GSNO. Growth was monitored in Bioscreen C microplate reader with agitation at 37°C. Data are representative of three independent experiments.](image)
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**FIGURE 4.** Hypersusceptibility of Hmp-overexpressing cells to oxidative stress. A, morphology of wild-type Salmonella carrying the hmp plasmid or empty vector (pSK) was examined by light microscopy with Nomarski optics (×1000) after overnight aerobic culture or standing culture in minimal medium. B, the H₂O₂ (2 mM) susceptibility of wild-type Salmonella carrying the hmp plasmid or empty vector (pSK) with or without pretreatment with 3 mM potassium cyanide (KCN) to inhibit respiration and 2,2'-dipyridyl (DP) to chelate iron. The chelator deferoxamine was also used with results identical to those obtained with 2,2'-dipyridyl. Data are representative of three independent experiments. *, p < 0.005, compared with pSK + KCN. C, H₂O₂ susceptibility of wild-type Salmonella carrying plasmids expressing wild-type hmp or mutant alleles with disruption of the flavoreductase (Y206A-S207A) or heme (H85A) domains was determined after pretreatment with 3 mM potassium cyanide (KCN) to inhibit respiration and 2,2'-dipyridyl (DP) to chelate iron. The chelator deferoxamine was also used with results identical to those obtained with 2,2'-dipyridyl. Data are representative of three independent experiments. *, p < 0.005, compared with pSK + KCN. D, relative heme content of cells overexpressing wild-type (WT), Y206A-S207A, and H85A mutant Hmp proteins. Log-phase cells (Abs₆₀₀ nm ~ 0.3) were collected, and the heme content of total lysates containing 10 μg of total protein was determined by a colorimetric assay as described under “Experimental Procedures.” Data represent the mean ± S.D. *p = 0.0005 compared with wild type.

overexpression of mutant Hmp proteins were challenged as described above. A Y206A-S207A flavoreductase mutation largely restored hydrogen peroxide resistance to wild-type levels, whereas an H85A heme mutation had no effect (Fig. 4C). To confirm that His-85 is required for heme binding, a colorimetric heme assay was performed with lysates prepared from Hmp-overexpressing cells. The heme content of bacteria overexpressing H85A mutant Hmp was dramatically reduced in contrast to those overexpressing the wild-type or Y206A-S207A mutant Hmp proteins (Fig. 4D). These results indicate that the FAD binding domain but not the heme domain is required for Hmp-mediated hypersusceptibility to oxidative stress in intact bacterial cells.

**Hmp Transcription Is Repressed under Iron Replete Conditions by NsrR but Not by Fur—**An earlier study showed that iron availability or inactivation of the fur gene encoding an iron-sensitive transcriptional repressor was reported to induce hmp expression (42), but these observations were recently retracted due to the discovery that the published studies erroneously employed an iroC-lacZ reporter fusion (43). Therefore, the regulatory mechanisms controlling hmp expression in Salmonella are uncertain.

To determine whether hmp expression is affected by NO, iron availability, and the Fur repressor, quantitative real-time reverse transcription-PCR was used to measure steady state hmp mRNA levels. As shown in Fig. 5A, hmp transcription is induced by either iron limitation with 200 μM 2,2'-dipyridyl or exposure to 1 mM spermine-NONOate. The addition of excess iron to 2,2'-dipyridyl-treated cells restored repression of hmp transcription. However, iron-dependent regulation of hmp transcription did not require Fur. The well characterized iroN gene (44) provided a positive control for Fur-dependent gene
regulation. A recent comparative genomic analysis of NO-responsive transcriptional networks predicted that a [2Fe-2S] cluster-containing IscR homolog (YjeB, NsrR) represses hmp transcription in most enterobacterial species (45). Therefore, an nsrR deletion mutation was constructed in S. typhimurium. Transcription of hmp was found to be derepressed 1000-fold by the nsrR mutation in iron-rich LB medium, comparable with mRNA levels observed in spermine-NONOate-treated wild-type cells (Fig. 5A). Regulation by 2,2′-dipyridyl was also abolished by the nsrR mutation, as predicted for an IscR homolog. Expression of the nsrR gene on a plasmid repressed hmp transcription in nsrR mutant cells, but this repression was completely abrogated by treating cells with spermine-NONOate (Fig. 5B). These findings suggest that iron limitation and NO-control hmp expression by reversible inactivation of the NsrR repressor in S. typhimurium.

**DISCUSSION**

Production of NO+ by iNOS plays an essential role in innate immunity to many microbial pathogens, including *Salmonella* (28, 46). However, as a successful pathogen, *S. typhimurium* possesses mechanisms to antagonize the antimicrobial actions of RNS. This study has examined the contribution of RNS-metabolizing enzymes to *Salmonella* pathogenesis. Although in vitro biochemical studies demonstrate metabolism of RNS by the flavohemoglobin, flavoredoxin, GSNO reductase, and cytochrome c nitrite reductase enzymes, only the flavohemoglobin Hmp was found to be essential for *Salmonella* virulence in an acute infection model. This suggests that aerobic detoxification of NO+ produced by the oxygen-dependent iNOS host enzyme is of greatest importance to *Salmonella* during acute infection. We have observed that AdhC can limit nitrosative stress during exposure to high NO+ concentrations in vitro (data not shown), but these concentrations may be non-physiological. Functional redundancy between NorV and NrfA may have prevented individual mutants from demonstrating a phenotype in our experiments. Moreover, it is conceivable that anaerobic detoxification of chemically generated NO+ or the products of nitrate/nitrite respiration (47, 48) may be important in settings not demonstrated under our experimental conditions.

A previous study demonstrated that the fungal FhI flavohemoglobin is required for virulence of *Cryptococcus neoformans* (49), indicating that the detoxification of host-derived NO+ is a function conserved among flavohemoglobins produced by diverse pathogenic microorganisms. By demonstrating an unequivocal contribution of Hmp to *Salmonella* virulence, we have been able to expand significantly upon an earlier report that Hmp modestly enhances *Salmonella* survival in cultured macrophages (50). Moreover, our finding that Hmp is required for persistent infection of *Salmonella* in mice suggests that host-derived RNS are important during microbial persistence as well as acute infection.

Single domain globins are produced by a small number of bacterial species such as *Vitreoscilla* sp., *Campylobacter jejuni*, and *Mycobacterium bovis* (51, 52). But the role of these globins is unclear. More frequently, bacterial globins contain an additional reductase domain at the C terminus, which resembles flavin reductase or ferredoxin-NADP reductase with binding sites for FAD and NAD(P)H. The current study has demonstrated that site-specific mutation of either the FAD-binding site (Y206A-S207A) or proximal His ligand of heme (H85A) abrogates the NO+-detoxifying function of the *Salmonella* flavohemoglobin Hmp. The Tyr-206 and Ser-207 residues contact the FMN moiety and influence the electrochemical potential of the prosthetic FAD group (53), which subserves the reduction of heme-bound NO+ to NO−. That is, the flavin reductase domain is essential for electron delivery from FAD to the heme ligand whether NO+ is converted to NO− under aerobic or microaerobic conditions or to N2O under anaerobic conditions (7). Of note, on the basis of relative activity, Hmp has been suggested to make only a minor contribution to NO− reduction in comparison to the flavoredoxynorV (8). However, a recent comparison of the NO− susceptibility of *hmp* and *norV* mutant *E. coli* strains suggests that both are functionally important under anaerobic conditions (54), whereas only the Hmp is functionally important under the microaerobic conditions that likely predominate in vivo (7).

In the absence of NO+, Hmp can transfer electrons from NAD(P)H to external electron acceptors such as O2, dihydropyridine, ferrisiderophores, ferric citrate, Fe(III)-hydroxamate, and cytochrome c (18–20, 55, 56). Purified *E. coli* Hmp can generate superoxide and hydrogen peroxide by a heme-dependent mechanism (16, 17), suggesting one possible pathway by which Hmp might contribute to oxidative stress. However, the present study suggests an alternative mechanism; that is, promotion of ferric iron reduction by reduced flavins. Ferric reductase activity of Hmp was originally detected in an analysis of soluble ferrisiderophore reductases (18) and is attributable to the reduction of flavins (19) that can reduce ferric iron and thereby accelerate Fenton chemistry (21). Therefore, Hmp might enhance oxidative stress both by producing superoxide at the heme ligand and by reducing FAD at the flavoreductase domain. Structural studies have shown that heme ligand in Hmp is stabilized by His-85, Tyr-29, and Gln-53 (13). The proximal F8 histidine (His-85 in *Salmonella* Hmp) in the heme pocket was found to be absolutely conserved in an alignment of 700 vertebrate and nonvertebrate globins and shown to be essential for heme binding in the protoglobin of *Aeropyrum pernix* (57). In this study an H85A mutation was found to abolish NO+ detoxification (Fig. 3) and heme binding by *Salmonella* Hmp (Fig. 4D) but not the enhancement of susceptibility to hydrogen peroxide, whereas a Y206A-S207A mutation in the FAD binding domain had little effect on heme binding but abrogated the pro-oxidant effects of Hmp (Fig. 4C). These observations suggest that the increased susceptibility of Hmp-overexpressing cells to hydrogen peroxide under non-respiring conditions results primarily from the heme-independent reduction of flavins at the flavoreductase domain, where electrons are transferred from NADH to free FAD.

In the presence of NO+, inhibition of bacterial respiration (58) can increase intracellular pools of NAD(P)H (21), which might enhance oxidative damage unless electrons are directed to ferric-nitrosyl Hmp (7) to promote reaction of heme-bound NO+ with O2 (Fig. 6, pathway 1). In this fashion Hmp can actually ameliorate oxidative stress. However, bacterial respiration
can also be inhibited in the absence of NO\textsuperscript{−}, e.g. by antimicrobial peptides produced by host cells (59). Under such circumstances, elevated levels of NAD(P)H can lead to reduction of Hmp-bound FAD. Intermolecular transfer of these reducing equivalents could promote oxidative injury. Consequently, expression of Hmp in the absence of NO\textsuperscript{−} may increase FAD- and iron-dependent damage by hydrogen peroxide (Figs. 4 and 6, pathway 2).

_Salmonella_ evidently avoids this potential toxicity by inducing _hmp_ transcription in the presence of NO\textsuperscript{−} and repressing expression when intracellular free iron concentrations are elevated. There have been some conflicting observations regarding the regulatory factors responsible for control of _hmp_ expression in enteric bacteria. Induction of the _E. coli_ _hmp_ gene in response to NO\textsuperscript{−} has been attributed to the MetR transcriptional regulator, which responds to depleted cellular homocysteine levels during nitrosative stress (60). However, other investigators did not find MetR to be required for NO\textsuperscript{−} induction of _hmp_ expression in _E. coli_ (61). Although the DNA sequences for MetR binding are fairly well conserved between the _E. coli_ and _Salmonella_ _hmp_ promoters, we did not find an effect of a _metR_ mutation on _Salmonella_ _hmp_ transcription under the experimental conditions used in this study (data not shown). A comparative genomics study has predicted regulation of _hmp_ by the transcriptional repressor NsrR (45), and this has very recently been experimentally confirmed in _E. coli_ (62). In _Salmonella typhimurium_, _hmp_ expression was originally reported to be induced by iron limitation or NO\textsuperscript{−} via a mechanism dependent on the Fur (iron uptake regulator) protein (42). In the present study we show that expression of the _Salmonella hmp_ gene is in fact induced by NO\textsuperscript{−} and repressed by intracellular free iron. However, we find _hmp_ regulation not to be mediated by Fur but, rather, by NsrR (Fig. 5). This provides a mechanism by which _hmp_ can be produced during nitrosative stress but suppressed under iron-replete conditions in the absence of NO\textsuperscript{−}. Precise regulation allows the flavohemoglobin Hmp to play a central role in NO\textsuperscript{−} detoxification without contributing to oxidative stress (Fig. 6). It is interesting to note that an analogous inverse relationship between iron and NO\textsuperscript{−} is also observed in the regulation of eukaryotic gene expression, in which iron-regulatory RNA-binding proteins are activated by NO\textsuperscript{−} and inhibited by iron (63). Such regulation is critical in an organism such as _S. typhimurium_ that must withstand exposure to both reactive oxygen species and RNS during its interactions with host phagocytes (29).

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