Cytochrome bd-Dependent Bioenergetics and Antinitrosative Defenses in Salmonella Pathogenesis

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ABSTRACT In the course of an infection, Salmonella enterica occupies diverse anatomical sites with various concentrations of oxygen (O_2) and nitric oxide (NO). These diatomic gases compete for binding to catalytic metal groups of quinol oxidases. Enterobacteraeceae express two evolutionarily distinct classes of quinol oxidases that differ in affinity for O_2 and NO as well as stoichiometry of H^+ translocated across the cytoplasmic membrane. The investigations presented here show that the dual functionality of bacterial cytochrome bd in bioenergetics and antinitrosative defense enhances Salmonella virulence. The high affinity of cytochrome bd for O_2 optimizes respiratory rates in hypoxic cultures, and thus, this quinol oxidase maximizes bacterial growth under O_2-limiting conditions. Our investigations also indicate that cytochrome bd, rather than cytochrome bo, is an intrinsic component of the adaptive antinitrosative toolbox of Salmonella. Accordingly, induction of cytochrome bd helps Salmonella grow and resist the presence of inhibitory NO. The combined antinitrosative defenses of cytochrome bd and the flavohemoglobin Hmp account for a great part of the adaptations that help Salmonella recover from the antimicrobial activity of NO. Moreover, the antinitrosative defenses of cytochrome bd and flavohemoglobin Hmp synergize to promote Salmonella growth in systemic tissues. Collectively, our investigations indicate that cytochrome bd is a critical means by which Salmonella resists the nitrosative stress that is engendered in the innate response of mammalian hosts while it concomitantly allows for proper O_2 utilization in tissue hypoxia.

IMPACT It is becoming quite apparent that metabolism is critically important to the virulence potential of pathogenic microorganisms. Bacterial cells use a variety of terminal electron acceptors to power electron transport chains and metabolic processes. Of all the electron acceptors available to bacteria, utilization of O_2 yields the most energy while diversifying the type of substrates that a pathogen can use. Recent investigations have demonstrated important roles for bd-type quinol oxidases with high affinity for O_2 in bacterial pathogenesis. The investigations presented here have revealed that cytochrome bd potentiates virulence of a clinically relevant bacterial pathogen by fueling bioenergetics of prokaryotic cells while protecting the respiratory chain against NO toxicity. The adaptive antinitrosative defenses afforded by cytochrome bd synergize with other NO-detoxifying systems to preserve cellular bioenergetics, thereby promoting bacterial virulence in tissue hypoxia.

Received 8 November 2016 Accepted 21 November 2016 Published 20 December 2016

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This article is a direct contribution from a Fellow of the American Academy of Microbiology. External solicited reviewers: Anthony Richardson, University of Pittsburgh; Andreas Bäumler, University of California, Davis.

Salmonella enterica serovar Typhimurium is a common cause of nontyphoidal salmonellosis in humans and domestic animals. In most healthy individuals, nontyphoidal Salmonella infections acquired from diverse vertebrate hosts via the fecal/oral route present as self-limiting gastroenteritis. Nonetheless, in immuno-compromised people bearing defects in CD4 T cell immunity or gamma interferon (IFN-γ) signaling, diverse strains of nontyphoidal Salmonella can cause life-threatening extraintestinal infections (1–3). Salmonella suffers the cytotoxicity of cationic peptides, as well as reactive oxygen and nitrogen species that are generated in the host response of vertebrate animals and humans. Nitric oxide (NO) is one of the most studied anti-Salmonella effectors of the innate response in mammalian cells (4). The oxidation of the guanidino group of L-arginine by the enzymatic activity of NO synthases generates NO and L-citrulline (5). Reactions of NO with superoxide, molecular oxygen (O_2), iron, or low-molecular-weight thiols produce an amalgam of antimicrobial reactive nitrogen species that include peroxynitrite, nitrogen dioxide, dinitrogen trioxide, and S-nitrosothiols. A collection of reactive nitrogen species can independently be generated upon the condensation of two molecules of acidified nitrite in the stomach and phagosomal lumen of macrophages (6–10). NO and its oxidative and nitrosative congeners exert antimicrobial activity against diverse eukaryotic and prokaryotic organisms. Cytochrome bd, DNA, lipoamide-dependent lipoamide dehydrogenase, and the regulatory proteins DksA and SsrB are some of the
few biomolecules known to be modified in Salmonella undergoing nitrosative stress (11–15). Despite the potent antimicrobial activity that NO can exert against Salmonella, this intracellular pathogen tolerates remarkably well the nitrosative stress engendered in the innate host response (8, 16). Diverse antinitrosative defenses help Salmonella cope with NO and its oxidative by-products. For example, the low-molecular-weight thiols homocysteine and glutathione scavenge reactive nitrogen species, whereas the denitrosylase activity of the flavohemoglobin Hmp detoxifies NO to nitrate (NO$_3^-$) (17–19). The combined actions of low-molecular-weight thiols and Hmp protect Salmonella against the nitrosative stress engendered in the innate host response of human and murine macrophages (20–24). Not only does Salmonella tolerate NO-mediated host defenses, but these pathogens can also take advantage of the redox properties of nitrogen oxides to colonize the gastrointestinal tract. For example, terminal cytochromes such as nitrate reductases energize cytoplasmic membranes by utilizing NO oxidative products as terminal electron acceptors (25).

Reduction of O$_2$ to water is the canonical function of aerobic terminal cytochromes of the electron transport chain, a process that generates an electrochemical gradient across cytoplasmic membranes and powers transport systems and ATP synthesis. Salmonella expresses two evolutionarily distinct classes of quinol oxidases. Cytochrome bd, a member of the cytochrome c oxidase family, is encoded in the cyoABCD operon, whereas cytochrome bd and cytochrome bd-II are encoded in the cydAB and cyxAB operons, respectively. Cytochrome bd and cytochrome bd-II contain heme d in place of the C$_{450}$ atom that occupies the catalytic site of cytochrome bo (26). Although less efficient than cytochrome bo, cytochrome bd also participates in the bioenergetics of the bacterial cell (26). The expression of cytochrome bd in S. enterica, Klebsiella pneumoniae, Mycobacterium tuberculosis, Shigella flexneri, group B Streptococcus, Listeria monocytogenes, and Bacteroides suggests a possible role for this quinol oxidase in bacterial pathogenesis (27). Accordingly, cytochrome bd promotes gastrointestinal and systemic fitness of Citrobacter rodentium and S. enterica serovar Typhimurium, respectively (28–30).

Expression of cytochrome bd in E. coli, Staphylococcus aureus, Bacillus subtilis, and M. tuberculosis in response to NO and the nitrosylation of the heme d in cytochrome bd of Salmonella raise the interesting possibility that, in addition to fueling the bioenergetics of the cell, cytochrome bd may be part of the antinitrosative arsenal of several pathogenic microorganisms (11, 31–35). This idea is suggested further by the fact that ΔcydAB E. coli and Δfur Salmonella, a strain that harbors low concentrations of cytochrome bd, are hypersusceptible to the bacteriostatic activity of chemically generated NO (16, 31, 36). However, given the copious antinitrosative defenses available to pathogenic bacteria (4), it remains uncertain whether the NO-detoxifying activity of cytochrome bd contributes to bacterial virulence. The following investigations have explored the extent to which cytochrome bd contributes to the antinitrosative defenses of Salmonella in culture and murine models of infection.

RESULTS

Contribution of quinol oxidases to Salmonella virulence. Salmonella expresses two major terminal quinol oxidases encoded within cyoABCD and cydAB operons. Compared to cydAB-encoded cytochrome bd, cytochrome bo has lower affinity for O$_2$ and NO but greater capacity to translocate protons across the cytoplasmic membrane (27, 36). Given the pronounced differences between these two quinol oxidases, we compared the capacities of ΔcyoABCD and ΔcydAB mutants to colonize the gastrointestinal tract of streptomycin-treated C3H/HeN mice. Similar amounts of ΔcyoABCD and ΔcydAB Salmonella were shed in feces of C3H/HeN mice compared to control mice infected with an isogenic wild-type strain (Fig. 1A). With the exception of the ΔcydAB mutant, which was recovered in lower numbers in colon, ΔcyoABCD- and ΔcydAB-deficient Salmonella appeared to be as capable as wild-type bacteria in colonizing the small and large intestines of streptomycin-treated C3H/HeN mice 3 days after oral (p.o.) inoculation. All strains tested also induced similar levels of inflammation in ceca of infected animals as indicated by the presence of edema in the submucosa, infiltration of polymorphonuclear cells in the lamina propria, and depletion of goblet cells (Fig. 1C and D). Despite these similarities, some differences in histopathology were noted. For instance, ceca of ΔcyoABCD Salmonella-infected mice contained fewer polymorphonuclear cells than wild-type controls, whereas the ceca of mice infected with ΔcydAB Salmonella contained more goblet cells than controls infected with either wild-type or ΔcyoABCD Salmonella. Collectively, our investigations are consistent with recently published data that showed the apparent dispensability of cytochrome bd during colonization of the gut (28).

Cytochrome bd contributes to Salmonella antinitrosative defenses. Previous work showed that Δfur Salmonella is hypersusceptible to the bacteriostatic activity of NO generated chemically in vitro or enzymatically in vivo (16). Diminished 420-nm and 560-nm absorption peaks in cytoplasmic membranes of Δfur Salmonella indicate that this mutant harbors reduced concentrations of both cytochrome bo and cytochrome bd. To investigate a possible role of these two terminal cytochromes in the antinitrosative defenses of Salmonella, we compared growth rates of ΔcyoABCD and ΔcydAB mutants deficient in cytochrome bo and cytochrome bd, respectively, in LB broth supplemented with diethylenetriamine (DETA) or the NO donor DETA NONOate (Fig. 2A). Wild-type and ΔcyoABCD Salmonella strains grew similarly in LB broth containing 5 mM DETA. The addition of 5 mM DETA inhibited the growth of ΔcydAB Salmonella and ΔcydAB mutants deficient in cytochrome bo and cytochrome bd, in contrast to wild-type and ΔcyoABCD isogenic controls, ΔcydAB Salmonella consistently grew more slowly in LB broth, suggesting that the high affinity for O$_2$ of cytochrome bd improves growth of Salmonella in hypoxic media. Salmonella bearing the ΔcydAB mutation was also hypersusceptible to the bacteriostatic activity of NO as suggested by the extended lag phase that followed DETA NONOate treatment. Cumulatively, these investigations indicate that cytochrome bd, rather than cytochrome bo, is an intrinsic constituent of the antinitrosative defenses of Salmonella.

Cytochrome bd protects respiration against NO. Since terminal cytochromes of the electron transport chain are some of the preferred targets of NO (4), we measured the rates of respiration in ΔcyoABC and ΔcydAB Salmonella exposed to spermine NONOate (Fig. 2C). Wild-type and ΔcyoABCD Salmonella strains grown

November/December 2016 Volume 7 Issue 6 e02052-16
to log phase in LB broth showed comparable respiratory activities. Addition of 50 \(\mu\)M NO donor spermine NONOate similarly repressed the respiratory activity of wild-type and \(\Delta\)cyoABCD Salmonella (0.54 versus 0.52 \(\mu\)M O\(_2\)/s, respectively). Respiratory rates improved in both wild-type and \(\Delta\)cyoABCD Salmonella 3.5 min after treatment (1.3 \(\mu\)M/s) but did not reach those recorded in resting cells (~1.90 \(\mu\)M/s). The \(\Delta\)cydAB Salmonella strain respired more slowly than unstimulated wild-type or \(\Delta\)cyoABCD controls, strains that likely take advantage of the superior performance of cytochrome bd under hypoxia. Compared to wild-type bacteria, the respiratory activity of the \(\Delta\)cydAB mutant was considerably more sensitive to the inhibitory effects of spermine NONOate. Furthermore, in contrast to wild-type and \(\Delta\)cyoABCD Salmonella, the respiratory activity of \(\Delta\)cydAB Salmonella did not seem to improve over time after the addition of spermine NONOate (0.67 \(\mu\)M/s). These findings indicate that cytochrome bd, but not cytochrome bo, affords protection to quinol oxidases against NO toxicity.

**Cytochrome bd and the flavohemoglobin Hmp lessen NO cytotoxicity.** Previous work identified the flavohemoglobin Hmp as the main antinitrosative defense of Salmonella (22). Because our investigations indicate that cytochrome bd is part of the anti-nitrosative arsenal of Salmonella, we deemed it important to compare the relative contributions of Hmp and cytochrome bd to the anti-nitrosative defenses of Salmonella. Toward this end, a \(\Delta\)cydAB::Km mutation was moved into \(\Delta\)hmp Salmonella strain AV0468. Differential spectrophotometry of whole bacterial cells indicates that \(\Delta\)hmp Salmonella expresses higher concentrations of cytochrome bd than wild-type controls as shown by the characteristic 650-nm heme d absorption peak (Fig. 3A). We initially
tested the susceptibility of hmp-deficient *Salmonella* strains to 5 mM DETA NONOate; however, at this concentration the NO donor completely inhibited growth of both Δhmp and Δhmp ΔcydAB::Km *Salmonella*. Therefore, we tested lower concentrations of DETA NONOate and the DETA parent compound. The addition of 1 mM DETA did not have much of an effect on the growth of wild-type *Salmonella* (Fig. 3B). As noted above for ΔcydAB *Salmonella*, the Δhmp ΔcydAB strain exhibited a slight but reproducible growth defect in LB broth. The addition of 1 mM DETA NONOate did not inhibit growth of wild-type *Salmonella*. However, 1 mM DETA NONOate extended in increasing order the lag phase of ΔcydAB, Δhmp, and Δhmp ΔcydAB::Km mutant *Salmonella* strains. Together, our investigations indicate that both Hmp and cytochrome bd protect *Salmonella* against NO, although quantitatively the flavohemoglobin appears to confer the greatest protection. Because Δhmp ΔcydAB::Km mutant *Salmonella* was more susceptible to NO than ΔcydAB or Δhmp isogenic strains, our investigations also indicate that Hmp and cytochrome bd independently add to the antinitrosative arsenal of *Salmonella*.

Transcriptional analysis showed that the cydA operon is induced in wild-type *Salmonella* shortly after the addition of 50 μM spermine NONOate (P < 0.0001) (Fig. 3C). Consistent with the spectroscopic analysis shown in Fig. 3A, Δhmp *Salmonella* expressed higher levels of cydA than wild-type controls (P = 0.0117). Although delayed compared to wild-type controls, NO also induced cydAB expression in Δhmp *Salmonella* (P < 0.0001). Cumulatively, these data indicate that cytochrome bd is part of the adaptive response of *Salmonella* to nitrosative stress.

**Cytochrome bd and Hmp independently protect the respiratory chain from the inhibitory activity of NO.** We next examined the rates of respiration of *Salmonella* strains deficient in Hmp and/or cytochrome bd. Wild-type *Salmonella* exposed for 1 min to 50 μM spermine NONOate exhibited reduced respiratory activity (Fig. 3D). As shown above, wild-type *Salmonella* recovered about two-thirds of its maximal respiratory activity a few minutes after NO treatment, consistent with the stimulation of a partial adaptive response. To test if *Salmonella* can fully adapt to NO, respiratory activity was independently measured 10 min after spermine NONOate treatment. Wild-type *Salmonella* completely recovered O2-consuming capacity 10 min after spermine NONOate treatment (1.47 versus 1.45 μM/O₂/s for nontreated and NO-treated specimens, respectively). As observed earlier (Fig. 2C), untreated ΔcydAB *Salmonella* exhibited lower rates of respiration than wild-type controls. We also noticed that the marked reduction in respiratory activity (0.45 μM/O₂/s) of ΔcydAB *Salmonella* treated with NO for 1 min was sustained for the duration of the experiment. Nonetheless, the respiratory activity of ΔcydAB *Salmonella* recovered to levels of untreated specimens 10 min after the addition of NO (0.70 and 0.69 μM/O₂/s for untreated and NO-treated samples, respectively), suggesting that *Salmonella* can eventually adapt to nitrosative stress in the absence of cytochrome bd. It should be noted that the respiratory activity of ΔcydAB *Salmonella* decreased...
after 10 min of culture, likely reflecting reduced affinity of cytochrome bo for O₂ as bacterial density increases over time.

Because the Δhmp mutant is highly susceptible to the antimicrobial activity of NO (Fig. 2) and because Hmp is a critical antinitrosative defense that promotes respiratory activity in Gram-negative bacteria undergoing nitrosative stress (37), we also examined the effects of NO on respiration of Δhmp Salmonella (Fig. 3C). Compared to ΔcydA isogenic controls, respiration of Δhmp Salmonella was more profoundly inhibited 1 min after exposure to 50 μM spermine NONOate (0.45 versus 0.23 μM O₂/s, respectively), suggesting that Hmp is more efficient at detoxifying NO than cytochrome bd. Interestingly, Δhmp Salmonella recovered more than 50% respiratory activity 10 min after NO treatment (1.70 versus 0.96 μM O₂/s in untreated and NO-treated specimens, respectively), indicating the existence of Hmp-independent means to detoxify NO.

We finally quantified the O₂-consuming capacity of a mutant lacking both hmp and cydA. The Δhmp ΔcydA::Km Salmonella strain AV09592 suffered as much repression of O₂ consumption as its Δhmp isogenic control 1 min after the addition of 50 μM spermine NONOate (0.20 versus 0.23 μM O₂/s, respectively). However, compared to Δhmp controls, the respiration of Δhmp ΔcydA::Km Salmonella remained more profoundly inhibited 10 min after NO treatment (1.48 versus 0.44 μM O₂/s). Together, these data indicate that cytochrome bd adds to the dominant NO-detoxifying activity of the flavohemoglobin Hmp. Because the Δhmp ΔcydA mutant partially recovered its respiratory activity 10 min after NO treatment, these investigations also point to the existence of Hmp- and cytochrome bd-independent means to detoxify NO.

**Contribution of Hmp and cytochrome bd to Salmonella pathogenesis.** Having established that both Hmp and cytochrome bd protect respiration of Salmonella experiencing nitrosative stress, we used two murine models to examine the extent to which these two antinitrosative defenses contribute to Salmonella pathogenesis. First, C3H/HeN mice were inoculated intraperitoneally (i.p.) with equal numbers of wild-type and Δhmp, ΔcydA, or Δhmp ΔcydA::Km Salmonella, and the competitive advantage of bacteria in the mixtures was determined by quantifying hepatic burden 5 days after infection. These investigations showed that Δhmp and ΔcydA ΔcydA::Km Salmonella are similarly attenuated (competitive indexes of about 0.1 and P > 0.05 compared to wild-type Salmonella) (Fig. 4A). In contrast, Δhmp ΔcydA::Km Salmonella had a competitive index of 0.01 compared to wild-type bacteria. The double mutant was found to be significantly more attenuated than either Δhmp (P < 0.01) or ΔcydA (P < 0.001) Salmonella. Together, these findings suggest a substantial degree of independence between Hmp and cytochrome bd in Salmonella pathogenesis. We also examined the relative contributions of Hmp and cytochrome bd in a live/dead model of acute Salmonella infection.

**FIG 3** Synergy of Hmp and cytochrome bd in the antinitrosative defenses of Salmonella. (A) Differential whole-cell spectra of wild-type (WT) and mutant Salmonella. The arrow shows the absorption peak of heme d. (B) Growth of Salmonella diluted to 2 × 10⁶ CFU/ml in LB broth after treatment with 1 mM DETA or DETA NONOate (dNO). Data represent the means ± standard errors of the means from 10 observations from two independent experiments. (C) Expression of cydA in wild-type and Δhmp Salmonella exposed to 50 μM spermine NONOate (sNO). Time after addition of NO (P < 0.0001) and bacterial strain (P = 0.0117) were found to statistically affect the expression of cydA as determined by two-way analysis of variance (n = 4 from two independent days). No cydA mRNA was detected in ΔcydA Salmonella (not shown). (D) Effect of NO on respiration. Bacterial cells were prepared as described in the legend to Fig. 2. Where indicated, bacteria were treated with 50 μM sNO for 1 or 10 min prior to the analysis of respiration. Untreated controls (C) are shown for comparison. Data are representative of 3 independent experiments.
Investigations indicate that Hmp and cytochrome bd after controls challenged with wild-type bacteria. Collectively, our treated, components of the antinitrosative toolbox of virulence of nitric oxide synthase (iNOS) inhibitor aminoguanidine increased the /H9004 was significantly (P < 0.0001) in C3H/HeN mice according to the log rank Mantel-Cox survival test. The data are from 5 to 7 mice per group.

FIG 4 Hmp and cytochrome bd in Salmonella pathogenesis. (A) The competitive index was measured in livers of C3H/HeN mice 3 days after i.p. inoculation with 2,000 CFU of a mixture containing wild-type (WT) and equal numbers of ΔcydAB::Km, Δhmp::Km, and Δhmp ΔcydAB::Km Salmonella. **, ***, P < 0.01; ***, P < 0.001. (B) Survival of Salmonella-infected C3H/HeN mice was recorded over time after i.p. inoculation. Selected groups of mice were continuously fed water containing 500 μg/ml of the iNOS inhibitor amino- guanidine (AG). The Δhmp Δhmp mutant strain was found to be attenuated (P < 0.0001) in C3H/HeN mice according to the log rank Mantel-Cox survival test. The data are from 5 to 7 mice per group.

(Fig. 4B). Strain AV09592 harboring mutations in both hmp and cydAB was significantly (P < 0.0001) more attenuated than wild-type, Δhmp, or ΔcydAB Salmonella. Administration of the inducible nitric oxide synthase (iNOS) inhibitor aminoguanidine increased the virulence of Δhmp ΔcydAB::Km Salmonella. Aminoguanidine-treated, Δhmp ΔcydAB::Km Salmonella-infected mice died a few days after controls challenged with wild-type bacteria. Collectively, our investigations indicate that Hmp and cytochrome bd are important components of the antinitrosative toolbox of Salmonella.

DISCUSSION

Salmonella must adapt to various concentrations of O₂ and NO in different anatomical sites during the course of an infection. Differential utilization of quinol oxidases with distinct affinities for O₂ and NO allows Salmonella to colonize the gastrointestinal tract and to establish infections in deep tissue (this study and reference 28). Our investigations have demonstrated that the ability of cytochrome bd to protect the electron transport chain against NO is a considerable component of the adaptive antinitrosative defenses of Salmonella in a murine model of acute systemic infection. The high affinity for NO of cytochrome bd (Kd [dissociation constant] of 0.55 nM) provides a molecular mechanism by which this quinol oxidase contributes to antinitrosative defenses (36). Preferential nitrosylation of cytochrome bd frees up cytochrome bo for respiration. In addition to having high affinity for NO, cytochrome bd dissociates faster from NO than most known cytochromes, including cytochrome bo (36). The fast dissociation of NO from heme d may explain why we noted that ΔcyoABCD Salmonella expressing cytochrome bd maintains excellent respiratory activity in the presence of NO. Compared to cytochrome bo, fast dissociation of NO from heme d may also underlie the lower sensitivity of cytochrome bd to this diatomic radical. Cytochrome bd is resistant not just to NO but also to hydrogen sulfide (38, 39). Expression of cytochrome bd may therefore preserve respiratory activity in hypoxic environments in the presence of sulfide and NO.

Cytochrome bd may mediate antinitrosative defense through nitrosoy and nitrate pathways. According to the nitrosyl pathway, ferrous iron in heme d binds NO in competition with O₂. The nitrosyl (Fe²⁺-NO) product resulting from this reaction dissociates with a Kₐd of 0.133 s⁻¹ (40). The nitrosyl pathway prevails at high e⁻ flux and low O₂ concentrations, conditions that appear to be encountered by Salmonella in hypoxic systemic tissues (29). Second, according to the nitrite pathway, the oxoferryl (Fe⁴⁺-==O) intermediate can react with NO with a second-order rate constant of 10⁵ M⁻¹ s⁻¹ (41). The NO₂⁻ anion formed at the catalytic site is ejected successfully from the Fe⁴⁺-NO₂⁻ intermediate, providing a direct mechanism for NO detoxification. The nitrite pathway, which proceeds under low e⁻ flux and high O₂ conditions, provides a rationale for the noncompetitive inhibition of cytochrome bd at low NO/O₂ ratios. Because the flavohemoglobin Hmp, whose enzymatic activity operates at high O₂ concentrations (42), plays a role in Salmonella pathogenesis (22), Salmonella is likely to encounter host-derived NO under high O₂ tensions. Therefore, it is possible that cytochrome bd detoxifies NO in vivo by the nitrite pathway. Whether cytochrome bd uses the nitrosoy or nitrite pathways depends on changing NO/O₂ ratios during the course of the infection.

Salmonella encounters NO and its congeners in the gastrointestinal tract and within mononuclear phagocytic cells (8, 9, 43). Salmonella is remarkably resistant to the antimicrobial activity of NO generated in the innate host response (8). Reaction of reactive nitrogen species with low-molecular-weight thiols such as homocysteine and glutathione contributes to Salmonella pathogenesis (20, 21). Salmonella can also detoxify NO to nitrous oxide (N₂O₃) or NO₃⁻ through the enzymatic activities of the anaerobic flavo-rubedoxin NorVW or the aerobic flavohemoglobin Hmp, respectively (22, 44, 45). Acute murine models of infection have shown that Hmp, not NorVW, defends Salmonella against nitrosoative stress generated in the host (22), suggesting that aerobic
conversion of NO to $\text{NO}_3^-$ is a biologically relevant pathway for detoxification of host-derived NO. Our investigations indicate that, together with Hmp, cytochrome bd is an important component of the adaptive antinitrosative arsenal of *Salmonella in vivo*. Our biochemical and microbiological approaches suggest that Hmp is more important than cytochrome bd in protecting the respiratory and replicative capacity of *Salmonella* exposed to chemically generated NO. The competitive assays recorded in mice indicate, however, that both Hmp and cytochrome bd contribute to similar extents to *Salmonella* pathogenesis. Several reasons may explain the ranking of importance for Hmp and cytochrome bd as antinitrosative defenses of *Salmonella* depending on the experimental conditions tested. First, limited O$_2$ concentrations in mice might favor NO detoxification by cytochrome bd, whereas high P$_{O2}$ tension in culture may favor Hmp enzymatic activity. Second, *Salmonella* may experience different O$_2$ and NO concentrations as the inflammatory response evolves over time in the course of the infection. Thereby, *Salmonella* may preferentially use Hmp or cytochrome bd according to the availability of O$_2$ and NO. Unique utilization of Hmp and cytochrome bd could explain why $\Delta hmp$ $\Delta cydAB$: Km *Salmonella* is significantly more attenuated than mutants lacking hmp or cydAB. Third, it is also possible that Hmp and cytochrome bd may perform redundant NO detoxification at high O$_2$ tensions. Thus, the absence of both antinitrosative defenses in $\Delta hmp$ $\Delta cydAB$: Km *Salmonella* accentuates susceptibility to NO.

Cytochrome bd may also add to *Salmonella* pathogenesis in ways that are independent of NO detoxification. The electrogenic quinol-O$_2$ oxidoreductase activity of cytochrome bd generates a proton motive force across the membrane that fuels oxidative phosphorylation (46). The importance of energetics in *Salmonella* pathogenesis is suggested by the observation that inhibition of iNOS did not completely restore virulence of $\Delta hmp$ $\Delta cydAB$: Km *Salmonella*. Moreover, our biochemical and microbiological analyses showed that $\Delta cydAB$ *Salmonella* has lower rates of respiration and growth than wild-type or $\Delta cyoABCD$ controls. The high affinity of cytochrome bd for O$_2$ could also allow *Salmonella* to colonize hypoxic areas in the host. In this sense, cytochrome bd promotes growth of *Salmonella* in systemic sites but seems to play a marginal role in colonization of gut mucosa (this work and references 28 and 30). These patterns likely reflect the fact that cytochrome bd works at a 5 to 10% O$_2$ tension in tissue but performs poorly at 0.8% O$_2$ in gut lumen (28). In addition to canonical energetic functions, the high affinity of cytochrome bd for O$_2$ could protect vulnerable [4Fe-4S] clusters in dehydratases and transcription factors such as fumarate-nitrate reduction regulator (FNR) from oxidative damage. Finally, cytochrome bd is an important source of oxidizing power that aids the DsbA-DsbB-ubiquinone complex with the formation of disulfide bonds and folding of periplasmic proteins (47). In this fashion, cytochrome bd could fuel DsbA-dependent folding of components of the *Salmonella* pathogenicity island 2 type III secretion system (48), a nanomachine that is essential for the intracellular replication of *Salmonella* as well as resistance of this facultative intracellular pathogen to oxygen-dependent and -independent antimicrobial host defenses.

In summary, our investigations indicate that dual functions of cytochrome bd in bacterial bioenergetics and antinitrosative defenses contribute to *Salmonella* pathogenesis in murine models of systemic infection (Fig. 5). Generation of electrochemical gradients across cytoplasmic membranes, oxidation and folding of periplasmic proteins, and detoxification of O$_2$ and NO represent some of the diverse mechanisms by which cytochrome bd may promote bacterial growth in inflammatory and normal tissue hypoxia.

**MATERIALS AND METHODS**

**Bacterial strains.** Strains and primers used in these investigations are listed in Tables S1 and S2 in the supplemental material. Mutations were constructed following the A Red recombinase system (49). In-frame deletions were verified by PCR analysis.

**Susceptibility to NO.** The effects of the polyamine diethylenetriamine (DETA) or the NO donor DETA NONOate on growth of wild-type and mutant *Salmonella* were measured spectrometrically on a Bioscreen C microbiology microtiter plate (Growth Curves USA, Piscataway, NJ). *Salmonella* cultures grown overnight in LB broth were diluted 1:500 in LB broth and treated with 5 mM NO donor DETA NONOate or the polyamine DETA control. Where indicated, some cultures were independently treated with 1 mM DETA NONOate. The half-life ($t_{1/2}$) of DETA NONOate at neutral pH is about 20 h. We estimate that 5 mM DETA NONOate produced a rather stable flux of 5 µM NO for 20 h that lasted the experiment (50). Bacterial growth was recorded as optical density at 600 nm (OD$_{600}$) every 15 min, while cultures were shaken at 37°C.

**Cytochrome spectrometry.** Wild-type and mutant *Salmonella* strains grown overnight in LB broth were subcultured in LB broth to an OD$_{600}$ of 0.5. Inner membranes were prepared as described by Husain et al. (11). Briefly, bacterial pellets were resuspended in 100 mM EDTA, 100 mM Tris-HCl buffer, pH 8.5. 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 3 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$_2$HPO$_4$, 150 mM KCl, 5 mM EDTA, 60 mM N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate buffer, pH 7.1.

**FIG 5** Bioenergetic and antinitrosative roles of cytochrome bd in *Salmonella* pathogenesis. Cytochrome bd promotes antinitrosative defenses by ensuring respiratory activity in the presence of NO (left) and by detoxifying NO (right). At high NO/O$_2$ ratios and high electron flow, the high affinity of cytochrome bd for NO ensures that cytochrome bo is free to respire in *Salmonella* undergoing nitrosative stress. In addition, the high K$_{m}$ of ferrous iron in heme d for NO allows cytochrome bd to reduce O$_2$ to water. 2H$^+$ and 1H$^+$ are translocated by the actions of cytochrome bo and cytochrome bd, respectively. At low NO/O$_2$ ratios and low electron flow, NO reacts with the oxoferryl intermediate in heme of cytochrome bd, yielding the oxidative product nitrite (NO$_2^-$). The dominant denitrosylase enzymatic activity of the flavohemoglobin Hmp detoxifies NO to NO$_3^-$, whereas high P$_{O2}$ tension in culture may favor Hmp enzymatic activity.

| SUSTAINED RESPIRATION | DETOXIFICATION |
|-----------------------|----------------|
| Cytochrome bo          | Hmp            |
| Cu                    | Fe             |
| $2H^+$                | NO             |
| $H^+$                 | NO$_2$         |
| Fe                    | NO$_3$         |
| O$_2$                 | Fe             |
| Hmp                   | NO$_2$         |
| Cytochrome bo          |                |
| Cu                    |                |
| $2H^+$                |                |
| $H^+$                 |                |
| Fe                    |                |
| O$_2$                 |                |
| Hmp                   |                |

*Note:* The width of the arrows represents, in heme of cytochrome bd, yielding the oxidative product nitrite (NO$_2^-$). The dominant denitrosylase enzymatic activity of the flavohemoglobin Hmp detoxifies NO to NO$_3^-$.
6.4. Supernatants containing inner membranes were collected, and the protein concentration was assayed using the bicinechonic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL). The protein concentration in the specimens was adjusted to 1.5 mg/ml in 75 mM K$_2$HPO$_4$, 150 mM KC$_2$, 5 mM EDTA, 10 mM ascorbate, and 60 mM N-dodecyl-NN-dimethyl-3-ammonio-1-propanesulfonate buffer, pH 6.4. Absorbance spectroscopy was collected in a Cary 50 Bio UV-visible spectrophotometer. Cytochrome content also was evaluated by difference spectroscopy. Briefly, stationary-phase wild-type, Δhmp, and ΔcydAB Salmonella strains grown overnight in LB broth were subcultured 1:100 in EG medium (E salts [1.66 mM MgSO$_4$, 9.5 mM citric acid monohydrate, 57 mM K$_2$HPO$_4$, 16.7 mM Na$_2$PO$_4$] supplemented with 0.4% [wt/ vol] glucose), pH 7.0, for 4 h. Bacterial densities were adjusted to an OD$_{600}$ of 0.1. Some of the specimens were oxidized with 10 mM ammonium persulfate for 10 min before measuring cytochrome content by UV-visible spectroscopy. Cytochrome content in bacterial cells was estimated by recording reduced-minus-oxidized spectra.

Transcriptional analysis. Wild-type, Δhmp, or ΔcydAB Salmonella strains grown overnight in LB broth were subcultured 1:100 in LB broth and grown at 37°C with shaking to an OD$_{600}$ of 0.5. The cultures were treated with 50 μM spermine NONOate for 3, 5, or 15 min at 37°C with shaking. Cultures were then combined 5:1 with a mixture of ice-cold phenol (5%)–ethanol (95%), incubated on ice for 10 min, and pelleted by centrifugation. RNA isolation was performed using the High Pure RNA isolation kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions for bacterial samples and included on-column DNase treatment. cDNA was prepared from 1 μg total RNA using 0.45 μM N6 random hexamer primers (Life Technologies, Carlsbad, CA) and 100 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI). The primers and probes used for quantitative PCR (qPCR) are listed in Table S3 in the supplemental material. Reaction mixtures were prepared using TaqMan Gene Expression Master Mix (Life Technologies) and were incubated at 50°C for 2 min and then 95°C for 10 min, prior to 40 cycles of 95°C for 15 s and 57°C for 1 min. The expression of cydA was normalized to the expression of the rpoD housekeeping gene.

O$_2$ measurements. Salmonella grown overnight in LB broth were diluted 1:100 in EG medium. Bacteria were grown at 37°C in a shaker incubator until they reached an OD$_{600}$ of 0.5. The cultures were diluted to an OD$_{600}$ of 0.2 in EG medium and equilibrated in a shaker incubator at 37°C for 3 min before they were transferred into an air-sealed, multiport measurement chamber equipped with an Iso-Oxy-2 O$_2$ probe. The evolution of O$_2$ in the cultures was recorded with an Apollo 4000 free radical analyzer (World Precision Instruments, Inc., Sarasota, FL). 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 data are expressed as micromolar concentrations of O$_2$.

Bacterial virulence in mice. Eight- to 10-week-old NRAMP1$^{-}$ C3H/HeN mice were bred at the animal facility of the University of Colorado School of Medicine according to Institutional Animal Care and Use Committee guidelines. C3H/HeN mice were treated with 20 mg/mouse 1 day before intragastric infection with 10$^8$ CFU of Salmonella prepared in phosphate-buffered saline (PBS) from overnight cultures grown in LB broth. Salmonella shedding was examined over time in fecal pellets. The abilities of wild-type and mutant Salmonella to colonize ileum, cecum, and colon were measured 5 days after infection. C3H/HeN mice were independently inoculated i.p. with ~2,000 CFU of a bacterial mixture containing equal numbers of wild-type and mutant Salmonella (51). After 3 days of infection, the bacterial burden in livers was quantified on LB agar plates containing the appropriate antibiotics. The competitive index was calculated according to the formula (strain 1/strain 2)output/(strain 1/strain 2)input. In addition, C3H/HeN mice were inoculated intraperitoneally with 1 × 10$^3$ to 3 × 10$^5$ CFU/mouse of wild-type or mutant Salmonella. Where indicated, the drinking water of selected groups of C3H/HeN mice was supplemented with 500 μg/ml of the INOS-specific inhibitor aminoguanidine. The survival of Salmonella-infected mice was recorded over time.

Histopathology. Ceca were scored for submucosal edema, neutrophil infiltration into the lamina propria, and goblet cell number per 400 × field as described previously (52). Ten fields per animal per tissue were examined. Images demonstrating representative fields were captured on an Olympus BX51 microscope equipped with a 4-megapixel Macrofire digital camera (Optronics, Goleta, CA) using the PictureFrame application 2.3 (Optronics). Composite images were assembled with the use of Adobe Photoshop. All images in the composite were handled identically.

Statistical analysis. One-way analysis of variance, followed by a Bonferroni posttest, was used to establish statistical significance. Differences in mouse survival of Salmonella infection were determined by a log rank Mantel-Cox test. A P value of <0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at mbio.asm.orglookup/suppl/doi:10.1128/mBio.02052-16/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.04 MB.
Table S3, DOCX file, 0.04 MB.

ACKNOWLEDGMENTS

We thank members of the Vázquez-Torres lab for discussions.

This work, including the efforts of Andrés Vázquez-Torres, was funded by the Veterans Administration (I01 BX002073), the National Institutes of Health (R01 AI54959), and the Burroughs Wellcome Fund.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

FUNDING INFORMATION

This work, including the efforts of Andrés Vázquez-Torres, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (R01 AI54959). This work, including the efforts of Andrés Vázquez-Torres, was funded by VA | Veterans Affairs Eastern Colorado Healthcare System (VAECHS) (I01 BX002073).

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