The NorR Regulon Is Critical for *Vibrio cholerae* Resistance to Nitric Oxide and Sustained Colonization of the Intestines

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ABSTRACT ***Vibrio cholerae*, the cause of an often fatal infectious diarrhea, remains a large global public health threat. Little is known about the challenges *V. cholerae* encounters during colonization of the intestines, which genes are important for overcoming these challenges, and how these genes are regulated. In this study, we examined the *V. cholerae* response to nitric oxide (NO), an antibacterial molecule derived during infection from various sources, including host inducible NO synthase (iNOS). We demonstrate that the regulatory protein NorR regulates the expression of NO detoxification genes *hmpA* and *nnrS*, and that all three are critical for resisting low levels of NO stress under microaerobic conditions *in vitro*. We also show that *prxA*, a gene previously thought to be important for NO detoxification, plays no role in NO resistance under microaerobic conditions and is upregulated by H_2O_2, not NO. Furthermore, in an adult mouse model of prolonged colonization, *hmpA* and *norR* were important for the resistance of both iNOS- and non-iNOS-derived stresses. Our data demonstrate that NO detoxification systems play a critical role in the survival of *V. cholerae* under microaerobic conditions resembling those of an infectious setting and during colonization of the intestines over time periods similar to that of an actual *V. cholerae* infection.**

IMPORTANCE **Little is known about what environmental stresses *Vibrio cholerae*, the etiologic agent of cholera, encounters during infection, and even less is known about how *V. cholerae* senses and counters these stresses. Most prior studies of *V. cholerae* infection relied on the 24-h infant mouse model, which does not allow the analysis of survival over time periods comparable to that of an actual *V. cholerae* infection. In this study, we used a sustained mouse colonization model to identify nitric oxide resistance as a function critical for the survival of *V. cholerae* in the intestines and further identified the genes responsible for sensing and detoxifying this stress.**

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Vibrio cholerae causes the disease cholera and represents a large global health problem in impoverished countries. Cholera continues to cause epidemics and has the ability to spread to new locations, having caused over 4,500 deaths in Haiti since the earthquake in 2010 (Centers for Disease Control and Prevention, Atlanta, GA). Cholera is characterized by profuse dehydrating diarrhea and can be treated with vigorous oral rehydration and supplementary antibiotics. Despite these interventions, cholera remains a source of considerable worldwide morbidity and mortality. Cholera toxin, which directly causes secretory diarrhea, and its transcriptional regulation are well understood (1, 2). However, the bacteria that cause cholera, or any intestinal infection, encounter chemical and physical barriers during the establishment and maintenance of colonization. The host-derived stresses that *V. cholerae* encounters while infecting a host are not well characterized, and even less well understood is how *V. cholerae* senses these stresses.

One of the toxic chemical species elaborated by the host during bacterial infection is nitric oxide (NO). NO is a toxic radical that disrupts the function of proteins containing cysteine residues, enzymes catalyzing iron-dependent reactions, and members of the electron transport chain (3). Furthermore, NO reacts with other small molecules produced by the immune system to form other toxic reactive nitrogen species (RNS) such as nitroxyl and peroxynitrite (4, 5). In the host, NO is generated by acidified nitrite in the stomach and by enzymes of the NO synthase (NOS) family, which derive NO from arginine (6). There are three isoforms of NOS, and the form associated with the immune system is inducible NOS (iNOS), which is capable of generating large quantities of NO in an inflammatory setting. Epithelial cells are known to express iNOS, as are immune cells such as macrophages and dendritic cells (6–9). Clinical studies have demonstrated that patients with cholera have increased NO metabolite levels in their serum and urine, as well as an increase in the expression of iNOS in their small intestines during *V. cholerae* infection, suggesting that *V. cholerae* encounters NO during infection of humans (10–12). To cope with NO produced during infection, many pathogenic bacteria have evolved mechanisms to convert NO into other, less

toxic, nitrogen oxides (3). The only enzyme predicted to have this activity in *V. cholerae* is HmpA (VCA0183), a member of the flavohemoglobin family of enzymes that is well characterized in other bacteria such as *Escherichia coli* (13). Under low-oxygen conditions such as those one might find in the gut, HmpA catalyzes the conversion of NO to N_2O or NO_3^-, both of which are less toxic to the bacterium (14). Within HmpA is an iron-heme moiety that directly catalyzes the reaction, as well as a flavin group and an NADPH oxidase domain that mediate the transfer of electrons to and from NO (14). HmpA homologs are important for detoxification of NO during infections with other bacterial pathogens such as *E. coli*, *Yersinia pestis*, *Staphylococcus aureus*, and *Salmonella enterica*, as well as *V. fischeri* colonization of its squid host (15–19). In *V. cholerae*, *hmpA* emerged as a gene expressed in both infant mice and rabbits in two different *in vivo* screens (20, 21). A recent study demonstrated that in the infant mouse model of *V. cholerae* infection, HmpA was important for resistance of NO generated in the stomach from acidified nitrite (22). However, since the suckling mouse model of cholera is limited to 24-h studies, it is unknown whether NO might be generated later during infection and present a second NO barrier to *V. cholerae* infection beyond the stomach. Furthermore, it remains unknown how the expression of *hmpA* is regulated. Here we demonstrate that *hmpA* expression is controlled by the NO sensor NorR (VCA0182), a predicted σ^{54}-dependent transcriptional regulator (23). A previous bioinformatic study predicted a NorR-binding site upstream of *hmpA* and also upstream of one other gene, *nnrS* (*vc2330*). The function of NnrS, a membrane protein, was previously unknown, but it may have a role in the metabolism of nitrogen oxides (24). We also demonstrate that the expression of *nnrS* is controlled by NorR and that *nnrS* is important for NO resistance *in vitro* when *hmpA* is deleted. In addition, we show that *hmpA* and *norR* are critical for long-term colonization of the adult mouse intestine.

RESULTS

NorR is required for NO-inducible expression of *hmpA* and *nnrS* and represses its own expression. The regulatory networks that control NO detoxification vary widely between bacterial species (13). *V. cholerae* has a limited repertoire of NO-related genes that includes *hmpA*, which encodes a flavohemoglobin, *nnrS*, a widely conserved gene of previously unknown function, and *norR*, which encodes a NO-responsive DNA-binding regulatory protein (23, 25, 26). A computational study predicted that NorR would control the expression of *hmpA* and *nnrS* (13). This is different from enteric species and other *Vibrio* species, in which NorR controls or is predicted to control the expression of the NO reductase gene *norVW*. There is no *norVW* homolog present in the *V. cholerae* chromosome. To determine the effect of NO on the expression of *hmpA*, *nnrS*, and *norR*, we constructed transcriptional reporter plasmids containing the promoters of these genes fused to the *lacZ* gene. Strains grown in minimal medium had low background transcription of *hmpA* and *nnrS*, but the addition of a 50 μM concentration of the NO donor DEA-NONOate [diethylammonium (Z)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate] resulted in a dramatic upregulation of both of these promoters (Fig. 1A and B). DEA-NONOate releases NO over a short period of time. Under aerobic conditions, there was no upregulation of either the *hmpA* or the *nnrS* promoter (data not shown), likely because the NO diffused out of the system or reacted with O_2. Under the closed-tube microaerobic conditions of this experiment, 50 μM DEA-NONOate did not inhibit the growth of any of the strains. In a *norR* deletion background, however, virtually no upregulation of the *hmpA* or t*nnrS* promoter was observed (Fig. 1A and B), suggesting that NorR is absolutely required for the activation of both of these promoters. These experiments were performed with minimal medium because the background activity of the *hmpA* and *nnrS* promoters was low. However, performance of the experiments with LB medium under microaerobic conditions still resulted in >10-fold upregulation of both promoters (see Fig. S1 in the supplemental material). Taken together, these data suggest that NorR controls the NO-inducible upregulation of both *hmpA* and *nnrS*. We further investigated how *norR* is regulated by comparing *norR-lacZ* expression in the wild-type and *norR* mutant strains with or without NO. The activity of the *norR* promoter was not altered by the addition of NO (Fig. 1C). Interestingly, *norR* promoter activity was significantly increased in the *norR* background (Fig. 1C). These data suggest that NorR represses its own expression independently of NO.

***norR*, *hmpA*, and *nnrS* are critical for NO resistance *in vitro*.** A recent study by Davies et al. (22) implicated *hmpA* as an important gene for resistance to NO under aerobic conditions in the presence of high (millimolar) concentrations of NO donors. To examine whether the NorR regulon, including *hmpA*, is important for resistance to NO in a microaerobic environment more similar to what bacteria are likely to encounter during infection of the small intestine, we performed growth curve assays with sealed 96-well plates. We added 10 μM (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate), which continuously releases NO with a half-life of 20 h, to the cultures and measured the optical density at 600 nm (OD_{600}) every 10 min at 37°C in a plate reader. The *hmpA*, *norR*, and *nnrS* single mutants and *hmpA nnrS* double mutant were examined. None of these mutations conferred a growth defect in the absence of NO (Fig. 2A). Similar to the results of Davies et al., who grew bacteria aerobically, deletion of *hmpA* resulted in a growth defect in the presence of NO (Fig. 2B). Deletion of *norR* resulted in a more severe defect, and interestingly, deletion of both *hmpA* and *nnrS* resulted in the most severe phenotype. The *nnrS* single mutation, however, did not result in an NO-sensitive defect. These data suggest that the NorR regulon, containing *hmpA* and *nnrS*, is critical for resistance to NO. Similar but less dramatic results were obtained using 10 μM spermine-NONOate, which releases NO with a half-life of approximately 39 min (data not shown). Almost no defect could be detected with micromolar concentrations of DEA-NONOate, which releases NO with a half-life of approximately 2 min. This suggests that during continuous exposure to NO, such as that which might occur during infection, physiologically relevant concentrations of NO (22, 27) are sufficient to affect the growth of *V. cholerae*. The importance of *nnrS* is revealed only in an *hmpA* mutant background, suggesting that it may play a redundant role in NO detoxification. Alternatively, NnrS may catalyze the detoxification of a related RNS. We tested whether *nnrS* mutants are more sensitive to peroxynitrite ($ONOO^-$), Angeli's salt (a donor of nitroxyl anion, NO^-), and nitrite (NO_2^-) but found no difference from the wild type (data not shown), suggesting that the role of *nnrS* in resistance to RNS is important but subtle. The function of *nnrS* is a subject of ongoing research.

***prxA* expression is induced by H_2O_2 and not by NO and is not important for NO resistance under microaerobic conditions.**

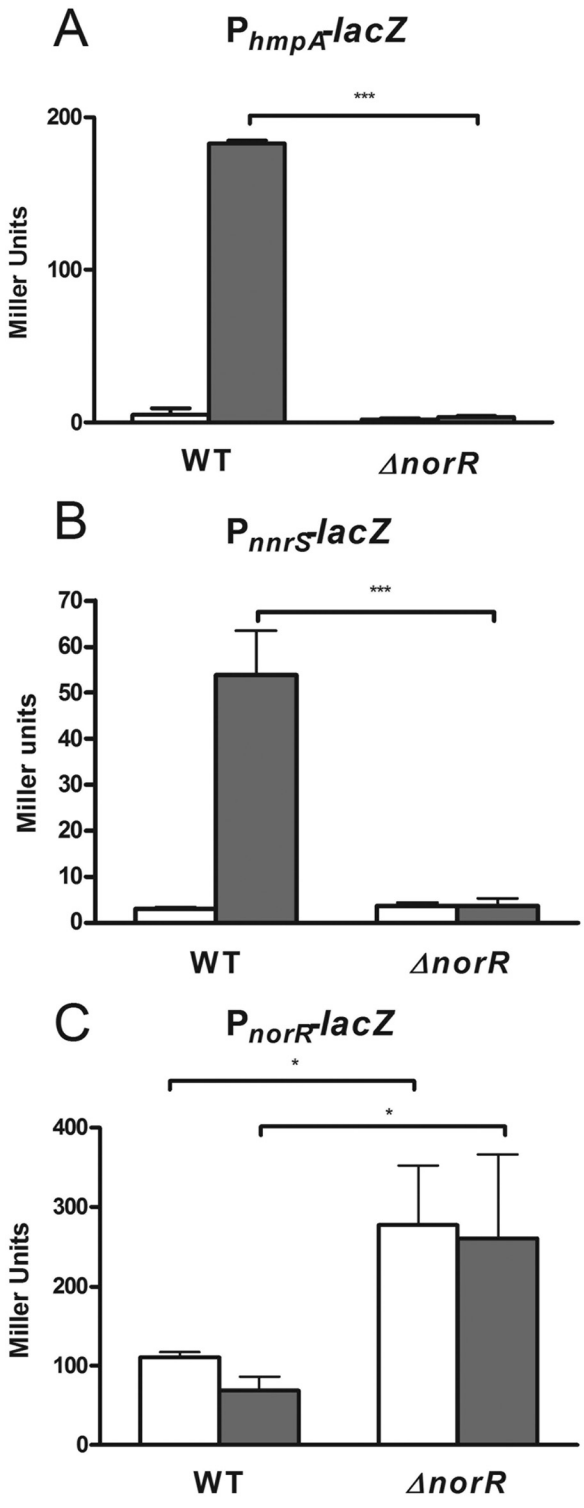

FIG 1 Effects of NO and NorR on the expression of NO detoxification genes. Strains containing a promoter-*lacZ* reporter were grown in minimal medium under microaerobic conditions. *hmpA* (A), *nnrS* (B), or *norR* (C) promoter activity was measured after the addition of a 50 μM concentration of the control compound diethylamine (white bars) or the NO donor DEA-NONOate (gray bars) and is reported in Miller units (34). Experiments were performed with wild-type (WT) strain C6706 or a strain containing a clean deletion of the entire *norR* open reading frame. Error bars represent standard deviations. *, $P < 0.05$; ***, $P < 0.001$ (for experiments performed in triplicate).

Davies et al. recently found that deletion of *prxA*, a gene that encodes a putative peroxiredoxin, resulted in sensitivity to NO (22). We examined the NO sensitivity of a strain in which *prxA* and adjacent gene *vc2638* from the same operon were deleted under microaerobic conditions in minimal medium. In their study, a high concentration of DEA-NONOate (1 mM) was used. This results in the full release of 2 mM NO over a period of approximately 10 min under aerobic conditions. However, under microaerobic conditions in minimal medium containing 10 μM DETA-NONOate, conditions which significantly inhibited the growth of strains lacking *hmpA* or *norR* (Fig. 2B), there was no detectable growth defect in a *prxA* mutant (Fig. 3A and B). To study whether *prxA* expression could be induced by NO, we constructed a reporter consisting of the *prxA* promoter fused to *lacZ*. The addition of 50 μM DEA-NONOate, which caused dramatic upregulation of *hmpA* and *nnrS* (Fig. 1), did not result in activation of the *prxA* promoter (Fig. 3C). However, addition of 100 μM H_2O_2 did result in upregulation of the *prxA* promoter in both wild-type bacteria and a strain lacking *norR*. The *prxA* gene is located divergent from the *oxyR* gene, which has been shown in other bacteria to mediate responses to oxidative stress (28). We speculate that the results of Davies et al. resulted not directly from NO but from other species generated under aerobic conditions during a burst of millimolar concentrations of NO from a short-lived NO donor.

***norR* and *hmpA* are critical for sustained colonization of the adult mouse intestine.** Previous experiments (22) tested the effect of *hmpA* deletion in an infant mouse model and demonstrated a moderate defect (competitive index = 0.13) that was partially dependent on the presence of acidified nitrite in the mouse stomach. We repeated these experiments and found a similar competitive index of 0.40 $\pm$ 0.01, confirming these results. However, the infant mouse model only allows for a 24-h experiment and is not suitable for study of the extended survival of a bacterial strain in the intestines. The incubation time of *V. cholerae* infection is typically 2 to 3 days, and symptoms can last a long time after this (Centers for Disease Control and Prevention), suggesting that *V. cholerae* may be exposed to challenges such as RNS for prolonged periods of time during infection. Furthermore, the majority of people inoculated with *V. cholerae* do not develop symptoms but continue to shed vibrios in their stool for days, a time when RNS may still be generated in the host (29, 30). To determine the importance of *hmpA*, as well as *norR* and *nnrS*, in the setting of long-term colonization, we employed an adult mouse model (29) in which we could monitor colonization levels by collecting fecal pellets.

We used a competition assay in our mouse studies. After treatment with streptomycin and neutralization of stomach acid, mice were coinoculated with a wild-type strain and a mutant strain. Either the mutant or the wild-type strain lacked the *lacZ* gene, allowing differentiation on plates containing 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal). At the end of each experiment, the small intestine of each mouse was homogenized and competitive indices were calculated from the homogenates. In each experiment performed, the competitive indices from intestinal homogenates were always virtually identical to those from the fecal samples (data not shown).

Interestingly, deletion of *hmpA* resulted in a colonization defect at 3 days postinoculation that worsened to nearly undetectable levels by 7 days, suggesting that HmpA is important for sus-

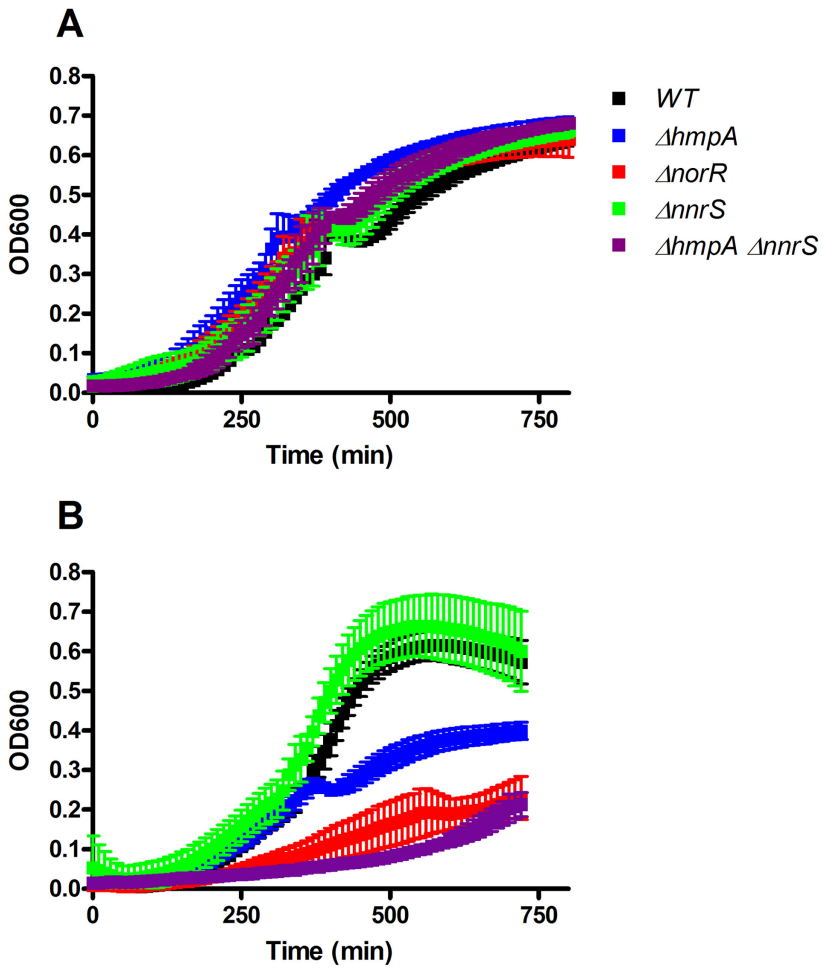

FIG 2 Role of the NorR regulon in NO resistance *in vitro*. To test the effect of NO on the growth of mutant strains, bacteria were inoculated in triplicate into a sealed 96-well plate in minimal medium at 37°C in the absence (A) or presence (B) of 10 μM DETA-NONOate. Growth was measured by determining the OD_{600} every 10 min. The strains used were as follows: black, wild type (WT); blue, Δ*hmpA* mutant; green, Δ*nnrS* mutant; red, Δ*norR* mutant; purple, Δ*hmpA* Δ*nnrS* double mutant. Error bars indicate standard deviations.

tained colonization of the intestines (Fig. 4A). A competitive index was considered below the limit of detection (denoted by the dotted line in Fig. 4A) if no *hmpA* mutant colonies were detected. The *norR* mutant displayed a more moderate but significant defect as well. As in the *in vitro* studies, the *nnrS* single deletion mutant displayed no colonization defect and perhaps even a slight advantage over wild-type bacteria in wild-type mice (Fig. 5A). We hypothesized that, similar to our *in vitro* data, this phenotype might be reversed in an *hmpA* mutant background and that the *hmpA nnrS* double mutant might have an even more severe defect than the *hmpA* single mutant. However, competition of the *hmpA nnrS* double mutant against wild-type bacteria displayed a profound defect similar to that of the *hmpA* single mutant (Fig. 5A). To determine if a smaller *nnrS*-mediated defect might be masked by the larger defect due to *hmpA* mutation, we competed the *hmpA nnrS* double mutant against the *hmpA* single mutant. We were surprised to find, however, that the double mutant did not fare significantly worse or better than the single *hmpA* mutant (Fig. 5B).

To assess the contribution of iNOS to the colonization defects observed, we repeated the experiments with $iNOS^{-/-}$ mice. By day 7 postinoculation, the severe colonization defect of the *hmpA* mutant was attenuated more than 10-fold in $iNOS^{-/-}$ mice (Fig. 4A), suggesting that iNOS presents a long-term challenge for *V. cholerae* that is dealt with by *hmpA*. The *norR* mutant displayed a similar effect, in which the defect observed in the wild type was completely attenuated in $iNOS^{-/-}$ mice (Fig. 4B). This again suggests that over the time period during which a *V. cholerae* infection occurs, iNOS-generated RNS present a significant challenge for *V. cholerae* to overcome. Unexpectedly, however, the *nnrS* single mutant displayed a small but significant defect in $iNOS^{-/-}$ mice (Fig. 4C). Furthermore, the competition defect of the *hmpA nnrS* double mutant was not mitigated in $iNOS^{-/-}$ mice as it was in the *hmpA* single mutant at 7 days postinoculation (Fig. 5A). With wild-type mice, the competitive index at day 7 of the *hmpA nnrS* double mutant was significantly higher than that of the *nnrS* single mutant ($P = 0.0397$). These data suggest that in our long-term colonization model, *nnrS* may actually be detrimental to detoxification of iNOS-derived stresses. The exact mechanism behind this requires further investigation.

DISCUSSION

Despite a wealth of research on the virulence factors that allow *V. cholerae* to cause disease, relatively little is known about the challenges that *V. cholerae* encounters during infection of the intestines and how it senses and overcomes them. In this study, we have identified how *V. cholerae* senses and responds to NO, a common challenge for intestinal pathogens. We have further demonstrated that one of the NO detoxification genes, *hmpA*, and its transcriptional activator NorR are critical for sustained colonization of the intestines of mice.

Previous bioinformatic analysis led to the identification of a remarkably limited repertoire of NO-related genes found in the *V. cholerae* genome, even compared to highly related *Vibrio* species (13). Using reporter assays, we demonstrated that the expression of two of these genes, *nnrS* and the flavohemoglobin-encoding gene *hmpA*, is highly inducible by the addition of NO to microaerobically growing cells. This upregulation was dependent on the σ^{54}-dependent transcriptional regulator NorR (23, 25, 26). Growth curve analysis demonstrated that these genes are essential for NO resistance *in vitro*. Intriguingly, a strain of *V. cholerae* lacking both *hmpA* and *nnrS* was the most attenuated for growth

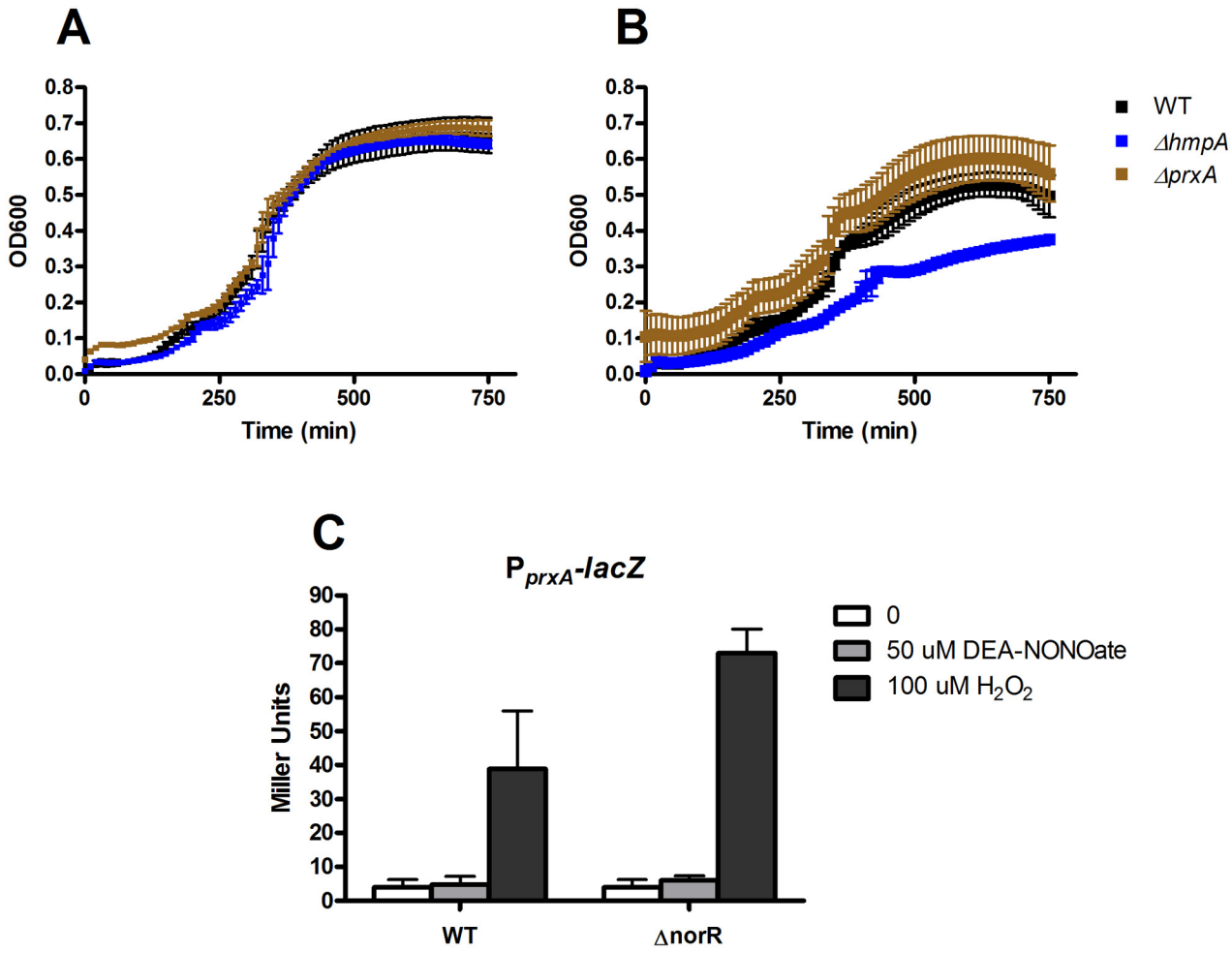

FIG 3 Importance of *prxA* in response to NO and H_2O_2 under microaerobic conditions. The growth of a strain of *V. cholerae* lacking *prxA* and its adjacent gene *vc2638* was compared to that of the wild type (WT) and a Δ*hmpA* mutant in the absence (A) or presence (B) of 10 μM DETA-NONOate under microaerobic conditions in minimal medium. In the experiment shown in panel C, wild-type and Δ*norR* mutant strains of *V. cholerae* containing a reporter plasmid that contains *lacZ* fused to the *prxA* promoter were grown in minimal medium. After the addition of 50 μM DEA-NONOate (gray bars), 100 μM H_2O_2 (black bars), or nothing (white bars), *prxA* promoter activity was measured by Miller assay.

in the presence of NO; concomitantly, deletion of *norR* resulted in a nearly equivalent growth defect in the presence of NO. These data demonstrated that HmpA is the principal detoxifier of NO but that NnrS may play an auxiliary role. The only study of NnrS published to date identified it as a heme- and copper-containing membrane protein in *Rhodobacter sphaeroides* (24). However, *nnrS* homologs are found in the genomes of human pathogens such as *Pseudomonas*, *Brucella*, *Burkholderia*, *Bordetella*, and *Neisseria*, suggesting that it may play NO detoxification roles in a variety of infectious settings. The exact function of NnrS is an area of current investigation in our laboratory.

The role of NO detoxification genes in *V. cholerae* pathogenesis has been examined in an infant mouse model in which bacteria are allowed to colonize the intestines for 24 h (22). After this brief period, there was a moderate colonization defect in the *hmpA* mutant attributed to the low pH of the stomach. We were interested in whether NO resistance could be important in colonization of the intestine over a time period resembling that of a human infection. Interestingly, we found that the importance of HmpA was much greater than previously thought; there were virtually no *hmpA* mutants recovered from fecal samples or small intestinal homogenates after 7 days. This defect was partially due to iNOS-derived stress, as the colonization defect was partially mitigated in *iNOS*$^{-/-}$ mice at 7 days. The remaining defect is not likely to be due to stomach acidity because the mice were administered bicarbonate prior to inoculation. Mice and humans possess two other NOS isoforms, neuronal NOS and endothelial NOS (31), which may also account for some of the defect that persists in *iNOS*$^{-/-}$ mice.

We were surprised to discover the effects of the *nnrS* mutation on colonization. Although the *hmpA nnrS* double mutant was severely inhibited *in vitro*, this mutant fared no better in *iNOS*$^{-/-}$ mice than in wild-type mice. Furthermore, the *nnrS* single mutant slightly outcompeted wild-type *V. cholerae* in wild-type mice but was attenuated in *iNOS*$^{-/-}$ mice. It is difficult to interpret these data, given the unknown function of NnrS, but we hypothesize that the complex metabolism of RNS results in the buildup of detrimental chemical products in some contexts. Furthermore, an acknowledged disadvantage of competition studies is that a defect in the *nnrS* mutant may be complemented *in trans* by the wild-

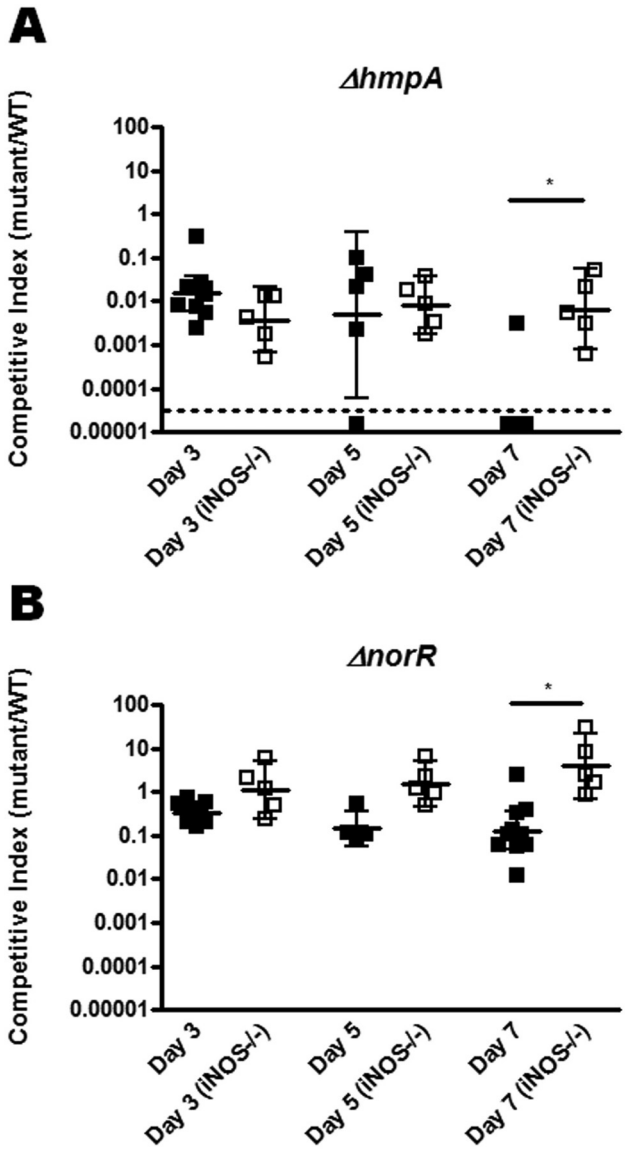

FIG 4 Importance of *hmpA* and *norR* for sustained colonization of the adult mouse. Six-week-old C57BL/6 (black squares) or C57BL/6 *iNOS*$^{-/-}$ (white squares) mice were coinfected with wild-type (WT) *V. cholerae* and either a Δ*hmpA* (A) or a Δ*norR* (B) mutant strain. Fecal pellets were collected on days 3, 5, and 7 postinoculation and plated on differential medium. The competitive index was calculated as the ratio of mutant to wild-type colonies normalized to the input ratio. Error bars indicate 95% confidence intervals. Data points below the dotted line indicate that no mutant colonies were detected. *, $P < 0.05$.

type coinoculated strain. Future studies may address this possibility. Given the *in vitro* importance of NnrS, however, we speculate that there are infectious settings in which NnrS is critical to the survival of *V. cholerae*. In addition, we were surprised to find that the *hmpA nnrS* double mutant had a far more severe colonization defect than the *norR* mutant in wild-type mice (Fig. 4), since NorR is absolutely required for the upregulation of *hmpA* and *nnrS* in response to NO (Fig. 1). One possible explanation for the discrepancy between the colonization defects is that baseline transcription of *hmpA* and *nnrS* in the *norR* deletion mutant, however low, is sufficient to detoxify a significant proportion of the NO stress found *in vivo*. Alternatively, signals other than NO, and thus regulators other than NorR, might cause the upregulation of *hmpA* and *nnrS in vivo*. This could allow better colonization efficiency than when *hmpA* and *nnrS* are deleted entirely. Our laboratory is currently working to find these alternative signals and regulators of *hmpA* and *nnrS*.

Davies et al. (22) recently demonstrated a growth defect in a strain of *V. cholerae* lacking the *prxA* gene, which encodes a putative peroxireductase. They used a large, short-lived bolus of NO under aerobic conditions and found that the strain exhibited a delayed log phase. In the presence of a low level of continuously released NO, a strain lacking *prxA* exhibited no defect compared to the wild type. Furthermore, the expression of *prxA* was not increased in the presence of NO but was dramatically increased in the presence of H_2O_2. We suspect that PrxA is important for resistance to reactive oxygen species that may have been generated under aerobic conditions in the presence of large amounts of NO, but we conclude that it plays no role directly related to NO detoxification.

In summary, we have demonstrated the importance of the NorR regulon in NO sensing and resistance to NO toxicity. Furthermore, we identified the importance of NO detoxification genes during extended colonization of the mouse intestine. Our work highlights the role of resistance to chemical stresses in the successful survival of *V. cholerae* during infection and ultimately its ability to cause disease.

MATERIALS AND METHODS

Bacterial strains and plasmids. The parent strain used in this study was *V. cholerae* O1 El Tor C6706. Sucrose counterselection (32) was used to generate all clean deletions. Promoter-*lacZ* transcriptional fusions were generated by cloning the approximately 500 bp proximal to the ATG start codon upstream of a promoterless *lacZ* gene in a plasmid (33). Strains were propagated in LB containing appropriate antibiotics at 37°C, unless otherwise noted.

Gene expression studies. For *in vitro* gene expression studies under microaerobic conditions, saturated overnight cultures in LB were inoculated 1:100 into minimal medium containing 79 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.65 mM $MgSO_4$, 0.07 mM $CaCl_2$, 0.018 mM $FeSO_4$, 0.013 mM $MnSO_4$, and 0.2% (wt/vol) glucose in filled, sealed glass vials. After 4 h of growth, 50 μM DEA-NONOate (from a 50 mM stock in dimethyl sulfoxide; Cayman Chemical) was added to the cultures. Diethylamine was used as a negative control. Two hours later, the OD_{600} of the cultures was measured and a Miller assay (34) was used to measure LacZ production. For experiments in LB, bacteria were inoculated 1:1,000, with 2.5 h of growth prior to NO addition and 1.5 h of growth thereafter.

Growth curves. To measure *in vitro* growth, strains from saturated LB cultures were inoculated 1:100 into 0.25 ml of minimal medium (described above) in a 96-well plate. Plates were sealed with an optically clear film and incubated at 37°C, and the OD_{600} was measured every 10 min by an automated plate reader (Bio-Tek Synergy HT). To measure the effect of NO on growth, 10 μM DETA-NONOate (Cayman Chemical) was included.

***In vivo* mouse colonization studies.** Mouse colonization competition studies were performed using a protocol modified from reference 29. Six week-old C57BL/6 or C57BL/6 *iNOS*$^{-/-}$ (strain B6.129P2-*Nos2*^{tm1Lau}/J) mice were obtained from The Jackson Laboratory. Two days before inoculation, 0.5% (wt/vol) streptomycin and 0.5% (wt/vol) glucose were added to the drinking water; this treatment was maintained throughout this experiment, with regular replacement every 2 to 3 days. One day before inoculation, food was removed from the cages. On the day of inoculation, stomach acid was neutralized with 0.05 ml 10% (wt/vol)

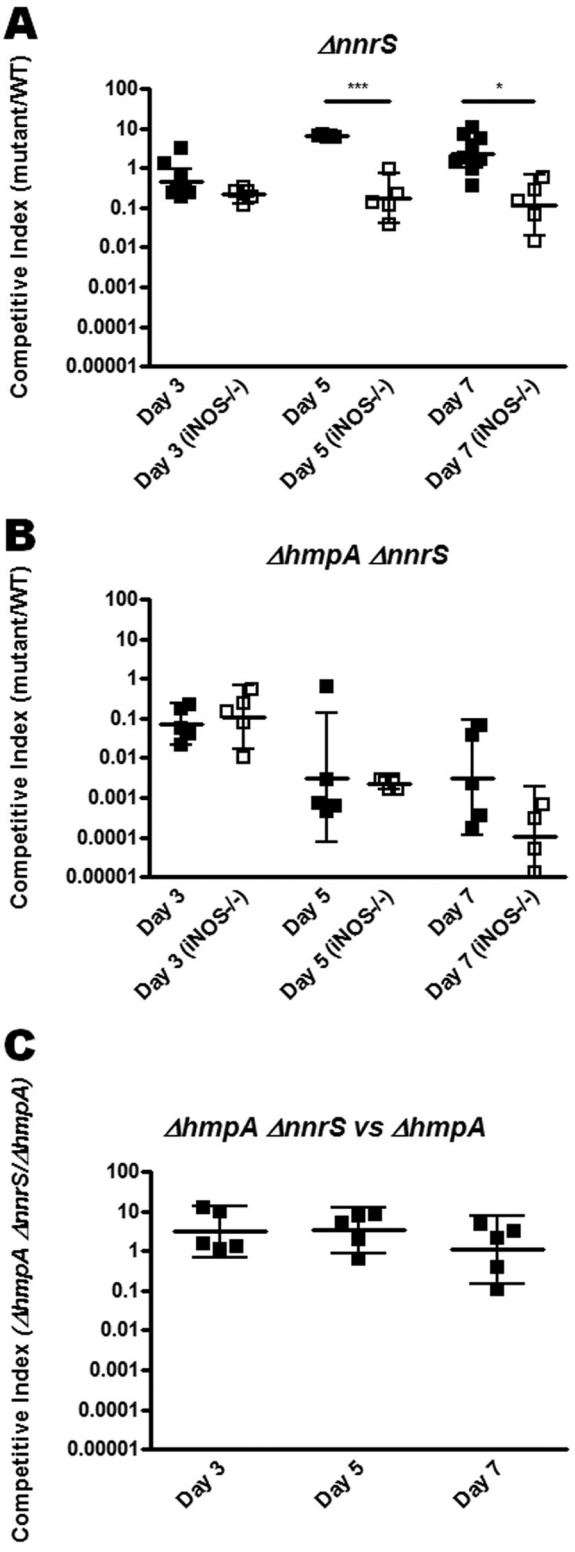

FIG 5 Effect of deletion of *nnrS* on colonization of wild-type and *iNOS*$^{-/-}$ mutant mice. As described in the legend to Fig. 4, mice were inoculated with a mixture of wild-type (WT) *V. cholerae* and a Δ*nnrS* (A) or a Δ*hmpA* Δ*nnrS* (B) mutant. In the experiment shown in panel C, mice were inoculated with a mixture of the *V. cholerae* Δ*hmpA* and Δ*hmpA* Δ*nnrS* mutants and the competitive index is reported as the ratio of Δ*hmpA* Δ*nnrS* to Δ*hmpA* mutant colonies normalized to the input. Error bars indicate 95% confidence intervals. *, $P < 0.05$; ***, $P < 0.001$.

$NaHCO_3$ by oral gavage. Twenty minutes later, 0.4 ml of a saturated culture of each of the two strains was mixed with 0.2 ml 10% (wt/vol) $NaHCO_3$, and 0.1 ml of this mixture was administered to each mouse by oral gavage. The size of the inoculum was determined by serial dilution and plating on LB plates containing 0.1 mg/ml streptomycin and 0.04 mg/ml X-Gal. Food was replaced 2 h after inoculation. On days 3, 5, and 7 postinoculation, two or three fecal pellets were collected from each mouse, resuspended in LB, serially diluted, and then plated on plates containing streptomycin and X-Gal. The competitive index was calculated as the ratio of mutant to wild-type colonies normalized to the ratio contained in the inoculum. At the end of the experiment, mice were sacrificed and competitive indices were calculated from homogenates of their small intestines.

Statistical analyses. For all experiments, a two-tailed Student *t* test was performed to determine statistical significance. Data points below the limit of detection were considered at the limit of detection for statistical analyses. A difference in means was considered statistically significant if the *P* value was <0.05.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00013-12/-/DCSupplemental.

Figure S1, DOCX file, 0.1 MB.

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