



OxyR2 Modulates OxyR1 Activity and *Vibrio cholerae* Oxidative Stress Response

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ABSTRACT Bacteria have developed capacities to deal with different stresses and adapt to different environmental niches. The human pathogen *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera, utilizes the transcriptional regulator OxyR to activate genes related to oxidative stress resistance, including peroxiredoxin PrxA, in response to hydrogen peroxide. In this study, we identified another OxyR homolog in *V. cholerae*, which we named OxyR2, and we renamed the previous OxyR OxyR1. We found that OxyR2 is required to activate its divergently transcribed gene *ahpC*, encoding an alkylhydroperoxide reductase, independently of H₂O₂. A conserved cysteine residue in OxyR2 is critical for this function. Mutation of either *oxyR2* or *ahpC* rendered *V. cholerae* more resistant to H₂O₂. RNA sequencing analyses indicated that OxyR1-activated oxidative stress-resistant genes were highly expressed in *oxyR2* mutants even in the absence of H₂O₂. Further genetic analyses suggest that OxyR2-activated AhpC modulates OxyR1 activity by maintaining low intracellular concentrations of H₂O₂. Furthermore, we showed that Δ *oxyR2* and Δ *ahpC* mutants were less fit when anaerobically grown bacteria were exposed to low levels of H₂O₂ or incubated in seawater. These results suggest that OxyR2 and AhpC play important roles in the *V. cholerae* oxidative stress response.

KEYWORDS *V. cholerae*, ROS resistance, AhpC, OxyR, reactive oxygen species

Vibrio cholerae is an aquatic bacterium that causes severe diarrheal disease in humans and affects thousands of people every year in regions of endemicity. Cholera infection is generally acquired through the ingestion of contaminated food or water. Once ingested, *V. cholerae* is able to survive the stomach acid shock and subsequently colonizes the mucosal layer of the small intestine, where it produces a cascade of virulence factors, including cholera toxin (CT), the agent primarily responsible for producing the severe symptoms of cholera infection (1, 2). The diarrhea facilitates *V. cholerae*'s escape from the human host back into its natural aquatic habitat (3, 4). As *V. cholerae* transitions from the aquatic environment to the human host and back into the aquatic environment, it must sense a variety of different signals and respond accordingly by modulating gene expression to effectively adapt to its new niche. Oxidative stress is one such environmental signal that *V. cholerae* can face. Oxidative stress can cause mutations and damage proteins, DNA, cell membranes, etc. (5). Organisms must therefore have mechanisms to remove reactive oxygen species (ROS) and repair any damage caused. Any environment where oxygen is present has the potential to produce ROS such as the superoxide anion, hydroxyl radical, and hydrogen peroxide (H₂O₂) (5). For example, an aerobically grown bacterial culture can contain ROS; there are diverse reactive species, including ROS, in marine environments

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(6); and *V. cholerae* can experience ROS during infection. Stool samples collected from patients infected with *V. cholerae* contained higher levels of ROS and decreased levels of host antioxidant enzymes compared with uninfected persons (7, 8), and a recent proteomics study of duodenal biopsy specimens of cholera patients indicated activation of oxidative stress-related enzymes (9), suggesting an increase in ROS levels during infection. Thus, oxidative stress is a challenge that *V. cholerae* must respond to both in aquatic environments and inside the human host.

Most bacteria have multiple scavengers that maintain levels of hydrogen peroxide (H_2O_2) at nanomolar concentrations (10). A widely conserved LysR-family transcriptional regulator, OxyR, has been shown to be involved in hydrogen peroxide resistance in many bacteria (11). The *Escherichia coli* OxyR is activated by hydrogen peroxide by either the modification of a key cysteine residue, C199, or formation of a disulfide bond between C199 and C206 (12–14). Activated OxyR then binds to the promoter of its target genes and activates their expression. Members of the *E. coli* OxyR regulon include genes encoding catalases KatG and KatE and alkylhydroperoxide reductase AhpC. AhpC belongs to a family of thiol peroxidases (peroxiredoxins), and together with its reducing partner AhpF, it scavenges H_2O_2 at low micromolar concentrations. Catalases are activated only when AhpC is saturated, at millimolar H_2O_2 concentrations (10). Several bacteria have AhpC, although not all have the reducing partner AhpF (e.g., *Helicobacter pylori* and *Salmonella enterica* serovar Typhimurium) (10). In *V. cholerae*, OxyR positively activates genes for peroxiredoxin PrxA and DPS (DNA-binding protein from starved cells), and these genes are critical for *V. cholerae* oxidative stress resistance (15, 16). In this study, we sought to find additional peroxiredoxins that may be involved in *V. cholerae* oxidative stress resistance. By analyzing the *V. cholerae* genome, we identified an *ahpC* homolog on the large chromosome. Divergently transcribed from the putative *ahpC* gene is a gene encoding another LysR-family regulator. We show that this gene is required for *ahpC* activation and that AhpC is involved in fine-tuning *V. cholerae* responses to ROS through OxyR. We here name the *ahpC* regulator OxyR2 and rename the previous OxyR OxyR1.

RESULTS AND DISCUSSION

OxyR2 activates *ahpC* expression, and a conserved cysteine residue is critical for OxyR2 function. We have shown previously that in *V. cholerae*, OxyR (VC2636) upregulates the expression of its neighboring gene *prxA* (*vc2637*), encoding a peroxiredoxin (17). Both OxyR and PrxA are important for H_2O_2 resistance (15). To better understand how *V. cholerae* protects itself from oxidative stress, we searched for other peroxiredoxins in the *V. cholerae* genome and identified a putative *ahpC* homolog, *vc0731*. Similar to the *oxyR-prxA* genetic structure, a gene (*vc0732*) that is divergently transcribed from *ahpC* encodes another LysR-family transcriptional factor (Fig. 1A). VC0732 bears significant sequence homology to OxyR (47% to *E. coli* OxyR and 49% to *V. cholerae* OxyR), including the conserved two-cysteine motif (Fig. 1B, boxed). As such, we have annotated VC0732 as OxyR2 and henceforth refer to VC2636 (OxyR) as OxyR1.

To explore the role of OxyR2 and AhpC in *V. cholerae* ROS resistance, we first examined *ahpC* transcriptional regulation. Using a P_{ahpC} -*luxCDABE* transcriptional fusion reporter, we found that *ahpC* was highly expressed in the wild type (WT) and that addition of hydrogen peroxide did not affect its expression (Fig. 1C), suggesting that *ahpC* expression is H_2O_2 independent. Deletion of *oxyR1* did not affect *ahpC* expression, whereas deletion of *oxyR2* abolished *ahpC* transcription (Fig. 1C). Expression of *oxyR2* in *trans* restored *ahpC* expression in the $\Delta oxyR2$ background (Fig. 1C). Finally, *ahpC* expression in $\Delta oxyR1 \Delta oxyR2$ double mutants was at a similarly low level as in $\Delta oxyR2$ mutants. These results suggest that OxyR2 activates *ahpC* in an H_2O_2 -independent manner.

To further analyze OxyR2 function, we mutated the first of the two conserved cysteine residues, Cys206, to serine and tested whether the OxyR2 mutant was still functional. In the *E. coli* OxyR, the first of the two cysteines (C199) has been shown to be involved in OxyR activation (12–14). The involvement of the second cysteine is

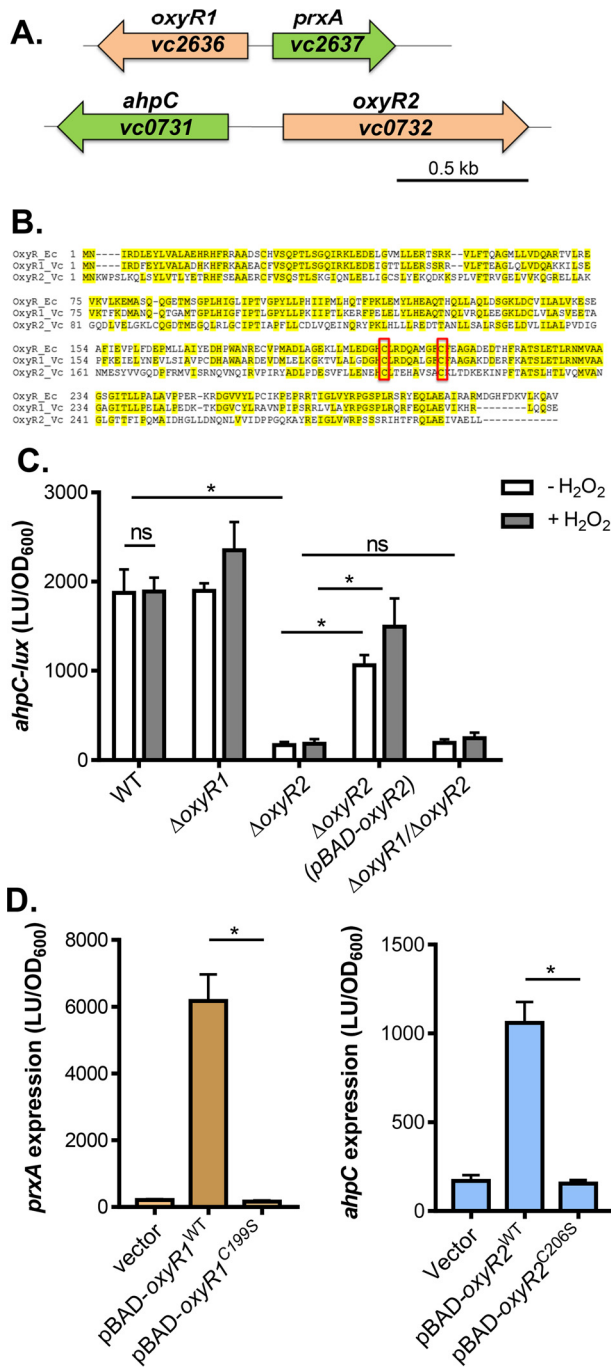


FIG 1 OxyR2 regulation of *ahpC*. (A) Schematic representation of the genetic structure of *oxyR1-prxA* and *oxyR2-ahpC* regulons. (B) Sequence alignment of *E. coli* OxyR (OxyR_Ec), *V. cholerae* VC2636 (OxyR1_Vc), and *V. cholerae* VC0732 (OxyR2_Vc). Conserved cysteine residues are boxed. (C) *ahpC-lux* expression. Wild type, Δ *oxyR1* Δ *oxyR2* mutants, or Δ *oxyR1* Δ *oxyR2* mutants containing the P_{*ahpC*}-*luxCDABE* transcriptional fusion plasmids were grown standing in AKI medium at 37°C for 4 h followed by shaking for 1 h until mid-log phase was reached. H₂O₂ (50 μ M final concentration) was added to indicated cultures, and cultures were shaken at 37°C for 2 h. Luminescence was then measured and reported normalized to OD₆₀₀. LU, luminescence unit. For complementation assays, 0.1% arabinose was included in the medium to activate pBAD-*oxyR2*. (D) The importance of cysteine residues in OxyR1 (left) and OxyR2 (right). pBAD-*oxyR1*^{WT} or pBAD-*oxyR1*^{C199S} was introduced into Δ *oxyR1* (pP_{*prxA*}-*luxCDABE*), and pBAD-*oxyR2*^{WT} or pBAD-*oxyR2*^{C206S} was introduced into Δ *oxyR2* (pP_{*ahpC*}-*luxCDABE*). Arabinose (0.1%) was used to induce the P_{BAD} promoter, and 50 μ M H₂O₂ (final concentration) was added to activate OxyR1. Data shown represent the mean and standard deviation from three biological replicates. ns, not significant; *, Student's *t* test *P* value of <0.05.

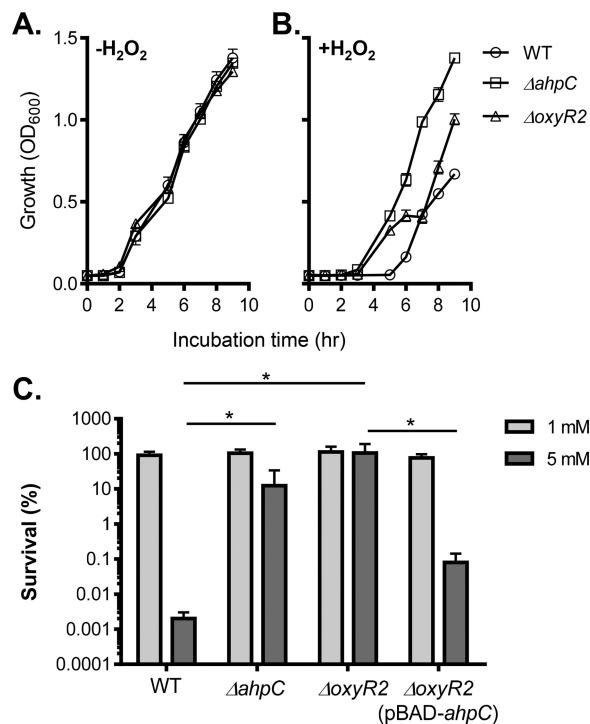


FIG 2 AhpC and OxyR2 effects on ROS resistance. (A and B) Growth of *V. cholerae* wild type and mutants at 37°C in the absence (A) and presence (B) of 150 μ M H₂O₂. Cultures were grown standing in virulence-inducing AKI medium at 37°C, and OD₆₀₀ was measured at the time points indicated. (C) Killing assay of *V. cholerae* wild type and mutants in the presence of 1 mM and 5 mM H₂O₂. Cultures were grown standing for 4 h followed by shaking for 2 h at 37°C. Arabinose (0.1%) was used to induce the P_{BAD} promoter. H₂O₂ was then added at the indicated concentrations (1 mM and 5 mM), and the cultures were incubated at 37°C with shaking for 2 h. Percent survival was calculated for each strain by comparing CFU of treated samples to that of untreated samples. Data shown represent the mean and standard deviation from three biological replicates. *, *P* value < 0.05.

debatable. As shown in Fig. 1D (left), OxyR1 C199 was critical; mutation of this residue significantly reduced OxyR1 activity. Similarly, OxyR2^{C206S} could not activate *ahpC* expression (Fig. 1D, right), suggesting that this cysteine residue is critical for OxyR2 function. However, unlike OxyR1, OxyR2 activity is not dependent on H₂O₂, implying that the mechanisms of thiol modification through C206 may be different in the two OxyRs.

ΔoxyR2 and ΔahpC mutants are more resistant to hydrogen peroxide. Next, we tested whether AhpC and OxyR2 are involved in ROS resistance in *V. cholerae*. We grew the wild type and ΔahpC and ΔoxyR2 mutants under virulence-inducing conditions (in AKI medium) (18) in the absence or in the presence of hydrogen peroxide. The virulence-inducing condition was used to mimic the *in vivo* condition that *V. cholerae* may encounter during the infection. Neither the ΔahpC nor the ΔoxyR2 mutant had any growth defect without addition of exogenous H₂O₂ (Fig. 2A). However, in the presence of H₂O₂, both ΔahpC and ΔoxyR2 mutants grew significantly better than the wild type (Fig. 2B). Of note, it is not clear why, in the presence of H₂O₂, ΔoxyR2 mutants appeared to have a plateau during the mid-log growth (Fig. 2B, triangles). It is possible that OxyR2 may regulate genes other than *ahpC*. To further confirm that ΔahpC and ΔoxyR2 mutants are more resistant to ROS, we exposed the mid-log-phase cultures of these strains to brief H₂O₂ treatment and examined their viability. We found that when bacteria were treated with 5 mM H₂O₂ for 2 h, over 99.9% of wild-type cells were readily killed, whereas both ΔahpC and ΔoxyR2 mutants were highly resistant (Fig. 2C). Expression of *ahpC* in ΔoxyR2 mutants partially restored H₂O₂ susceptibility (Fig. 2C). These results indicate that *V. cholerae* strains that lack AhpC or its cognate activator OxyR2 are hyperresistant to ROS.

RNA sequencing reveals opposing roles for OxyR1 and OxyR2 in regulation of ROS-resistant genes. To investigate whether the increased ROS resistance of Δ *ahpC* and Δ *oxyR2* is linked to OxyR1 regulation, we performed transcriptome analysis using RNA sequencing (RNA-seq). We grew wild-type, Δ *oxyR1*, and Δ *oxyR2* strains in AKI medium until mid-log phase (determined by a growth curve analysis) and exposed cultures to 0 and 50 μ M H₂O₂ for 60 min. RNA was extracted and subjected to RNA-seq analysis. The analysis identified expression of over 3,600 coding DNA sequence (CDS) tags in each sample. Biological replicates were tightly clustered (data not shown), indicating consistency between replicates. We analyzed differential expression of genes in the WT versus the mutant in the presence of H₂O₂. We identified a total of 58 genes that were at least 2-fold differentially expressed in the Δ *oxyR1* mutant and 222 genes that were differentially expressed in the Δ *oxyR2* mutant (see Data Set S1 in the supplemental material). In addition to regulation of ROS resistance-related genes (see below), OxyR1 also affects transcription of a two-component regulatory system, VC1084-VC1086. Deletion of VC1086 has been shown to have slightly enhanced colonization (19). Moreover, OxyR1 negatively regulates genes needed for CTX phage production, such as the VC1453-VC1454 and VC1462-VC1463 genes (20). Interestingly, in Δ *oxyR2* mutants, the expression of several virulence genes, such as *tcpA* (*vc0828*) and *toxT* (*vc0838*), was downregulated. Further studies to fully understand OxyR1- and OxyR2-regulated genes are ongoing. Next, we compared OxyR1-regulated genes with OxyR2-regulated genes using a Venn diagram and a volcano plot (Fig. 3A and B). We found that a number of ROS resistance-related genes (Fig. 3B, diamonds), such as *vc0139* (*dps*), *vc1560* (*katG*), and *vc2637* (*prxA*), were highly induced in the Δ *oxyR2* mutant background but had reduced expression in the Δ *oxyR1* mutants, suggesting that these genes are activated by OxyR1 but that their expression is negatively affected by OxyR2. Figure 3C shows the reads of selected genes under different conditions. The RNA-seq data corroborated our finding that *ahpC* expression is dependent on OxyR2 but independent of H₂O₂ (Fig. 3C). The expression of both *prxA* and *katG* was also significantly higher in the presence of H₂O₂ in an OxyR1-dependent manner (Fig. 3C), confirming previous findings (15, 17). Both *prxA* and *katG* transcriptome levels were significantly higher in the Δ *oxyR2* mutants, either in the absence or in the presence of H₂O₂, again implying that OxyR2 may negatively affect OxyR1-activated gene expression.

OxyR2-activated AhpC modulates OxyR1 activity. To further validate the RNA-seq data, we used a P_{*prxA*}-*luxCDABE* transcriptional reporter to measure *prxA* expression in the wild type. We have shown previously that OxyR1-activated *prxA* expression is H₂O₂ dependent (15, 17). Deletion of *oxyR2* strongly induced *prxA* expression even in the absence of H₂O₂ (Fig. 4A), consistent with the RNA-seq data. To investigate how OxyR2 negatively affects *prxA* expression, we first examined whether OxyR2 regulates *prxA* expression directly. We found that in *E. coli*, *V. cholerae* OxyR2 could activate *ahpC* expression independently of H₂O₂ (Fig. 4B), indicating that OxyR2 functions in *E. coli* and OxyR2 activation of *ahpC* may be direct. However, in *E. coli*, *prxA* expression was not affected by OxyR2 (Fig. 4B), suggesting that OxyR2 may not transcriptionally regulate *prxA* directly. To examine whether OxyR2 effects on *prxA* expression occur through OxyR2-activated AhpC, we examined *prxA* expression in the absence of H₂O₂ in different *V. cholerae* mutants. We found that, similarly to that in the Δ *oxyR2* mutant, *prxA* expression was strongly induced in both Δ *ahpC* mutants and Δ *ahpC* Δ *oxyR2* double mutants, whereas expression of *ahpC* in Δ *oxyR2* mutants reduced *prxA* induction (Fig. 4C), suggesting that the OxyR2 effect on *prxA* expression may occur through AhpC. Furthermore, deletion of *oxyR1* abolished *prxA* expression (Fig. 4C), even in the *oxyR2* mutation background, suggesting that OxyR1 is required and OxyR2-AhpC may modulate OxyR1 activity and thereby the expression profiles of the OxyR1 regulon.

In *E. coli*, it has been shown that AhpC is the primary scavenger of endogenous H₂O₂ as it can efficiently scavenge trace H₂O₂ (21). Thus, *ahpC* mutants accumulate sufficient hydrogen peroxide to induce the OxyR regulon, including genes encoding catalase, which is the predominant scavenger when H₂O₂ concentrations are high. Based on the

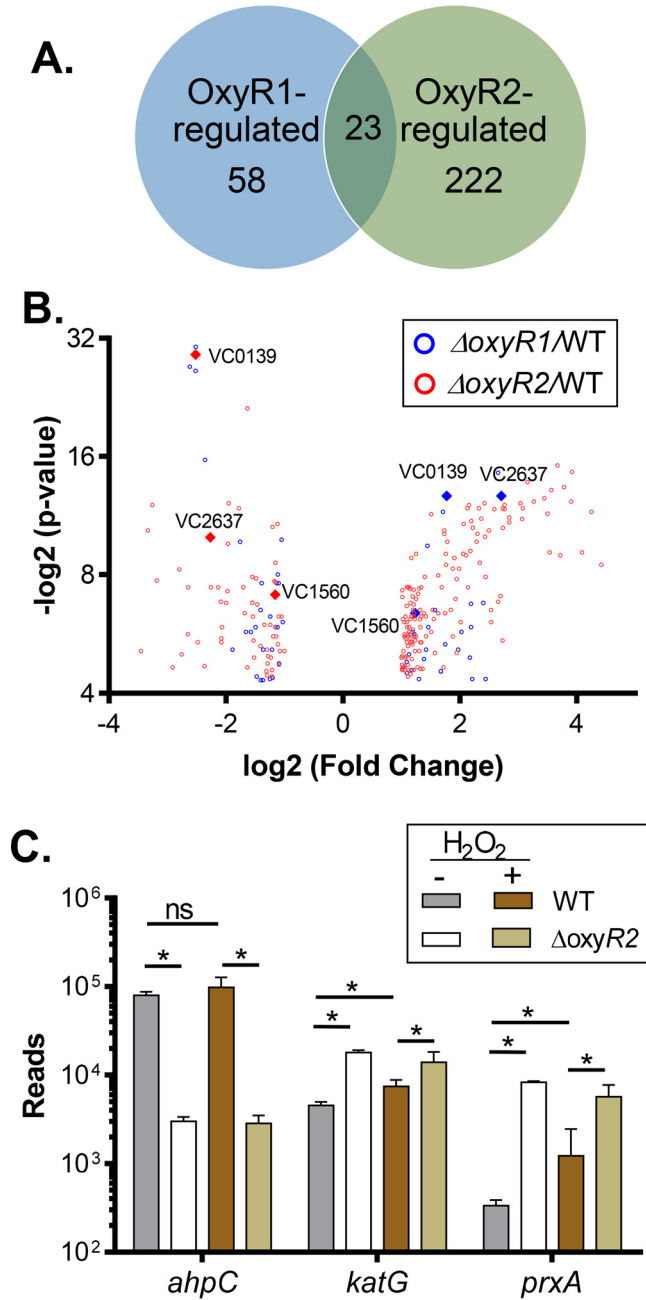


FIG 3 Determination of OxyR1- and OxyR2-regulated genes by RNA sequencing. (A and B) Venn diagram (A) and volcano plot (B) of differentially expressed genes (fold change of >2, P value of <0.05) in $\Delta oxyR1$ (blue) and $\Delta oxyR2$ (red) strains in the presence of 50 μM H_2O_2 . Diamonds represent oxidative stress-related genes common to the two data sets. (C) Normalized RNA-seq reads of selected genes in the wild-type and $\Delta oxyR2$ strains in the presence and absence of 50 μM H_2O_2 . Data shown represent the mean and standard deviation from two biological replicates. *, P value < 0.05; ns, not significant.

studies in *E. coli* and the above results, we speculate that OxyR2-activated AhpC modulates OxyR1 activity by maintaining low intracellular concentrations of H_2O_2 . Below a certain threshold of H_2O_2 concentration, OxyR1 and its regulon remain inactive and AhpC serves as the primary scavenger for H_2O_2 . However, if that threshold is surpassed, due to either an exogenous increase in H_2O_2 levels or the absence of OxyR2 and/or AhpC, then the OxyR1 regulon is activated. This may explain why $\Delta ahpC/oxyR2$ mutants are more resistant to H_2O_2 than the wild type (Fig. 2) as accumulation of endogenous H_2O_2 results from the absence of AhpC “preinduction” of OxyR1-activated genes, such as catalases and PrxA.

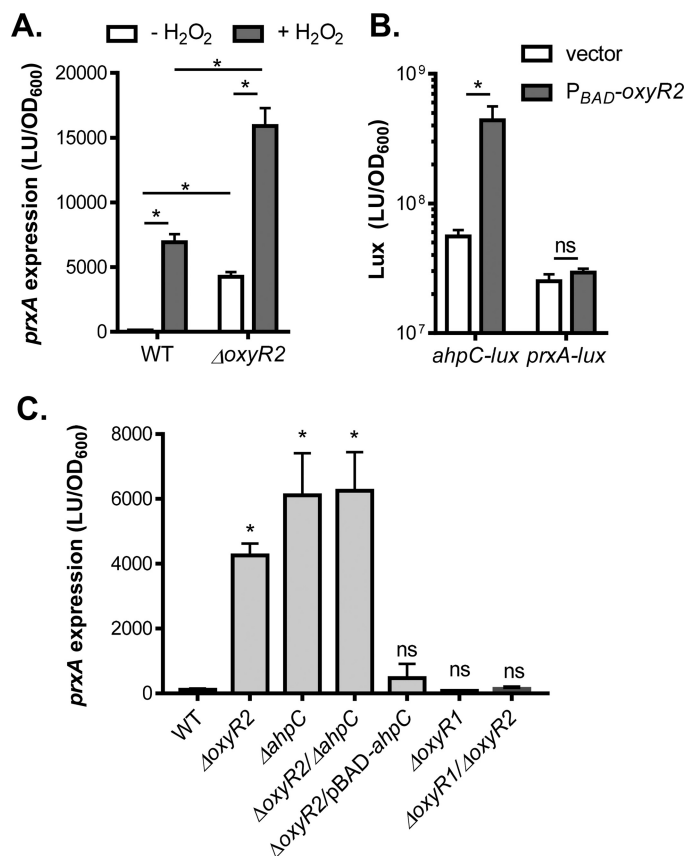


FIG 4 OxyR2-AhpC effects on *prxA* expression. (A) Confirming RNA-seq data using P_{*prxA*}-*luxCDABE* reporters. The wild type and the $\Delta oxyR2$ mutant containing the P_{*prxA*}-*luxCDABE* transcriptional fusion plasmids were grown standing in AKI medium at 37°C for 4 h followed by shaking for 1 h. H₂O₂ (50 μ M final concentration) was added to indicated cultures, and cultures were shaken at 37°C for 2 h. Luminescence was then measured and reported normalized to OD₆₀₀. (B) OxyR2 effects on *ahpC* and *prxA* expression in *E. coli* DH5 α containing pBAD24 (vector control) or pBAD-*oxyR2* and P_{*ahpC*}-*luxCDABE* or P_{*prxA*}-*luxCDABE* reporter plasmids was grown in LB containing 0.3% arabinose until reaching an OD₆₀₀ of 0.4. Luminescence was then measured and normalized to OD₆₀₀. (C) *prxA* expression in different *V. cholerae* mutants. The wild type and different mutants indicated containing the P_{*prxA*}-*luxCDABE* plasmids were grown under the conditions described for panel A (without H₂O₂). Luminescence was then measured and reported normalized to OD₆₀₀. Data shown represent the mean and standard deviation from three biological replicates. *, *P* value < 0.05; ns, no significance; LU, luminescence unit.

OxyR2-regulated AhpC contributes to *V. cholerae* environmental survival but not *in vivo* colonization. To investigate the physiological role of OxyR2-activated AhpC in *V. cholerae*, we first examined whether deletion of *oxyR2* or *ahpC* affects *V. cholerae* colonization. In an infant mouse competition model (22), we found that both $\Delta ahpC$ and $\Delta oxyR2$ mutants colonized as well as the wild type (Fig. 5A). Similar results were obtained when we performed $\Delta ahpC/\Delta oxyR2$ mutant competition assays using a streptomycin-treated adult mouse model (23) (Fig. 5B), suggesting that OxyR2-AhpC is not required for *V. cholerae* colonization, at least under the conditions tested. Since *V. cholerae* encounters oxygen-limiting conditions and host-derived oxidative stress during colonization of the host small intestines (9, 24), we grew the wild type and $\Delta ahpC$ and $\Delta oxyR2$ mutants anaerobically and exposed them to different levels of H₂O₂. At very low (≤ 10 μ M) or very high (≥ 1 mM) H₂O₂ concentrations, the mutants grew similarly to the wild type (Fig. 5C). However, at an intermediate level of H₂O₂ (100 μ M), both $\Delta ahpC$ and $\Delta oxyR2$ mutants displayed growth defects, suggesting that under oxygen-limiting conditions, AhpC may be required to remove harmful levels of ROS. Finally, we tested whether OxyR2-AhpC is important for *V. cholerae* survival in seawater. To mimic the environment that *V. cholerae* encounters during the transition from oxygen-poor guts to oxygen-rich aquatic reservoirs, we grew the wild type and $\Delta ahpC$

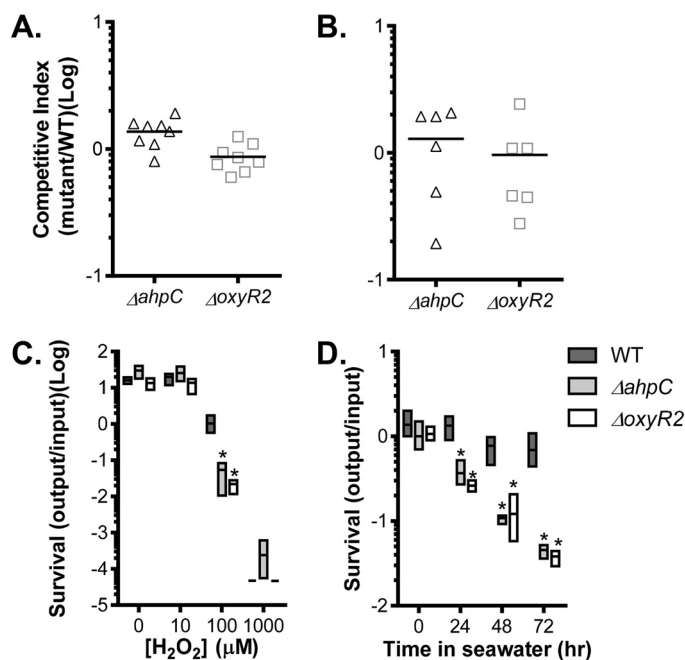


FIG 5 Physiological roles of OxyR2-AhpC. (A) Infant mouse colonization. Approximately 10^6 cells of the wild type (*lacZ*) and $\Delta oxyR2$ or $\Delta ahpC$ mutants (*lacZ*⁺) were intragastrically inoculated into 6-day-old CD-1 mice in a 1:1 ratio. After a 12-h incubation, CFU from small intestines was determined by serial dilution and plating on LB agar. The data shown are from three independent experiments, and each symbol represents CFU recovered from one mouse intestine. The horizontal line represents the average number of cells recovered. (B) Streptomycin-treated adult mouse model. Five-week-old CD-1 mice were treated with streptomycin, and a saturated culture of wild type (*lacZ*) and mutants (*lacZ*⁺) was mixed in a 1:1 ratio and intragastrically administered to each mouse. Fecal pellets were collected from each mouse after 3 days, resuspended in LB, serially diluted, and then plated on plates containing X-Gal. The competitive index (CI) was calculated as the ratio of mutant to wild-type colonies normalized to the input ratio. The horizontal line represents the average CI. (C) Anaerobic survival. AKI medium with indicated concentrations of H₂O₂ was inoculated with anaerobically grown cultures and grown in the anaerobic chamber at 37°C for 24 h. Samples were serially diluted and plated to enumerate cell counts. Growth was measured and represented as a ratio of output/input. (D) Seawater survival. The wild type, $\Delta ahpC$, or $\Delta oxyR2$ strain was grown in AKI medium anaerobically at 37°C for 4 h. Bacterial cells were then collected, washed, and then resuspended in artificial seawater and incubated at 22°C. At the indicated intervals, viable cells were determined by serial dilutions and plating on LB plates. Data shown represent the mean and standard deviation from at least three biological replicates. *, *P* value < 0.05.

and $\Delta oxyR2$ mutants at 37°C under oxygen-limiting conditions and then incubated the bacterial cells in seawater at 22°C. We found that the number of viable cells of $\Delta ahpC$ and $\Delta oxyR2$ mutants was significantly reduced compared to the wild type (Fig. 5D). The viable cells of the mutants were similar to the wild type when we incubated aerobically grown cells in seawater (data not shown). Taken together, these results suggest that OxyR2-AhpC plays an important role in detoxifying low levels of environmental oxidative stresses.

In this study, we identified a second OxyR homolog, OxyR2, in *V. cholerae* that is required to activate the alkylhydroperoxide reductase gene *ahpC*. We hypothesize that OxyR2-AhpC removes low levels of ROS and modulates OxyR1 activity in *V. cholerae*. OxyR2-regulated AhpC may be particularly important for *V. cholerae* to better adapt to environmental niches that pose low levels of oxidative stress such as in inflammatory intestines and exiting from the gut to aquatic environments (Fig. 5C and D). In *Vibrio vulnificus*, there are two OxyR homologs, which control different peroxiredoxin genes (25). *V. vulnificus* OxyR2 senses H₂O₂ with high sensitivity (26). Coexistence of OxyR1 and OxyR2 homologs is widespread in many other bacteria (25), implying the importance of fine-tuning ROS resistance for optimal environmental adaptation.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All *V. cholerae* strains used in this study were derived from O1 El Tor C6706 (27) and are listed in Table 1. All strains were propagated at 37°C in LB medium

TABLE 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Characteristic or sequence (5' to 3')	Reference, source, or purpose
Strains		
C6706	<i>V. cholerae</i> O1 El Tor wild-type strain	27
$\Delta oxyR1$ mutant	VC2636 in-frame deletion mutant	15
$\Delta oxyR2$ mutant	VC0732 in-frame deletion mutant	This work
$\Delta ahpC$ mutant	VC0731 in-frame deletion mutant	This work
$\Delta oxyR2/\Delta ahpC$ mutant	VC0732 and VC0731 double deletion mutant	This work
$\Delta oxyR1/\Delta oxyR2$ mutant	VC2636 and VC0732 double deletion mutant	This work
Plasmids		
pWM91	Suicide vector	28
pBBR-lux	Plasmid containing a promoterless <i>luxCDABE</i> reporter	29
pBAD24	Plasmid containing an arabinose-inducible P_{BAD} promoter	30
pBAD-oxyR1	P_{BAD} -oxyR1 WT in pBAD24	15
pBAD-oxyR2	P_{BAD} -oxyR2 WT in pBAD24	This work
pBAD-oxyR1 ^{C199S}	P_{BAD} -oxyR1 cysteine 199→serine mutation in pBAD24	This work
pBAD-oxyR2 ^{C206S}	P_{BAD} -oxyR2 cysteine 206→serine mutation in pBAD24	This work
pBAD-ahpC	P_{BAD} -ahpC WT in pBAD24	This work
pPrxA-lux	P_{prxA} - <i>luxCDABE</i> in pBBR-lux	15
pAhpC-lux	P_{ahpC} - <i>luxCDABE</i> in pBBR-lux	This work
Primers		
oxyR2-1	TCACTCGAGCAGGAATGAACCCACGGAAAG	$\Delta oxyR2$
oxyR2-2	GTTCAGCCACGAGGCTTGGCCATTATTCA	
oxyR2-3	GCCAAGCCTCGTGGCTGAACGTGTATAGCG	
oxyR2-4	TAAGCGCGCGTGTGGGTTAATTCAGCAGT	
ahpC-1	CCGCTCGAGCTTGATGCGCCGTACGAATG	$\Delta ahpC$
ahpC-2	TTAGGAGCAAAAATAAGACTCTTAACCCCG	
ahpC-3	AGTCTTATTTTTGCTCCTAAGCAATTTTGT	
ahpC-4	AAGCGCCGCCTTCGCGCAACAATAAGTGC	
PahpC-1	ATCGAGCTCACCCGAGTAAATACCAGAGG	<i>ahpC-lux</i>
PahpC-2	GGATCCATTTTTTGTCTCCTAAGCAA	
pBAD-oxyR2-1	GGGTACCATGAATAAATGGCCAAGCCT	pBAD-oxyR2
pBAD-oxyR2-2	GCTCTAGACTATAACAGTTCAGCCACTA	
pBAD-oxyR2-3	ATGTTTCAGTTAAAGAGTGCCTGTTTTCGAGTAAA	pBAD-oxyR2 ^{C206S}
pBAD-oxyR2-4	AAACGAGCACTCTTAACTGAACATGCCGTATC	
pBAD-oxyR1*-1	CCCAAGCTTTTACTCGCTTTGCTGTAAGC	pBAD-oxyR1 ^{C199S}
pBAD-oxyR1*-2	GGAGATGGGCATTCCCTGCGCATCAAGCGTTA	
pBAD-oxyR1*-3	TTGATCGCGCAGGCAATGCGCATCTCCTAAAGC	
pBAD-oxyR1*-4	CGGAATTCATGAACATTCGTGATTTTGAATAC	
pBAD-ahpC-1	TGCTCTAGATTATTTTAGGTGAGCAGAGT	pBAD-ahpC
pBAD-ahpC-2	CCGGAATCTTGCTTAGGAGCAAAAAAT	

containing appropriate antibiotics and 1.5% agar where necessary. In-frame deletions of *oxyR2* (*vc0732*) and *ahpC* (*vc0731*) were constructed by cloning the flanking regions of these genes into the suicide vector pWM91 containing a *sacB*-counterselectable marker (28). The resulting constructs were introduced into *V. cholerae* by conjugation, and deletion mutants were selected for double homologous recombination events. The *ahpC* transcriptional fusion luminescence reporters were constructed by cloning the *ahpC* promoter sequences into the pBBR-lux plasmid, which contains a promoterless *luxCDABE* reporter (29). Constructions of the *oxyR1* (*vc2636*) in-frame deletion and the *prxA* (*vc2637*) *lux* reporter have been previously described (15, 17). OxyR1 and OxyR2 cysteine→serine mutations were constructed by overlap extension PCR (24). For expression of *oxyR1*, *oxyR2*, and their cysteine mutant derivatives, PCR-amplified fragments of *oxyR1* or *oxyR2* were cloned into pBAD24 (30), which contains an arabinose-inducible P_{BAD} promoter. A similar strategy was also used for construction of pBAD-ahpC. The plasmids and primers used in this study are described in Table 1.

***V. cholerae* ROS resistance assays.** *V. cholerae* strains were grown overnight at 37°C on LB plates with appropriate antibiotics and normalized to an optical density at 600 nm (OD_{600}) of 1.0 in liquid medium. A 1:1,000 dilution of normalized culture was used to inoculate fresh AKI medium (18). H_2O_2 (150 μ M final concentration) was added to each culture, and the cultures were incubated at 37°C standing in a 96-well plate. OD_{600} was measured using a microplate reader at the indicated time points.

For hydrogen peroxide killing assays, *V. cholerae* strains were grown overnight at 37°C on LB plates with appropriate antibiotics and normalized to an OD₆₀₀ of 1.0 in liquid medium. A 1:1,000 dilution of normalized culture was used to inoculate fresh AKI medium. Cultures were grown standing for 4 h followed by shaking for 2 h at 37°C. H₂O₂ was then added to appropriate concentrations (1 mM and 5 mM), and the cultures were incubated at 37°C with shaking for 2 h. Viable cells were determined by serial dilutions and plating on LB plates. Percent survival was calculated for each strain by comparing CFU of H₂O₂-treated samples to that of untreated samples.

For testing ROS resistance under anaerobic growth conditions, *V. cholerae* strains were grown overnight at 37°C in LB with appropriate antibiotics in an anaerobic chamber (Coy Laboratories) and normalized to an OD₆₀₀ of 1.0 in fresh liquid medium. A 1:1,000 dilution of normalized culture was used to inoculate fresh AKI medium containing indicated concentrations of H₂O₂. Cultures were incubated in the anaerobic chamber at 37°C for 24 h. Viable cells were determined by serial dilutions and plating on LB plates.

RNA sequencing. Overnight cultures of wild-type, $\Delta oxyR1$, and $\Delta oxyR2$ strains were inoculated 1:1,000 into fresh AKI medium. Cultures were grown without shaking for 4 h and subsequently treated with or without 50 μ M H₂O₂ for 1 h at 37°C. RNA was extracted from cultures using TRIzol and an RNeasy minikit (Qiagen) according to the manufacturer's instructions and was subjected to RNA sequencing. RNA sequencing was conducted at PrimBio Research Institute LLC (Exton, PA, USA). Briefly, the rRNA-depleted RNA was used to construct a cDNA library by using the Ion Total RNA-seq kit (Life Technologies). Amplified PCR products from cDNA libraries were then loaded on Ion P1 chips for Ion Torrent RNA-seq. Following the proton run, the raw sequences were aligned to the *V. cholerae* N16961 genome. Aligned BAM files were used for further analysis. BAM files, separated by the specific barcodes, were uploaded to the Strand NGS software (San Francisco, CA). After filtering, the aligned reads were normalized and quantified using the DESeq algorithm by Strand NGS. The standard *t* test was used to determine significantly differentially expressed genes based on two replicates for each condition.

Measuring transcriptional expression using Lux reporters. Wild-type, $\Delta oxyR1$, and $\Delta oxyR2$ strains and their cysteine mutant derivatives containing either P_{ahpC}-luxCDABE or P_{prxA}-luxCDABE transcriptional fusion plasmids were grown overnight at 37°C on LB plates with appropriate antibiotics and normalized to an OD₆₀₀ of 1.0 in liquid medium. Strains were inoculated at 1:100 into fresh AKI medium containing appropriate antibiotics and grown standing at 37°C for 4 h followed by shaking for 1 h. H₂O₂ (50 μ M final concentration) was then added, and cultures were incubated for an additional 2 h with shaking. Samples were then collected from growing cultures, and luminescence was read using a microplate reader and normalized for growth against optical density at 600 nm. Lux expression is reported as light units per OD₆₀₀.

In vivo colonization assays. All animal experiments were carried out in strict accordance with the animal protocol number 805287 that was approved by the IACUC of the University of Pennsylvania. The infant mouse colonization assay was performed as previously described (31) by inoculating approximately 10⁵ *V. cholerae* cells per mouse into 6-day-old suckling CD-1 mice in a 1:1 ratio of mutant (*lacZ*⁺) to wild type (*lacZ*). At 12 h postinfection, mice were sacrificed and their intestinal homogenates were collected. Samples were serially diluted and plated on LB agar plates containing appropriate antibiotics and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) to differentiate between wild-type and mutant strains.

The streptomycin-treated adult mouse model was used as previously described (23, 32). Briefly, 5-week-old CD-1 mice were provided with drinking water containing 0.5% (wt/vol) streptomycin and 0.5% sucrose. One day after streptomycin treatment, approximately 10⁸ cells each of two strains (wild type and mutant) were mixed at a 1:1 ratio and intragastrically administered to each mouse. Three days after they were inoculated, fecal pellets were collected from each mouse, resuspended in LB, serially diluted, and then plated on plates containing X-Gal. The competitive index was calculated as the ratio of mutant to wild-type colonies normalized to the input ratio.

Environmental survival assays. The wild-type, $\Delta ahpC$, or $\Delta oxyR2$ strain was grown overnight at 37°C in LB with appropriate antibiotics and normalized to an OD₆₀₀ of 1.0 in fresh liquid medium. A 1:1,000 dilution of normalized culture was used to inoculate fresh AKI medium and grown anaerobically at 37°C for 4 h. Bacterial cells were then collected, washed with phosphate-buffered saline (PBS) buffer, and then resuspended in artificial seawater (ASW) (33) and incubated at 22°C. At the indicated intervals, viable cells were determined by serial dilutions and plating on LB plates.

Accession number(s). Sequencing data for RNA-seq experiments are accessible at SRP095131 in the Sequence Read Archive (SRA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00929-16>.

DATA SET S1, XLSX file, 0.09 MB.

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