H-NOX–mediated nitric oxide sensing modulates symbiotic colonization by *Vibrio fischeri*

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The bioluminescent bacterium *Vibrio fischeri* initiates a specific, persistent symbiosis in the light organ of the squid *Euprymna scolopes*. During the early stages of colonization, *V. fischeri* is exposed to host-derived nitric oxide (NO). Although NO can be both an antimicrobial component of innate immunity and a key signaling molecule in eukaryotes, potential roles in beneficial host–microbe associations have not been described. *V. fischeri hnoX* encodes a heme NO/oxygen-binding (H-NOX) protein, a member of a family of bacterial NO- and/or O_2-binding proteins of unknown function. We hypothesized that H-NOX acts as a NO sensor that is involved in regulating symbiosis-related genes early in colonization. Whole-genome expression studies identified 20 genes that were repressed in an NO- and H-NOX–dependent fashion. Ten of these, including hemin-utilization genes, have a promoter with a putative ferric-uptake regulator (Fur) binding site. As predicted, in the presence of NO, wild-type *V. fischeri* grew more slowly on hemin than a *hnoX* deletion mutant. Host-colonization studies showed that the *hnoX* mutant was also 10-fold more efficient in initially colonizing the squid host than the wild type; similarly, in mixed inoculations, it outcompeted the wild-type strain by an average of 16-fold after 24 h. However, the presence of excess hemin or iron reversed this dominance. The advantage of the mutant in colonizing the iron-limited light-organ tissues is caused, at least in part, by its greater ability to acquire host-derived hemin. Our data suggest that *V. fischeri* normally senses a host-generated NO signal through H-NOX_{Vf} and modulates the expression of its iron uptake capacity during the early stages of the light-organ symbiosis.

symbiosis | iron uptake | transcriptional analysis | colonization

Small molecules have important signaling functions in microbe–microbe and microbe–host interactions (1). One such molecule, nitric oxide (NO), plays a key role in both cellular defense and signaling. When produced at high concentrations by activated macrophages and tissues, it is a powerful antimicrobial (2, 3). In contrast, when produced at low concentrations, NO can serve as a signaling molecule; for instance, in eukaryotic endothelial cells, it regulates activities such as vasodilation, and in bacteria that contain specific NO sensors, it facilitates responses to environmental conditions (4, 5). A family of putative sensor proteins widely distributed among bacteria binds O_2 and/or NO within a Fe(II)-heme domain that displays a high sequence identity to a domain of the eukaryotic soluble guanylate cyclase (sGC) (6). Thus, these putative sensors are collectively called heme NO/oxygen-binding (H-NOX) proteins. Several proteins in this family, such as those in *Vibrio cholerae* and *Thermoanaerobacter tengcongensis*, have been characterized both for structure (7, 8) and their ligand-discrimination properties (9). Nevertheless, the physiological role of H-NOX proteins has not been described in any bacterium. Members of the H-NOX family are either stand-alone proteins or are fused to other domains related to signaling functions. In addition, the *hnoX* gene is most often found in a predicted operon that includes a putative histidine kinase. This arrangement suggests that a H-NOX–mediated sensing mechanism may be involved in the bacterium's response to environments containing NO or O_2 (6, 10).

One such environment occurs in the mutualistic symbiotic association between the Hawaiian bobtail squid, *Euprymna scolopes*, and the marine bioluminescent bacterium, *V. fischeri* (11). The bacterial symbionts are housed in the crypts of the host's light-emitting organ, and they grow as a monospecific culture along the apical surfaces of the crypt epithelia (12). After reaching a high cell density in the organ, the bacteria induce their *lux* operon, producing light that is used by the host in an antipredatory behavior called counterillumination. In exchange for this bioluminescence, the host squid supplies nutrients that support symbiont proliferation (13).

The nascent light organ of a newly hatched juvenile squid is free of symbionts. During the initiation of the symbiosis, *V. fischeri* present in the ambient seawater gather as an aggregate in the mucus shed by superficial epithelia on the nascent organ. The bacteria in the aggregate then migrate to and into pores on the organ surface, moving through ducts to the deep crypts where the symbiont population is established (12). During this transit, the migrating *V. fischeri* are exposed to host-derived NO produced both by vesicles embedded in the secreted mucus and later, by the epithelium lining of the ducts (2), where reactive oxygen species are also produced (14). Given its antimicrobial and signaling properties, the generation of this host-derived NO has been proposed to be involved in the early stages of the association not only as a specificity determinant but also as a symbiotic signal (2, 12).

Analysis of the *V. fischeri* genome sequence (15) revealed the presence of a gene (*VF_A0071*) encoding a H-NOX homolog (H-NOX_{Vf}) that is located just upstream of a putative histidine kinase (HK; *VF_A0072*). H-NOX regulation of a histidine kinase in *Shewanella oneidensis* has been reported (10). We hypothesized that H-NOX_{Vf} plays a role in NO sensing and subsequent regulation of symbiosis-related genes. In this study, we examined the role of H-NOX_{Vf} as a sensor for NO both in culture and in the symbiosis. In this paper, we show that H-NOX_{Vf} binds NO, but not O_2, in vitro, and these are properties that are consistent with serving as an NO sensor. We also show that ~20 genes are negatively regulated in both a H-NOX_{Vf}– and NO-dependent manner and that H-NOX_{Vf}–mediated NO sensing modulates the effectiveness of symbiosis initiation.

Results

Ligand-Binding Properties of H-NOX_{Vf}. Sequence alignments and homology models of the *V. fischeri* H-NOX with other proteins bearing H-NOX domains indicated that H-NOX_{Vf} lacks the distal-pocket tyrosine that stabilizes oxygen binding in the H-NOXs (16), which leads to the prediction that H-NOX_{vf} will not form an O_2 complex. Indeed, the purified H-NOX_{Vf} protein formed stable Fe(II)-NO and Fe(II)-CO complexes (Fig. 1 and Table 1) but

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The authors declare no conflict of interest.

Data deposition: The whole-genome transcriptional data reported in this paper have been deposited in the GenBank database (accession no. GSE15522).

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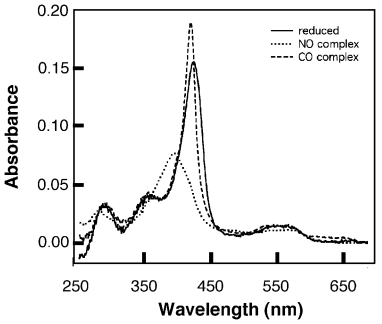

Fig. 1. Electronic absorption spectra of protein complexes of the H-NOX protein from *V. fischeri*. Purified H-NOX$_{Vf}$-Fe(II) (solid), -Fe(II)-CO (dotted), and -Fe(II)-NO (dashed) at concentrations of ~1 μM are shown.

showed no measurable affinity for O_2. The Fe(II) unligated complex displayed no noticeable spectral change when exposed to air for over 30 min, attesting to the aerobic stability of this protein and its slow oxidation rate. Based on these results, it is unlikely that H-NOX$_{Vf}$ functions in vivo as a redox sensor, although this possibility cannot be completely ruled out. The H-NOX$_{Vf}$ k_{off} for NO is slow (Table 2), similar to the k_{off} of sGCs (17) and other bacterial H-NOX proteins (7), and assuming a k_{on} for NO like that seen for NO in other H-NOXs, the K_d for NO would be in the picomolar range. Taken together, these results indicate a possible role for H-NOX$_{Vf}$ as a high-affinity NO sensor.

Certain Iron-Uptake Genes Are Repressed in a H-NOX$_{Vf}$– and NO-Dependent Manner. Because *V. fischeri* encounters host-derived NO during its colonization of the squid light organ (2), we asked if H-NOX$_{Vf}$ might convey this information about the symbiotic environment to the bacterium, leading to changes in the symbiont's gene expression. To determine which *V. fischeri* genes might be regulated by NO only in the presence of H-NOX$_{Vf}$, we compared the transcriptional profile of the *hnoX*-insertion mutant, YLW1 (Table S1; Fig. S1), with that of wild-type cells when the cultures were exposed to NO (diethylamine NONOate) for 30 min. To test if the *hnoX* insertion was not polar on the downstream HK gene, we used qRT-PCR analysis and showed that there was only a small (1.2- to 1.4-fold) difference between the NO-induced transcriptional response of the HK gene in the *hnoX* mutant and its parent.

Evidence that both the mutant and wild type produced a transcriptional response to NO treatment came from their elevated (>100-fold) expression of *VF_2316*, the *V. fischeri* homolog of the *Escherichia coli* NO dioxygenase-encoding *hmp,* a gene previously shown to be induced by NO exposure (18). A four-way contrast analysis with the cutoff for a false-discovery rate set at 0.05 was performed to identify those genes whose regulation required the presence of both NO and H-NOX$_{Vf}$ (Table S2). Although *V. fischeri* homologs of genes typically associated with NO detoxification were induced by the presence of NO, they were not differentially regulated between the wild-type and *hnoX*-insertion mutant (Table S2), suggesting that H-NOX$_{Vf}$ is not required for a typical bacterial defensive response to NO (Fig. S2).

In contrast, 40 other genes were differentially expressed in the wild-type strain on NO exposure, whereas their expression remained relatively unchanged in the *hnoX* mutant strain (Table S2). This group contains 20 genes that were up-regulated by NO treatment and another 20 genes that were down-regulated (Table S3). The promoter sequences of each of these two sets of genes were aligned to search for shared regulatory sequence elements, and a conserved palindromic motif was identified in 10 of 20 down-regulated genes (Fig. S3). This motif is similar to the binding site of the master iron-responsive regulator ferric-uptake regulator (Fur) of *E. coli* (19). Corroborating this observation, the annotation of these predicted targets indicated that 8 of 10 are in loci that have functions related to iron acquisition or use (Table S3). To determine if the effect of NO exposure on these putative Fur targets could be generalized to the entire Fur regulon, the genome sequence of *V. fischeri* ES114 was searched for additional putative Fur-binding sites using a position-specific weight-matrix model derived from the initial 10 targets. The expression patterns of the top 48 Fur targets from this analysis show a strong correlation with each other (Fig. 2), suggesting that the expression level of the entire Fur regulon is affected by the NO treatment in a H-NOX$_{Vf}$–dependent manner. Thus, in response to nonlethal concentrations of NO, H-NOX$_{Vf}$ plays a role in down-regulating the expression of iron-use genes either directly or indirectly by modulating their repression by Fur.

Table 1. UV-visible peak positions for Fe(II) complexes of H-NOX proteins (at 20 °C)

Protein	Soret	α	β	Reference
Fe(II) unligated				
sGC	431	555	555	40
H-NOX$_{Vc}$	429	568	568	7
H-NOX$_{Vf}$	428	568	568	This study
Fe(II)-CO				
sGC	423	541	567	40
H-NOX$_{Vc}$	423	541	566	7
H-NOX$_{Vf}$	423	538	571	This study
Fe(II)-NO				
sGC	398	537	572	40
H-NOX$_{Vc}$	398	540	573	7
H-NOX$_{Vf}$	398	539	571	This study

Table 2. NO dissociation rates ($s^{-1} \times 10^{-4}$) for H-NOX proteins

Protein	k_{m1} (k_{off})	k_{m2}	Reference
sGC	3.6 ± 0.8	166 ± 44	17
H-NOX$_{Vf}$	21 ± 0.6	100 ± 10	this study

Exposure to NO Suppresses *V. fischeri* Growth on Hemin. Among the genes shown to be down-regulated in NO-treated wild-type cells, but not the *hnoX*-insertion mutant, were those encoding putative TonB-dependent hemin receptors (*VF_1234* and *VF_A0331-A0333*), a hemin transporter (*VF_1220-1222*), and hemin-degradation proteins (*VF_1226-1228*) (Fig. 2 and Table S2). Thus, we predicted that, when exposed to NO, wild type would grow more slowly than the *hnoX* mutant in an iron-depleted minimal medium supplemented with hemin. To test this hypothesis using a nonpolar deletion of the *hnoX* gene, the mutant, YLW37 (*ΔhnoX*), was examined (Table S1). Both wild type and mutant were able to use hemin as the sole iron source, and the addition of NO initially delayed the growth of both cultures (Fig. 3*A*) (3). However, when growth resumed, the NO-treated wild-type cells grew at a significantly lower rate than the *ΔhnoX* mutant cells (Fig. 3*B*). Taken together, these data suggest that (*i*) NO exerts a suppressive effect on hemin uptake and use by wild-type cells and (*ii*) this suppression requires the presence of H-NOX$_{Vf}$. As predicted, the NO-dependent growth-rate phenotype of the *ΔhnoX* mutant could be genetically complemented by introduction of the *hnoX* gene *in trans*, which decreased the rate of growth back to that of wild-type cells (Fig. 3*B*).

The *hnoX* mutant had the same low growth rate as wild type in a low iron medium lacking hemin, both in the presence and absence of NO (Fig. S4). This result is consistent with the observation that the regulation of putative nonheme ferric- and ferrous-iron transporter operons (*VF_2149-2151* and *VF_0833-*

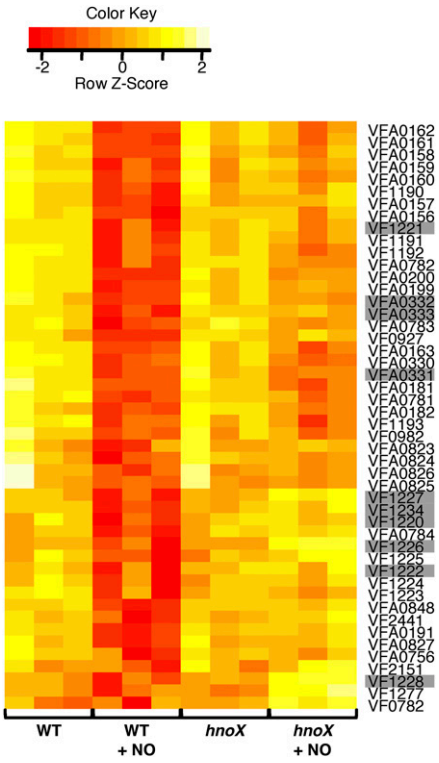

Fig. 2. Heat map summarizing the expression profile of all genes of *V. fischeri* ES114 whose promoter regions are predicted to contain a Fur-binding site. Genes are identified by their locus tags (rows), and experimental treatment triplicates are grouped in vertical columns. WT, wild-type cells; *hnoX*, *hnoX*-insertion mutant cells; NO, nitric-oxide treatment. The expression-level values were normalized to their Z scores for each row (red, low expression level; white, high expression level). The hemin-related genes are indicated by gray boxes.

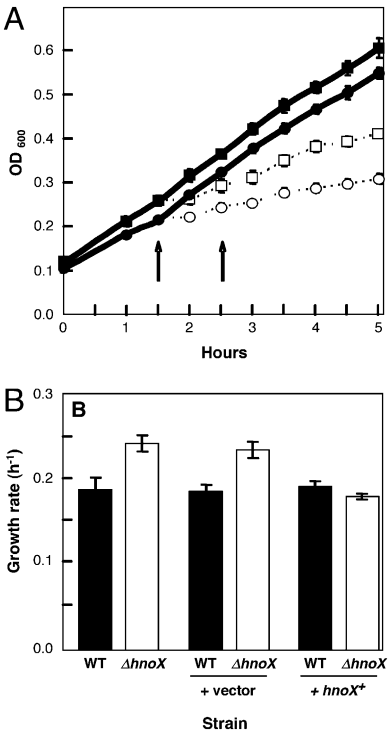

Fig. 3. Responses of wild-type *V. fischeri* and the Δ*hnoX* mutant to the addition of NO during the growth on hemin. (*A*) An iron-depleted, minimal-salts medium containing hemin (10 μM) as the sole iron source was inoculated with either the wild-type strain ES114 (circles) or the Δ*hnoX*-insertion mutant (squares), and growth was monitored by optical density (OD_{600}) at 28 °C with shaking. One-half of each culture (open symbols) was treated two times with the NO generator DEA-NONOate (arrows) as growth was monitored. Error bars indicate the SEM of triplicate cultures in a single experiment. Similar results were obtained in two other experiments. (*B*) The growth rates of the cultures in panel *A* were determined for the period between 2 and 5 h after inoculation in either the absence (solid bars) or presence (open bars) of DEA-NONOate. Growth rates were also determined for the wild type and Δ*hnoX* mutant, carrying either the plasmid vector (pVSV105) or the vector with an intact copy of the *hnoX* gene (pComhnoX; *hnoX*+). Plasmid carriage itself had no effect on the growth rates of either of the strains (Fig. S6). Error bars indicate the SEM of the mean growth rates calculated from three separate experiments; *t* test analysis indicated a significantly faster growth rate in the presence of NO for the Δ*hnoX* and Δ*hnoX* + vector.

0835) was neither under Fur control (Fig. 2) nor dependent on H-NOX_{Vf} (Table S2).

Deletion of the *hnoX* Gene Results in an Increased Symbiotic Competence. We reasoned that, during the colonization process, H-NOX_{Vf} serves as a sensor of host-derived NO for *V. fischeri*, and thus, loss of *hnoX* might give rise to symbiotic defects. However, the Δ*hnoX* mutant was actually more proficient in the initiation of colonization, particularly at a low inoculum density. Specifically, juveniles infected with the Δ*hnoX* mutant became luminescent sooner, reaching a higher maximum bioluminescence within 18 h, than did wild-type infected animals (Fig. 4*A*). Because the onset of luminescence is a good marker for colonization (20), the observation that the Δ*hnoX* mutant produces the same level of luminescence per cell as wild type in culture (Fig. S5) indicated that the mutant was more effective at colonizing. This greater degree of infectivity was tested by determining the inoculum dose that resulted in colonization of 50% of the animals (ID_{50}). In this assay, only about 125 cfu/mL of the Δ*hnoX* mutant were required to colonize one-half of a juvenile cohort, whereas the ID_{50} of the wild type was ~10-fold higher (Fig. 4*B*).

To better understand the nature of this increased efficiency, we performed competition experiments, which provided a direct measurement of the relative symbiotic proficiency of the wild-type and Δ*hnoX* mutant. When juvenile squids were inoculated with a mixed culture containing the two strains at a 1:1 ratio, the mutant outcompeted the wild type by an average of 16-fold (Fig. 4*C*); that is, after 24 h, the population of *V. fischeri* in the light organ was ~94% mutant. The competitive advantage of the Δ*hnoX* mutant diminished over the course of the next day, dropping to a 3-fold advantage (i.e., becoming 75% of the population) by 48 h post-inoculation. As predicted, the competitive advantage of the Δ*hnoX* mutant was reversed by the introduction of an intact copy of the *hnoX* gene, decreasing the relative competitiveness index (RCI) value at 48 h from 3.1 to 1.1 (Fig. 4*C*).

This result suggested that *V. fischeri* cells have an increased competence in initiating symbiotic colonization in the absence of H-NOX_{Vf}, the putative NO sensor. As described above, in the presence of NO, wild-type cells repress certain genes involved in iron acquisition, including those for hemin uptake and use. Thus, we reasoned that the competitive advantage of the Δ*hnoX* mutant might be, at least in part, caused by its greater ability for iron accumulation, perhaps in the form of host-derived hemin. Such a capability would be predicted to support a faster growth rate in the iron-limited tissues of the light organ. This hypothesis was tested by supplementing the seawater containing newly hatched squids with an excess of hemin (0.2 μM) or free iron (10 μM), thereby offsetting the mutant's putative advantage in scavenging low levels of this nutrient. Under either of these conditions of iron surplus, there was no longer evidence of a competitive advantage for the Δ*hnoX*

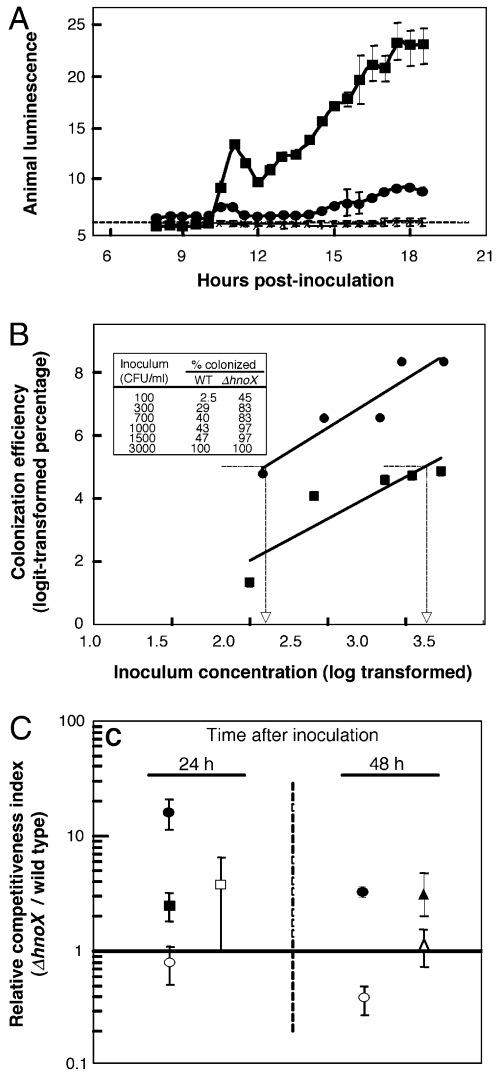

Fig. 4. Colonization by the *V. fischeri ΔhnoX* mutant. (*A*) Newly hatched juvenile squid ($n = 22$) were inoculated with either wild-type (circles) or mutant (squares) bacteria, and the appearance of luminescence was monitored periodically. Uninoculated squid (X) did not produce light above background (dashed line). One luminescence unit is equivalent to $\sim 4 \times 10^5$ quanta/sec. (*B*) Juvenile squid were exposed to different dosages (*Inset*) of either the wild-type (circles) or mutant (squares) bacteria. Colonization efficiency was determined by mathematically estimating the inoculation dosage at which 50% of the host animals became colonized (arrows) as indicated by the appearance of luminescence by 48 h (*Materials and Methods*). The r^2 values of both regressions (solid lines) were >0.8. (*C*) Juvenile squid ($n = 30$) were exposed to a mixed inoculum (~1:1) of the wild type and mutant at a total concentration of 3,000 cfu/mL. The ratio of the two strains in the light-organ population (relative competitive index) (39) at 24 and 48 h postinoculation was measured with no additions (closed circles), 0.2 μM hemin (closed square), 10 μM $FeCl_3$ (open circles), or 100 μM S-methyl-L-thiocitrulline (SMTC), an inhibitor of host NO synthase (2) (open squares) added at the time of inoculation. Open or closed triangles indicate carriage of either a complementing *hnoX*-encoding plasmid or the vector control, respectively.

mutant 24 h after inoculation (Fig. 4*C*). In fact, as time passed, the wild type had an advantage over the mutant in the presence of added iron; specifically, the RCI dropped significantly below 1 to a value of 0.4 by 48 h. To further test the hypothesis, we experimentally lowered the concentration of NO encountered by colonizing *V. fischeri* cells. *S*-methyl-L-thiocitrulline (SMTC) is a general NOS inhibitor, and therefore, the addition of SMTC during colonization would be expected to diminish the NO-induced suppression of hemin acquisition in the wild type and thereby, strengthen its symbiotic proficiency. Indeed, the addition of SMTC to the seawater increased the relative colonization competence of the wild type by 4-fold (Fig. 4*C*). It is worth noting that supplementation with either hemin or the NOS inhibitor did not compromise the squid's capacity to support normal levels of *V. fischeri* growth in the light organ (Fig. S7). Taken together, these results support the hypothesis that, during the colonization process, H-NOX_{Vf} senses host-derived NO, represses the bacterium's ability to acquire hemin, and suppresses rapid growth of the symbiont.

Discussion

Although well-characterized in vitro (7, 8, 21), the biological functions of the widely distributed bacterial H-NOX proteins in general and the NO-binding domain that they share with sGC in particular have remained an open question. Using the symbiosis between *V. fischeri* and the bobtail squid, we have established a connection between H-NOX_{Vf} and four bacterial activities: (*i*) sensing of environmental NO, (*ii*) transcriptional regulation of a set of genes, including those in the Fur regulon, (*iii*) suppression of the ability to use hemin, and (*iv*) modulation of the effectiveness of colonization. Thus, H-NOX_{Vf} couples the presence of a host-generated signal (NO) with an essential symbiont activity (colonization).

H-NOX_{Vf} Senses NO and Regulates the Cell's Iron Metabolism. H-NOX_{Vf} is not involved in regulating the bacterium's NO-detoxification capacity; nevertheless, it does influence the expression of *V. fischeri* genes associated with specific iron-acquisition proteins. Expression of the genes associated with the uptake of free (ferrous or ferric) iron is unaffected by H-NOX_{Vf}, suggesting that this is not the natural source of iron that limits growth in the light organ. Thus, it is not surprising that wild-type *V. fischeri* and an *hnoX* mutant grow equally well either in medium containing ferric iron as the source of iron or in the host when ferric iron is added in excess (Fig. 4*C*). In contrast, the transcription of genes encoding hemin transport is repressed in an NO- and H-NOX_{Vf}–dependent manner (Fig. S8). Thus, when growing in culture with hemin as the major iron source but only in the presence of NO, the absence of H-NOX_{Vf} regulation provides the *ΔhnoX* mutant with an advantage (Fig. 3*B*).

The mechanism or pathway underlying regulation of iron metabolism by H-NOX_{Vf} is unknown; nevertheless, because essentially all *V. fischeri* genes predicted to have a Fur box in their promoters are regulated by H-NOX_{Vf}–NO (Fig. 2), it is possible that this complex somehow targets the same regulon as this iron-binding, Fe-uptake regulator. NO has been reported to directly nitrosylate Fur-bound iron in *E. coli*, thereby abolishing its ability to repress transcription under iron-replete conditions (22, 23). However, the presence of NO produces the opposite effect in *V. fischeri*: a H-NOX–dependent increase in the repression of iron-uptake genes. Thus, *V. fischeri*, and perhaps other H-NOX–encoding bacteria, regulate iron metabolism by a mechanism not found in *E. coli*. We propose that this mechanism is a signal transduction pathway involving H-NOX_{Vf} and its associated HK, as well as an unknown response regulator (10), and it counters the effect of Fur nitrosylation.

Why Does the *hnoX* Mutant Dominate During Initiation of Symbiosis? The most striking finding in this study was the competitive advantage that a *V. fischeri ΔhnoX* mutant exhibits early in the colonization of the juvenile squid light organ (Fig. 4*C*). How might the presence of H-NOX_{Vf} provide an initial disadvantage to the symbiont but, eventually, prove advantageous?

During the onset of colonization by *V. fischeri*, the light organ is an iron-limited growth environment that requires the bacterium to scavenge this nutrient (24). However, although iron is essential for growth, a high concentration of free intracellular iron in the reducing environment of the cell can be toxic because of its role in generating hydroxyl radicals through the Fenton reaction. For this

reason, iron homeostasis is tightly regulated in bacteria (25). Fenton chemistry involves iron, H_2O_2, and a source of reducing equivalents. Evidence that *V. fischeri* encounters H_2O_2 during initiation of colonization includes the presence of halide peroxidase activity in the tissues of the light organ (14) and the colonization defect of a *V. fischeri katG* mutant, which was deficient in a periplasmic catalase (26). Thus, the role of H-NOX$_{Vf}$–NO may be to sense the light-organ environment, initially suppressing hemin accumulation (and growth rate) in the presence of host-generated oxidants. *V. fischeri* encounter NO even before they are exposed to host-derived oxidative stress in the ducts (12); hence, it is possible that these cells are primed for a subsequent exposure through the role of H-NOX$_{Vf}$ in NO sensing and down-regulation of iron accumulation. Then, 24–48 h later, when the presence of these oxidants has been reduced (2, 27), the symbionts would be depressed for hemin acquisition. In this way, whereas the *ΔhnoX* mutant might have an initial colonization advantage because of its ready accumulation of hemin, these bacteria could experience a higher mutation rate that eventually results in a loss of fitness. Such a scenario may explain, at least in part, why the RCI of the mutant dropped from 16- to 3-fold after the first 24 h post-inoculation (Fig. 4*C*). In the context of this hypothesis, it is interesting that two genes involved with *V. fischeri* cystine transport are also down-regulated 3- to 5-fold by H-NOX$_{Vf}$ in an NO-dependent manner (Table S2). An increased level of cystine would result in the generation of cysteine, which potentiates oxidative DNA damage in *E. coli* (28). Free cysteine, if present, would provide the essential source of reducing equivalents to drive DNA-damaging Fenton chemistry. As similar mechanisms of DNA damage are likely to apply in *V. fischeri*, a successful symbiont would be expected to initially maintain its concentrations of both cellular iron and cystine/cysteine low. As noted above, light-organ symbionts are provided with an abundance of host-derived amino acids (13); therefore, it is intriguing that genes associated with nitrogen starvation are also up-regulated in a NO- and H-NOX–dependent fashion (Table S2). Further investigation into the *V. fischeri* /squid symbiosis, as well as discovery of H-NOX/NO-regulated genes in other bacteria, will provide additional clues to indentify the diversity of biological functions performed by this family of proteins.

Conclusion. For bacteria to adapt to a symbiotic lifestyle, they must be able to correctly sense and successfully respond to the suite of host-derived signals that indicate they have arrived in their specific target tissue. *V. fischeri* apparently has adapted its homolog of the widely distributed family of NO-sensing H-NOX–domain proteins to such a role. Thus, the continued study of H-NOX$_{Vf}$ signaling in *V. fischeri* will provide a paradigm for both the regulatory mechanism of these proteins and their potential biological roles.

Materials and Methods

Bacterial Strains and Growth Conditions. The *hnoX*-insertion mutant (YLW1) was constructed by plasmid integration (29), and the *ΔhnoX* in-frame deletion mutant (YLW37) was constructed by allelic exchange (30) (Table S1). When exposed to NO, *V. fischeri* was grown in minimal salts (MS) medium, which contained (per liter) 500 mL 2× artificial seawater stock (31) supplemented with 1 mL 5.4% K_2HPO_4, 50 mL 1 M Tris-HCl buffer (pH 7.5), and 449 mL tap water. There was no significant loss in the viability of mutant or wild-type cultures when exposed up to 500 μM of the NO generator DEA-NONOate (Cayman Chemical) (Fig. S2); 10 mM *N*-acetyl-D-glucosamine (MP Biomedicals) served as the carbon and nitrogen source, and when desired, the iron in the medium was depleted by the addition of 50 μM of the deferrated iron chelator EDDHA (24).

Spectroscopic Characterization of H-NOX$_{Vf}$. Protein complexes were prepared as described previously (7, 16, 32, 33). Purified protein was desalted into the spectral buffer [50 mM triethanolamine (10), 50 mM NaCl at pH 7.5] using a PD10 desalting column (GE Healthcare) in an anaerobic glove bag. The protein was then oxidized with 20-fold molar excess of $K_3Fe(CN)_6$ and desalted to give the oxidized protein. Oxidized protein was reduced with a 50-fold excess of $Na_2S_2O_4$ and desalted to give the reduced complex. NO was added by providing a 10-fold excess of DEA-NONOate to the reduced-protein preparation, and the protein was desalted to give the ferrous–NO complex. The H-NOX$_{Vf}$ spectra were recorded at 20 °C on a Cary 3E spectrophotometer.

Expression Studies and Data Analysis. The wild type and *hnoX* mutant were freshly grown in LB-salt medium at 28 °C with an OD_{600} of 1.0. The precultures were diluted 1:100 and grown in MS medium to the early exponential phase (OD_{600} 0.2–0.3). One-half of each culture was exposed to DEA-NONOate (80 μM), which has a half-life of 16 min at 23 °C, and the other one-half of each culture was left untreated as control. After 30 min, cells from all of the cultures were fixed with RNAprotect Bacteria Reagent (Qiagen). Total RNA was isolated and purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Procedures for total RNA labeling and hybridization to the *V. fischeri* Affymetrix chip (34) were performed based on the protocols from *E. coli* Genome Project at the University of Wisconsin at Madison. Three independent experiments were carried out on separate days.

The microarray data were normalized using the RMAExpress version 1.0 software (http://rmaexpress.bmbolstad.com/) with background adjustment, quantile normalization (35), and the Probe Level Model summarization method (36). Genes differentially expressed between experimental treatments were detected with a false discovery rate of 0.05 using the *limma* package (37) in the R statistical environment (38). Data were deposited to GenBank under accession number GSE15522.

Growth Under Low Iron Conditions. *V. fischeri* were grown in MS medium supplemented with 50 μM deferrated ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid) and 10 μM hemin (Sigma-Aldrich). Cultures were continuously exposed to NO by supplementation with two dosages of 80 μM DEA-NONOate. Culture OD_{600} was recorded until stationary phase, and then, growth rates were calculated for the period between 2 and 5 h after NO exposure.

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