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**Vibrio fischeri** flavohaemoglobin protects against nitric oxide during initiation of the squid–**Vibrio** symbiosis

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**Summary**

Nitric oxide (NO) is implicated in a wide range of biological processes, including innate immunity against pathogens, signal transduction and protection against oxidative stress. However, its possible roles in beneficial host–microbe associations are less well recognized. During the early stages of the squid–**Vibrio** symbiosis, the bacterial symbiont **Vibrio fischeri** encounters host-derived NO, which has been hypothesized to serve as a specificity determinant. We demonstrate here that the flavohaemoglobin, Hmp, of **V. fischeri** protects against NO, both in culture and during colonization of the squid host. Transcriptional analyses indicate that hmp expression is highly responsive to NO, principally through the repressor, NsrR. Hmp protects **V. fischeri** from NO inhibition of aerobic respiration, and removes NO under both oxic and anoxic conditions. A Δhmp mutant of **V. fischeri** initiates squid colonization less effectively than wild type, but is rescued by the presence of an NO synthase inhibitor. The hmp promoter is activated during the initial stage of colonization, during which the Δhmp strain fails to form normalized aggregates of colonizing cells. Taken together, these results suggest that the sensing of host-derived NO by NsrR, and the subsequent removal of NO by Hmp, influence aggregate size and, thereby, **V. fischeri** colonization efficiency.

**Introduction**

Nitric oxide (NO) is a small, freely diffusible molecule that is implicated in a wide range of biological processes including innate immunity, signal transduction and protection against oxidative stress (Fang, 2004). Due to its reactivity towards diverse cellular constituents such as [Fe–S] clusters and haem, NO is used by many eukaryotes as an antimicrobial agent. In addition, when combined with reactive oxygen species (ROS), which are produced by NADPH oxidase during the ‘oxidative burst’, NO can also form other radicals of even greater cytotoxicity (Fang, 2004). Therefore, NO and ROS are regarded as early and effective host immune responses to invasive microorganisms. In contrast, at low (nM) concentrations, NO is an important signalling molecule that triggers specific physiological responses in mammalian cells, mainly by interacting with the haem-NO/Oxygen-binding (HNOX) domain of the soluble guanylate cyclase (sGC) (Cary et al., 2006). Similarly, in prokaryotes, low concentrations of NO are sensed by certain transcriptional regulators (e.g. NsrR and NorR), resulting in the initiation of specific cellular responses (Spiro, 2007). Moreover, the wide distribution of homologues of the eukaryotic HNOX domain among prokaryotic lineages (Iyer et al., 2003) suggests that NO sensing via HNOX proteins may be important in bacteria as well, including **Vibrio fischeri** (Wang et al., 2010).

Bacteria have evolved specific mechanisms to detoxify NO, whether it is endogenously generated during nitrite reduction, or exogenously imposed by host defences. In *Escherichia coli*, flavohaemoglobin (Hmp) detoxifies NO both aerobically as an NO dioxygenase or O₂ nitroxylase and, to a lesser extent, anaerobically as an NO reductase (Poole and Hughes, 2000). Hmp plays a critical role during pathogenesis, for example, in intraphagosomal survival and virulence of *Salmonella typhimurium* in mice (Stevanin et al., 2002; Bang et al., 2006), in protecting the respiration of *Erwinia chrysanthemi* from NO inhibition (Boccara et al., 2005), and in resistance of *Staphylococcus aureus* to host innate immunity (Richardson et al., 2006).

While hmp expression is generally upregulated by NO and nitrosating agents such as S-nitrosothiols, the underlying mechanism can vary. For instance, *E. coli*
NsrR, an [Fe–S]-containing regulatory protein, represses hmp transcription in the absence of NO (Spiro, 2007), while hmp expression in *Vibrio cholerae* is predicted to be regulated by NorR, a sigma 54-dependent activator (Rodionov et al., 2005). NO induces hmp expression either by attacking the [Fe–S] cluster in NsrR (Tucker et al., 2010), or nitrosylating the non-haem iron in NorR, which relieves intramolecular repression and leads to activation of gene expression (Arai et al., 2005; D’Autreaux et al., 2005). NorR can also activate the expression of genes encoding another important NO-detoxifying activity, the flavonubredoxin NorV (FiRD) and its redox partner NorW in *E. coli* (Gardner et al., 2002; Hutchings et al., 2002). Interestingly, cytochrome c nitrite reductase (NrfA), also works in combination with NorVV to protect *S. typhimurium* against NO killing in anoxic environments (Mills et al., 2008). Thus, synthesis of one or more of these different NO detoxification systems enables a bacterium to survive high concentrations of NO over a range of oxygen concentrations.

Recent studies have revealed the existence of functional commonalities in the mechanisms underlying pathogenic and beneficial host–microbe associations (Hooper et al., 2001; Koropatnick et al., 2004; Rawls et al., 2004). However, in contrast to the well-described role of NO in defending against pathogens, its function(s) in beneficial interactions are poorly understood. NO is known to be present in nodules of the model legume *Medicago truncatula* during its interactions with symbiont *Sinorhizobium meliloti* (Mathieu et al., 1998; Baudouin et al., 2006); however, only recently has an hmp mutant been shown to display a reduced nitrogen-fixation efficiency in planta (Meilhoc et al., 2010). The role(s) of NO in the symbiosis between the marine luminous bacterium *V. fischeri* and the Hawaiian bobtail squid *Euprymna scolopes* has been better described (Davidson et al., 2004; Wang et al., 2010). In this symbiosis, *V. fischeri* resides along the apical surfaces of epithelia lining the crypts of the host’s light-emitting organ, populating it as a monospecific culture. The symbiont’s bioluminescence is exploited by the squid in an anti-predatory behaviour called counterillumination; in return, the squid provides nutrients to the symbiont (Graf and Ruby, 1998; Wier et al., 2010). Because the nascent light organ of a newly hatched squid is free of symbionts, each generation of juveniles must become inoculated by *V. fischeri* present in the ambient seawater. During initiation of the symbiosis, *V. fischeri* cells gather as an aggregate in mucus shed by the epithelial surface of the organ. After 2–4 h, the symbionts disaggregate and migrate towards and into pores on the organ’s surface, moving through ducts to the deep crypts, where the symbiont population is established within 12 h (Nyholm and McFall-Ngai, 2004). At the aggregation stage, the bacteria are first exposed to a relatively low level of host-derived NO produced by vesicles embedded in the secreted mucus, while later they again encounter NO at higher levels as they traverse the light-organ ducts (Davidson et al., 2004), where ROS are also produced (Small and McFall-Ngai, 1999). Given its antimicrobial and signalling properties, this host-derived NO has been proposed to act as both a specificity determinant and a symbiotic signal (Nyholm and McFall-Ngai, 2004; Wang et al., 2010). Interestingly, while the location and relative intensity of NO exposure have been well described only during the first few hours to days of the symbiosis, the presence of NO synthase (NOS), and likely, NO in the symbiont-containing tissues continue throughout the life of the host (Davidson et al., 2004).

Analysis of the *V. fischeri* genome sequence (Ruby et al., 2005) has revealed the presence of genes encoding homologues of Hmp (VF_2316) and NorWV (VF_1782-1781), as well as the regulators NsrR (VF_2315) and NorR (VF_1783), and a bacterial homologue of HNOX (VF_A0071). Thus, we hypothesize that *V. fischeri* both senses and detoxifies host-derived NO encountered in the early stages of the symbiosis. Previously, we have established that the *V. fischeri* HNOX protein senses NO, differentially regulates the expression of the Fur regulon and modulates colonization proficiency (Wang et al., 2010). Here, we report Hmp is required for NO resistance both in pure culture and during animal colonization, specifically: (i) NO induces expression of *V. fischeri* hmp, mainly through NsrR, (ii) Hmp removes NO under both oxic and anoxic conditions, and (iii) hmp expression is activated at the aggregation stage of colonization, and the capacity to remove NO by Hmp controls the size and symbiotic efficiency of these bacterial aggregates.

**Results**

*V. fischeri* hmp expression responds to NO through its negative regulator NsrR

A previous whole-genome transcriptomic study showed that the *V. fischeri* hmp gene was upregulated >120-fold in response to NO (Wang et al., 2010). In the present study, when wild-type *V. fischeri* was exposed aerobically to 80 μM of the NO generator DEA-NONOate, hmp was induced >170-fold (Fig. 1A) as measured by quantitative real-time PCR (qRTPCR), confirming the previous study’s microarray result. An even stronger response (>300-fold) was observed under anoxic conditions, perhaps because the NO being generated was not scavenged by oxygen (Gilberthorpe et al., 2007). In contrast, only in the absence of oxygen did NO significantly induce the expression of norV (Fig. 1B).

A bioinformatic study of the *V. fischeri* genome sequence predicted the presence of a binding site for
Expression of hmp depends on NO under both aerobic and anaerobic conditions. Wild-type cells were grown in minimal-salts medium plus GlcNAc, either in the presence or in the absence of O₂. The relative expression levels (NO-treated/untreated) of either hmp or norV transcripts were determined by qRT-PCR. Data points are the means (± 1 SEM) calculated from three biological replicate experiments.

Based on the negative regulation of hmp by NsrR (Table 1), and its likely mechanism of NO-sensing (Tucker et al., 2010), we made two predictions: (i) pre-treatment of wild-type cells with a low dose (e.g. 40 μM) of NO will relieve hmp repression, thereby resulting in a greater resistance to subsequent NO challenge, and (ii) because hmp expression is constitutive in the ΔnsrR mutant (Table 1), this strain will be ‘blind’ to the pre-treatment, but still able to cope with the NO stress. As predicted, aerobically grown wild-type cells pre-treated with NO were resistant to NO challenge while, without the pre-treatment, they exhibited a short (approximately 1 h) delay before recovering (Fig. 2B). In contrast, the ΔnsrR mutant was resistant to NO challenge, even in the absence of pre-treatment (Fig. 2B). These data indicate that hmp expression levels correlate directly with the capacity for aerobic NO resistance.

Hmp ameliorates the toxic effects of NO under both oxic and anoxic culture conditions

Because hmp expression is highly responsive to NO, both in the presence and in the absence of oxygen, while norV was significantly induced only under anoxic conditions (Fig. 1), we asked what the physiological roles of the two systems were during NO stress. Four deletion mutants (ΔnsrR, Δhmp, ΔnorV and Δhmp-norV) had the same aerobic growth rate as wild type (data not shown). In contrast, the addition of an NO generator (100 μM DEA-NONOate) during early-exponential growth (OD₆₀₀ 0.2–0.4) resulted in an immediate growth arrest only in the Δhmp and Δhmp-norV strains (Fig. 2A). After 2 h, both these strains had recovered and began growing like wild type, suggesting that, at this concentration, the effect of NO is simply bacteriostatic. A determination of the total colony-forming units (cfu) of cultures during the NO exposure supported this conclusion (Fig. S1). Consistent with the previous observation that NorVW contributes only to anaerobic NO detoxification in E. coli and S. typhimurium (Gardner et al., 2002; Mills et al., 2008), an imposed aerobic NO challenge did not halt the growth of the V. fischeri ΔnorV mutant (Fig. 2A).

Based on the negative regulation of hmp by NsrR (Table 1), and its likely mechanism of NO-sensing (Tucker et al., 2010), we made two predictions: (i) pre-treatment of wild-type cells with a low dose (e.g. 40 μM) of NO will relieve hmp repression, thereby resulting in a greater resistance to subsequent NO challenge, and (ii) because hmp expression is constitutive in the ΔnsrR mutant (Table 1), this strain will be ‘blind’ to the pre-treatment, but still able to cope with the NO stress. As predicted, aerobically grown wild-type cells pre-treated with NO were resistant to NO challenge while, without the pre-treatment, they exhibited a short (approximately 1 h) delay before recovering (Fig. 2B). In contrast, the ΔnsrR mutant was resistant to NO challenge, even in the absence of pre-treatment (Fig. 2B). These data indicate that hmp expression levels correlate directly with the capacity for aerobic NO resistance.

No difference in anaerobic growth was observed with any of the deletion mutants in the absence of NO challenge (Fig. S2). However, in contrast to the growth profile obtained under oxic conditions (Fig. 2A), both the Δhmp and ΔnorV strains were arrested for 2 h after the challenge, and growth of the double mutant (Δhmp-norV) was even more severely inhibited by NO addition (Fig. 2C). The delayed recovery of the three mutants suggests that,
as with *E. coli* and *S. typhimurium* (Mills et al., 2008), in *V. fischeri* both Hmp and NorV contribute to removing NO under anoxic conditions. The protective effect of NO pre-treatment was also evident under anoxic growth conditions. In contrast to the 1.5 h lag observed in wild-type cells without pre-treatment, a pre-exposure to 5 μM DEA-NONOate, while initially leading to a partial growth retardation, did rescue cells from the strong growth inhibition caused by a subsequent, stronger NO challenge (Fig. 2D). This rescue probably results from the increased expression of *hmp* and *norV* (Fig. 1). Interestingly, in contrast to the full resistance seen under oxic conditions (Fig. 2B), the Δ*nsrR* mutant remained sensitive to an anaerobic challenge by NO (Fig. 2D). This observation suggests that Hmp itself, even when constitutively expressed, is not sufficient for removal of NO in the absence of oxygen. Thus, while Hmp may be the major NO-scavenging mechanism in the presence of oxygen, Hmp and NorV apparently work in combination under anoxic conditions.

**Hmp protects aerobic respiration from NO inhibition**

Owing to its reactivity with the haem groups of proteins, NO transiently and reversibly inhibits the activity of bacterial oxidases by competing with oxygen for binding (Stevanin et al., 2000; Borisov et al., 2004). To ask whether *V. fischeri* Hmp protects aerobic respiration from this inhibition, we determined the NO concentration that gave rise to half-maximal inhibition of oxygen consumption by cell suspensions (Fig. S3). For wild-type *V. fischeri*, this concentration was 2.4 ± 0.08 μM, while the Δ*hmp* strain (0.58 ± 0.02 μM) was fourfold more sensitive. This increased sensitivity, which is close to that reported for an *E. coli* hmp mutant (Stevanin et al., 2000), supports the conclusion that Hmp protects *V. fischeri* from NO-dependent inhibition of respiration.

We next compared the inhibitory effect of NO on respiration in wild-type, Δ*hmp*, Δ*norV*, Δ*hmp-norV* and Δ*nsrR* strains. Before exposure to NO, the wild-type, Δ*hmp* and Δ*nsrR* strains consumed oxygen at a similar rate.
V. fischeri NO protection in symbiosis

(Fig. 3A); however, the addition of NO immediately inhibited oxygen uptake by wild-type and Δhmp strains (traces 1 and 3, Fig. 3A). In contrast, respiration by the ΔnsrR strain, in which hmp expression is constitutive (Table 1), was resistant to NO exposure (trace 2, Fig. 3A). In addition, the period of inhibition for both the Δhmp and Δhmp-norV strains was fivefold longer than that of the wild-type and ΔnorV strains (Table 2), further implicating Hmp as the major contributor to NO removal under these conditions. Of note, when either wild-type or Δhmp cells carried pYLY29, which encodes a wild-type copy of hmp, they became hyper-resistant to NO inhibition (Table 2), possibly due either to the high (10–15) copy number of the plasmid or to NsrR titration by the native hmp promoter in the plasmid construct (Dunn et al., 2010). As expected, because hmp expression is highly responsive to NO (Fig. 1), pre-treatment of either wild-type or ΔnorV strains with NO resulted in loss of sensitivity of respiration to NO (trace 1, Fig. 3B; Table 2). Surprisingly, pre-treatment with NO allowed both the Δhmp and Δhmp-norV strains to continue to consume oxygen, albeit at a reduced rate (trace 3, Fig. 3B; Table 2). This intriguing result is consistent with NO-mediated induction (via NsrR) of the NO-resistant alternative oxidase (Dunn et al., 2010).

Hmp confers colonization efficiency in the squid–vibrio symbiosis

We hypothesized that Hmp and/or NorV serve to detoxify host-derived NO during the establishment of symbiosis by V. fischeri. Using inoculation conditions under which approximately 50% of juvenile squids were colonized by wild type, only 40%, 22% and 17% of the animals were colonized by the ΔnorV, Δhmp and Δhmp-norV strains respectively (Table 3), suggesting a lower colonization efficiency. In addition, the Δhmp-norV and Δhmp strains consistently took longer to initiate symbiotic bioluminescence and colonization compared with either the wild-type or ΔnorV strains (Table 3). Thus, while both Hmp and NorV may function in colonization, the contribution of Hmp appears more important. We further compared the infectivity of wild-type and Δhmp strains by determining the inoculum dose that resulted in colonization of 50% of the animals (ID50). In this assay, about 670 cfu ml\(^{-1}\) of wild type were required to colonize half of a cohort of squids, while the ID50 of Δhmp was approximately twofold higher (Fig. S4). Interestingly, the ΔnsrR mutant did not colonize more effectively than wild type in the assay (Table 3), suggesting that a constitutive expression of hmp early in colonization does not confer a significant colonization advantage.

The relative symbiotic proficiency of each of the mutants was also compared with wild type using a competitive-colonization assay. When juvenile squids were exposed to a two-strain inoculum, wild type out-competed the Δhmp or Δhmp-norV strains by three- to fourfold (Table 3). In the natural environment, where juvenile squids are likely to be colonized by only one or a few V. fischeri cells (Lee and Ruby, 1994; Wollenberg and Ruby, 2009), a three- to fourfold advantage would result in a significant increase in fitness over time. Just as the ΔnsrR strain did not show

### Table 2. Extent of inhibition of oxygen consumption by NO challenge.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Without pre-treatment</th>
<th>With NO pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.1 ± 0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Δhmp</td>
<td>14.9 ± 0.5</td>
<td>Partial inhibition†</td>
</tr>
<tr>
<td>ΔnorV</td>
<td>3.1 ± 0.1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Δhmp-norV</td>
<td>15.3 ± 0.1</td>
<td>Partial inhibition</td>
</tr>
<tr>
<td>ΔnsrR</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>WT-pVSV105</td>
<td>3.8 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>WT-pYLY29</td>
<td>&lt;0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Δhmp-pVSV105</td>
<td>16.3 ± 1.3</td>
<td>ND</td>
</tr>
<tr>
<td>Δhmp-pYLY29</td>
<td>&lt;0.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

a. Mean (± SEM) extent of inhibition by NO challenge (from three repeated experiments); wild type (WT) and mutant strain were each tested either after receiving an NO pre-treatment or after no pre-treatment.

b. Partial inhibition means that oxygen consumption was not inhibited, but continued at a reduced linear rate.

ND, not determined.
higher efficiency in the single-strain colonization, it also did not out-compete wild type (Table 3, Fig. S5). If the diminished competitiveness of the Δhmp or Δhmp-norV strains is due to their reduced ability to withstand host-derived NO stress, we reasoned that decreasing host-derived NO production would protect these two mutants. This hypothesis was tested by performing competition experiments in the presence of S-methyl-L-thiocitrulline (SMTC), a nitric-oxide synthase (NOS) inhibitor that effectively reduces NO levels in the light organ of juvenile squid (M. Altura, pers. comm.). Consistent with the prediction, SMTC treatment enhanced the relative colonization competence of the two mutants (Table 3). Similarly, genetic complementation of the Δhmp strain with a wild-type copy of hmp (on pYLW29) also reversed its colonization defect (data not shown). Thus, the ability to remove NO stress during the establishment of the symbiosis contributes to colonization efficiency, and is evident within the first 24 h.

The hmp promoter responds to NO in host mucus through NsrR

Because hmp expression increases in response to NO in culture (Fig. 1), we asked whether the bacteria encounter sufficient NO in the mucus outside the light organ to activate this response during the initial stage of the colonization. We introduced a plasmid (pYLW45) containing: (i) the hmp promoter fused to a promoterless gfp, and (ii) a constitutively expressed rfp, into both the wild-type and the ΔnsrR strains. The appearance of fluorescence in these strains was monitored by a micropipette reader and laser-scanning confocal microscopy. Cells were monitored first in seawater to determine whether the constructs would faithfully report the presence of NO, and then in mucus as the bacteria initiated symbiosis. In the wild type containing pYLW45, GFP expression was detected when 10–100 μM DEA-NONOate was added to seawater, with the response peaking between 2.5 and 3 h (Fig. S6), consistent with the reported maturation time of GFP (Ward, 2005). In the ΔnsrR mutant suspended in seawater, the gfp reporter was constitutively expressed, and at higher levels than those observed in wild type (data not shown).

When observed by confocal microscopy, significantly higher GFP fluorescence in wild-type cells was detected when the NO generator DEA-NONOate was added to seawater (Fig. 4). As predicted, the reporter was constitutively expressed in ΔnsrR cells, irrespective of the presence or absence of the NO generator (Fig. 4). When the two strains carried the vector control (pVSV209) instead, only RFP was detected (data not shown).

Similarly, hmp promoter activity was induced as the wild-type V. fischeri cells initially encountered juvenile squid (Fig. 4). Within the first 3–4 h of aggregating in host mucus, wild-type cells carrying pYLW45 began producing GFP, and the fluorescence intensity appeared to be at a similar level as that observed in the same strain after exposure to NO in seawater. Again, the reporter gfp was expressed at high levels in ΔnsrR cells, regardless of the test conditions (Fig. 4). In aggregates formed by the wild-type and ΔnsrR strains carrying the vector control (pVSV209), only RFP fluorescence was detected (data not shown). Thus, the hmp promoter of V. fischeri is activated during the aggregation stage of colonization, and NsrR is involved in its de-repression. Of note, pre-treating squids with 100 μM of the NOS inhibitor SMTC lowered the GFP intensity in wild-type cells, although not significantly, perhaps because the treatment did not lower the concentration of NO below the sensing sensitivity level of NsrR.

### Table 3. Colonization characteristics of mutants defective in NO detoxification.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage of squids becoming luminous</th>
<th>Hours until colonization detected</th>
<th>Competitive colonization as RCI&lt;sup&gt;a&lt;/sup&gt; (WT/mutant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No treatment</td>
</tr>
<tr>
<td>WT</td>
<td>50 ± 15</td>
<td>9.3 ± 0.1</td>
<td>--</td>
</tr>
<tr>
<td>ΔnorV</td>
<td>40 ± 5</td>
<td>9.7 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Δhmp</td>
<td>22 ± 5</td>
<td>10.2 ± 0.2</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>Δhmp-norV</td>
<td>17 ± 3</td>
<td>12.0 ± 1.2</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>ΔnsrR</td>
<td>60 ± 20</td>
<td>9.6 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

a. More effective colonization is indicated by a higher percentage of squids becoming luminous at 24 h, and by a more rapid onset of colonization as indicated by onset of detectable squid bioluminescence; values that are significantly different from wild type (WT) are in bold font; each experiment was repeated two to three times with similar results.

b. A relative colonization index (RCI) > 1.0 indicates that the WT outcompetes the mutant after 24 h; RCI values that are significantly different from 1.0 are in bold font; each experiment was repeated at least three times with similar results. Mean RCI (± 1 SEM) from the three experiments is shown.

c. Addition of the NOS inhibitor S-methyl-L-thiocitrulline (SMTC).

ND, not determined.
The $hmp$ promoter responds to host-derived NO during the aggregation stage of the symbiosis. Cells of wild type (black bars) or $\Delta nsrR$ (white bars), carrying the $hmp$ promoter-reporter plasmid pYLVW45, were added to seawater, or used to inoculate newly hatched squids. To induce the $hmp$ promoter in the seawater experiment, 80 $\mu$M DEA-NONOate was added to seawater. To lower the intrinsic level of NO production in the mucus, some squids were pre-treated with 100 $\mu$M NOS inhibitor SMTC for 2–3 h before inoculation with $V. fischeri$. Between 3 and 4 h after inoculation, the levels of induced GFP fluorescence and constitutively expressed RFP fluorescence in both strains were monitored using confocal microscopy. The ratio of GFP/RFP fluorescence generated under different conditions was calculated. Significant differences between the ratio found in seawater and the other treatments are indicated: * $P < 0.05$; ** $P < 0.01$. The experiments were repeated three times with similar results. Data points are means ($\pm$ 1 SEM) calculated from three biological replicates.

**Discussion**

In this study, we have used the squid–vibrio symbiosis to establish that: (i) $V. fischeri$ Hmp is an NO-inducible protein that confers protection against NO both in vitro and during host colonization, (ii) NsrR is the major NO-responsive regulator of $hmp$ expression, and (iii) early during symbiosis, exposure to NO in host mucus serves as a signal that may prepare the colonizing bacteria for a stronger NO stress encountered as the symbiont migrates through the light-organ duct (Davidson *et al*., 2004). To our knowledge, this is the first report of the physiological function of Hmp in the Vibrionaceae; more significantly, it demonstrates a novel process by which a bacterial symbiont both responds to and copes with NO during its initial contact with the host.

Genes encoding NO-detoxification systems are widely distributed among bacteria (Rodionov *et al*., 2005), and allow the cells to survive NO that is either endogenously generated, or imposed externally by host tissues. Interestingly, while the organization of the genes encoding the major NO-detoxification systems (i.e. $hmp$ and norVW) is conserved among Vibrio species, there are apparent differences in the architecture of their regulatory networks. For instance, in $V. cholerae$, $hmp$ expression is predicted to be controlled by the positive regulator NorR, but not by the repressor NsrR (Rodionov *et al*., 2005). Thus, within the Vibrionaceae, the induction of $hmp$ expression by NO may involve different regulators, indicating the need to look beyond the well-studied enteric bacteria for novel regulatory mechanisms. For example, $hmp$ was recently reported to be a novel target for HapR regulation in $V. cholerae$ (Tsou *et al*., 2009), suggesting that quorum sensing may play an unanticipated role in this bacterium’s NO-stress response.

The $hmp$ promoter region of $V. fischeri$ has predicted binding sites both for NsrR, the repressor considered in this study, and for NarP (Rodionov *et al*., 2005). NarQP is
a two-component sensor regulator that senses and responds to the presence of nitrate and nitrite in *E. coli* (Stewart, 2003). While it is not known whether *V. fischeri* encounters these anionic forms of nitrogen during the initiation of colonization, after the association is established, the symbionts apparently generate energy by anaerobic nitrite respiration as they proliferate in the light-organ crypts (Wier et al., 2010). Interestingly, *hmp* expression in *V. fischeri* was upregulated about 10-fold during nitrate respiration (J. Schwartzman, pers. comm.), possibly through NarQP modulation or NsrR that senses NO from nitrate metabolism. It is tempting to hypothesize that NarQP-mediated *hmp* activation prepares the bacterium for detoxification of any endogenously produced NO during its respiration of nitrate.

Because cytochrome *c* nitrite reductase (Nrf) has been implicated in the respiratory detoxification of NO by *E. coli* and *S. typhimurium* (Poock et al., 2002; Mills et al., 2008), we also asked whether the *V. fischeri* Nrf plays a similar role. Mutants deleted for the entire *nrf* operon (VF_1548-VF_1555) alone, or in combination with either *hmp* or *norV*, were constructed. Interestingly, the Δ*nrf* strain behaved similarly to wild type in the anoxic challenge experiment described above, while the Δ*hmp-nrf* and Δ*norV-nrf* strains reacted like the Δ*hmp* and Δ*norV* mutants respectively (data not shown). It is possible that the presence of at least one NO-detoxification system (i.e. Hmp or NorV) masked the activity of Nrf as an NO reductase in *V. fischeri*; alternatively, *nrf* may simply not be expressed under the growth conditions used. In any case, our inability to obtain a Δ*hmp-norV-nrf* triple mutant (even in the absence of added NO) may indicate the need by *V. fischeri* to maintain at least one intact mechanism for response to NO stress.

Apart from *hmp*, two other genes, *nnrS* and *aox*, are predicted to belong to the NsrR regulon in *V. fischeri* (Rodionov et al., 2005). NnrS is believed to be involved in denitrification (Bartnikas et al., 2002), although its physiological role remains unknown. Recently we reported that *Aox*, the alternative oxidase, is an NO-resistant oxidase in *V. fischeri* (Dunn et al., 2010). Not surprisingly, in wild-type cells, the expression of *aox*, like *hmp*, is highly upregulated in response to NO (Dunn et al., 2010; Wang et al., 2010). Thus, we predicted that the ability of NO pre-treatment to protect respiration in both Δ*hmp* and Δ*hmp-norV* strains (Fig. 3B; Table 3) was due to...
de-repression of aox. Indeed, while the Δhmp strain was only partially inhibited by NO after pre-treatment, oxygen consumption by a Δhmp-aox mutant ceased completely for about 9–10 min before returning to normal (data not shown). This observation suggests that Aox continues to consume oxygen in the presence of NO (trace 3, Fig. 3B; Dunn et al., 2010), supporting its role as an inducible NO-resistant oxidase.

Because NsrR represses two enzymes that provide resistance to NO (Dunn et al., 2010; Wang et al., 2010), and the ΔnsrR strain appeared to be more resistant to NO challenge under culture conditions, we were surprised to find that the ΔnsrR strain did not out-compete wild-type bacteria in a competitive colonization of juvenile squid. One possible explanation is that the ΔnsrR strain becomes more sensitive to the oxidative stress (Gilberthorpe et al., 2007) that is encountered during the establishment of symbiosis (Weis et al., 1996; Visick and Ruby, 1998; Small and McFall-Ngai, 1999). Specifically, although Hmp plays an important role in NO resistance, it is also a potent generator of ROS such as superoxide anion (O2−) (Bang et al., 2006; Gilberthorpe et al., 2007). Thus, an Hmp-over-producing strain like ΔnsrR might be more susceptible to oxidative killing, either directly by O2− or its derivative hydrogen peroxide (H2O2), or indirectly by providing H2O2 for halide peroxidase-mediated synthesis of highly toxic hypochlorous acid (Small and McFall-Ngai, 1999). Thus, although the V. fischeri ΔnsrR strain has an increased resistance to host-derived NO stress, it may be exposed to a more severe oxidative killing by H2O2 and hypochlorous acid produced by the duct epithelium of the light organ (Weis et al., 1996).

In the first few hours of the squid–vibrio symbiosis, where both host-derived NO and a respiratory burst are simultaneously present (Davidson et al., 2004; Small and McFall-Ngai, 1999), NO may serve not only as a specificity determinant, but also as an inducer of oxidative-stress protection in the symbiont. For instance, in response to NO induction, V. fischeri suppresses both iron uptake/utilization and cystine transport, probably to limit potential oxidative damage (Wang et al., 2010).

Because the presence of NO in the light organ tissues continues throughout the life of the host (Davidson et al., 2004; Wier et al., 2010), we are currently developing techniques for raising juvenile squids to adulthood, allowing an evaluation of the long-term role of V. fischeri Hmp. Based on the studies described here, we propose the following model for NO-signalling during the initiation of symbiosis: as they first encounter NO during their aggregation outside the light organ, V. fischeri cells de-repress hmp, thereby preparing themselves to survive the high concentrations of NO encountered during their subsequent migration along the ducts that lead to the light organ’s crypts (Davidson et al., 2004). At the same time, exposure to NO present in the mucus activates HNO signalling, temporarily suppressing the iron uptake/utilization capacity of the bacteria (Wang et al., 2010) until after the host’s ROS production has been curtailed (Small-Howard, 2004). Safely within the crypts, the symbiont population reaches a high enough density that it induces luminescence, lowers oxygen tension, and reduces host-produced NO via anaerobic detoxification mechanisms such as Hmp and NorVV. Thus, the initial exposure to NO in the mucus apparently serves as a ‘pre-treatment’ that results in better survival of the oxidative stress conditions within the ducts. In this way, host-derived NO may serve as a signal that is exploited by V. fischeri to help it endure the hostile conditions (both NO and oxidative stress) encountered during the establishment of its symbiosis.

**Experimental procedures**

**Strains, media and growth conditions**

The strains, plasmids and PCR primers used in this study are listed in Table S1. Cultures of *E. coli* were incubated at 37°C in either LB (Miller, 1992) or BHI (Difco Laboratories) media. When needed, chloramphenicol (Cam) and kanamycin (Kan) were added to LB medium at 20 and 50 μg ml−1 respectively. Erythromycin (Erm) was added to BHI medium at 150 μg ml−1. Cultures of *V. fischeri* were grown at 28°C in LBS medium (Bose et al., 2007), solidified with 1.5% (w/v) Bacto-Agar (Difco Laboratories) as required. Cam, Erm and Kan were added to LBS at 5, 5 and 100 μg ml−1 respectively. Before exposure to NO, *V. fischeri* cells were grown in a minimal-salts medium that contained (per litre) 500 ml of 2× artificial seawater stock (Boettcher and Ruby, 1990) supplemented with 1 ml of 5.4% K2HPO4, 50 ml of 1 M Tris-HCl buffer (pH 7.5) and 449 ml of tap water. Ten-millimolar NONOate (Cayman Chemicals) were reconstituted in 10 mM Tris-HCl buffer and equilibrated with argon for five cycles. Anaerobic cultures were incubated statically at 28°C. Stock solutions of the NO donors DEA-NONOate and Proli-NONOate (Cayman Chemicals) were reconstituted in 10 mM NaOH just before use. At 25°C and pH 7.4, the two NO generators release 1.5 and 2.0 equivalents of NO, and have half-lives of 16 min and 5 s respectively.

**Genetic manipulations**

The deletion mutants used in this study were constructed by allelic exchange as previously described (Dunn and Stabb, 2008). Briefly, approximately 1.5 kb of DNA upstream of the start codon of the gene to be deleted was PCR-amplified and fused to an approximately 1.5 kb DNA fragment downstream of the stop codon using an engineered restriction site (Nhel). This procedure resulted in the replacement of the gene with a 6 bp restriction-enzyme recognition site between the start and stop codons. The hmp promoter-reporter plasmid

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(pYLW45) was made by cloning approximately 300 bp of DNA upstream of the Hmp start codon into pVSV209 (Table S1) at the Sall–Avrl restriction sites, generating a transcriptional promoter fusion to a promoterless gfp. We constructed the hmp-complementing plasmid (pYLW29) by cloning the wild-type DNA fragment that encompasses 300 bp upstream of the start codon of Hmp and 20 bp downstream of the stop codon into pVSV105 at the XbaI–SacI restriction sites. Plasmids containing the RP4 origin of transfer were introduced into V. fischeri isolates using tri-parental mating as previously described (Stubb and Ruby, 2002).

Quantitative real-time PCR (qRT-PCR)

Vibrio fischeri strains were grown by shaking at 28°C in LBS medium to an optical density at 600 nm (OD600) of approximately 0.8, as measured with a Biophotometer (Eppendorf). Twenty microlitres of the culture was subcultured into 20 ml of mineral-salts medium containing GlcNAc in a 125 ml Erlenmeyer flask. This culture was grown to an OD600 of approximately 0.8, as measured with a Biophotometer (Eppendorf).

NO inhibition of oxygen consumption

A fresh culture (3 ml) of each V. fischeri strain was grown in LBS medium to an OD600 of approximately 0.8 with shaking at 28°C. Thirty microlitres of this pre-culture was subcultured into 30 ml of mineral-salts medium containing GlcNAc in a 250 ml Erlenmeyer flask. Cells in the early exponential phase (OD600 ~ 0.3) were harvested at 6000 r.p.m. for 10 min at 4°C. The pellet was washed and resuspended in about 500 μl of modified HEPES buffer (50 mM HEPES, pH 7.4, 300 mM NaCl, 5 mM KCl and 1 mM each of CaCl2, MgCl2, NaH2PO4, and d-glucose) (Stevanin et al., 2000). The final volume of the buffer was adjusted to assure that the suspension was of approximately the same cell density for all of the test strains.

Oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England). Specifically, 50 μl of a cell suspension were mixed with 650 μl of modified HEPES buffer and maintained at 25°C in a water-jacketed electrode chamber. Each assay contained approximately 2.8 mg (dry weight) of cells. The sensitivity of E. coli respiration to NO depends on both NO concentration and the oxygen tension at which the NO is added (Stevanin et al., 2000). Therefore, we ran pilot experiments on wild-type V. fischeri cells to determine the optimal testing conditions. In line with previous studies (Stevanin et al., 2000), NO (released from Proli-NONOate; final concentration 5.5 μM) was injected into a culture of respiring cells in the electrode chamber when the oxygen tension was between 40 and 200 μM. The rate of oxygen consumption was measured immediately before and after NO addition. We found that the degree of inhibition was strongly dependent on the oxygen concentration at the time the NO was added: at a higher concentration, the inhibitory effects were less severe (data not shown). We chose to run experiments at an oxygen concentration of 80 μM, where a clear inhibitory effect occurred when the NO generator was added; however, once the inhibition was reversed, oxygen consumption resumed.

The fractional inhibition of respiration (I) was calculated according to the formula

\[ I = 1 - \left( \frac{O_2}{O_2 \text{ consumption rate after NO addition/rate before addition}} \right) \]

I was plotted against NO concentration and, using Kaleidagraph (Synergy Software), the data were fit to the Hill equation:

\[ I = \frac{[\text{NO}]^n}{K + [\text{NO}]^n} \]

where K is the concentration of NO that gives rise to half-maximal inhibition, and n is the Hill coefficient. When respiration was completely inhibited by NO (i.e. there was no further change in the oxygen concentration after NO addition), the length of inhibition was calculated as the period between NO addition and the point obtained by extrapolation.
of the residual respiration rate after inhibition is relieved (Stevanin et al., 2000). If oxygen consumption still continued after NO addition, but only at a reduced rate, it was recorded as ‘partial inhibition’. To test the protective effects of pre-treating the cells with NO, cultures were exposed to 80 μM DEA-NONOate for 30 min before collection for respiration measurements.

Single-strain colonization assay

Approximately 22 newly hatched juvenile squids were inoculated with ~1500 cells of the different V. fischeri strains per ml. After a 3 h exposure time, each squid was transferred to 4 ml of filter-sterilized seawater (FSW) in individual scintillation vials. The onset of bioluminescence was monitored in an LKB scintillation counter (PerkinElmer), modified to operate as an automated photometer. Aposymbiotic animals, which were never exposed to any V. fischeri, were included as negative controls. Twenty-four hours post-inoculation, the percentage of luminous squids, and the time each strain took to initiate luminescence, were calculated: the onset of luminescence has been shown to be a good marker for colonization (Ruby and Asato, 1993). Wild-type and mutant strains used in this study produced the same level of luminescence per cell in culture (data not shown).

The minimal infection dose (MID) was determined as previously described (McCann et al., 2003). To calculate the efficiency of infection (ID50), we determined the percentage of animals that became colonized as a function of the inoculum dose. In these calculations, we assumed that the log inoculum is paired with the log of percent of animals colonized, and applied the log-logistic model to estimate the ID50 values of the wild-type and Δhmp strains.

Competitive colonization assay

Competition experiments between co-inoculated strains were carried out as previously described (Dunn and Stabb, 2008). Wild-type and mutant strains were marked with a stable plasmid carrying either the gfp (pVS102) or rfp (pVS102) gene (Table S1). The ratio of cfu in the mixed inoculum could be easily determined by the colour of the colonies after 48 h. The relative competitiveness index (RCI) was calculated by dividing the ratio of the cfu of wild type to the mutant in each light organ by the ratio of these strains in the initial inoculum. An RCI > 1 indicates that wild type out-competes the mutant in the colonization of host animals. In each experiment, approximately 30 squids were inoculated, and subsequently scored for bioluminescence as an indicator for colonization at 24 h post-inoculation. Only colonized animals were sacrificed for RCI determination. When desired, 100 μM of the NOS inhibitor SMTC was added to the seawater throughout the experiments. Supplementation with SMTC did not compromise the host’s capacity to support normal levels of symbiont growth in the light organ (data not shown).

Induction of the hmp promoter in NO-supplemented seawater and during symbiotic aggregation

To determine whether the V. fischeri hmp promoter responds to exogenously added NO in seawater, cultures of either the wild-type or ΔntrR strains (~10⁶ cfu ml⁻¹), carrying either pYLW45 or pVSV209, were exposed to between 10 nM and 100 μM DEA-NONOate. Triplicate 100 μl samples of the NO-treated cell suspensions were aliquoted into a 96-well microplate, and monitored every 30 min for optical density and GFP fluorescence intensity using a microplate reader (Tecan Group, Männedorf, Switzerland). Between each data collection point, the plate was incubated at 28°C with shaking. When either strain carried the vector control containing the promoterless gfp (pVSV209), no significant GFP signal was detected. The final level of GFP fluorescence was obtained by first subtracting this vector-control fluorescence (background), and then normalizing by cell density (OD₆₀₀).

Either wild-type or ΔntrR cells, each carrying pYLW45, were used to inoculate between 5 and 10 newly hatched juvenile squids at 10⁶ cfu ml⁻¹. The level of Induction of the hmp promoter, and the resultant GFP signal, were determined during aggregation as follows. At between 1.5 and 4.5 h post-inoculation, squid were washed three times in FSW, and anaesthetized in a 1:1 solution of 7.5% MgCl₂ in FSW (Nyholm et al., 2000). After dissection, fluorescent emission was visualized by LSM 510 confocal laser-scanning microscope (Carl Zeiss Microimaging). As a control, cells were inoculated into FSW (10⁶ cfu ml⁻¹), induced by the addition of 80 μM DEA-NONOate and, after 2–3 h, a 3 μl sample was examined by confocal microscopy. Cells that did not receive any treatment were also examined. The intensity of fluorescence of GFP and constitutively expressed RFP in the aggregating cells was recorded and extracted by the computer software (Zeiss LSM 510 ver. 4.20). Comparisons between the test conditions and the seawater control were performed using Student’s t-test. The statistical analysis was made with Excel software.

Observation of aggregates in the mucus

Five to 10 newly hatched juvenile squids were inoculated with approximately 10⁶ cfu (per ml) of V. fischeri strains that were marked with constitutively expressed gfp (pVS102) or rfp (pVS102) to allow their visualization by confocal microscopy during the initiation of colonization. Samples were processed as described above, except that squids were stained with 1 μM CellTracker Orange CMRA (Molecular Probes, Invitrogen) and 10 μg ml⁻¹ Wheat Germ Agglutinin Alexa Fluor 633 conjugant (WGA-633) (Molecular Probes, Invitrogen) for 20 min before examination. After dissection, squid tissue, secreted mucus and the aggregates formed by V. fischeri were observed. Images were recorded by the Axioplan 2 Imaging system attached to the scope. To study the effects of NOS inhibitor on aggregate size, squids were treated with 100 μM of the NOS inhibitor SMTC for 2–3 h before exposure to V. fischeri strains. After inoculation, the presence of SMTC was maintained by addition of the inhibitor to the inoculated seawater. For competitive colonization experiments, juvenile squid were co-inoculated with a 1:1 ratio of differentially marked wild-type and mutant strains at a final concentration of 10⁶ cfu ml⁻¹. All other procedures were performed as described above.
Acknowledgements

We thank Elizabeth Heath-Heckman for help and advice with the confocal microscopy and data analysis of GFP intensity, and Spencer Nyholm for confocal images. This work was supported by National Science Foundation Grant IOS-0817232 (M.M.-N. and E.G.R.) and National Institutes of Health Grant R01 RR 12294 (E.G.R. and M.M.-N.), NSF Grant MCB-0702858 (S.S.), and NSF Grant MCB-0803181 and the University of Oklahoma (A.K.D.).

References


Supporting information

Additional supporting information may be found in the online version of this article.

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Table S1. Strains, plasmids and primers used in this study

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YLW48  ES114 carrying pYLW45, KanR and promoterless CmR
YLW42  ES114 carrying pYLW29, CamR
YLW40  ES114 carrying pVSV209, KanR and promoterless CmR
YLW38  ES114ΔnorV
YLW25  ES114 carrying pVSV105, CamR
YLW20  ES114Δhmp
YLW117 ES114Δhmp-norV carrying pVSV102, KanR
YLW116 ES114Δhmp-norV carrying pVSV208, CmR
YLW113 ES114Δhmp-norV

**Plasmids**

pAKD711  ΔnsrR allele, R6Kγ and ColE1 replication origins, RP4 oriT, ermR, kanR
           (Dunn et al., 2010)

pCR-BluntII TOPO PCR-cloning vector, KanR
           Invitrogen Inc.

pEV5104  oriV<sub>R6Kγ</sub>, ori<sub>RP4</sub> RP4-derived conjugative helper plasmid, KanR
           (Stabb, 2002)

pVSV102  oriV<sub>R6Kγ</sub>, ori<sub>RP4</sub> oriV<sub>pES213</sub>, Dsgfp-tagged, KanR,
           (Dunn et al., 2006)

pVSV105  oriV<sub>R6Kγ</sub>, ori<sub>RP4</sub> oriV<sub>pES213</sub>, complementation vector,
           CamR
           (Dunn et al., 2006)

pVSV208  oriV<sub>R6Kγ</sub>, ori<sub>RP4</sub> oriV<sub>pES213</sub>, rfp-tagged, CamR,
           (Dunn et al., 2006)

pVSV209  oriV<sub>R6Kγ</sub>, ori<sub>RP4</sub> oriV<sub>pES213</sub>, KanR-constitutively expressed rfp, transcriptional terminators-(AvrII, Sall, StuI)-promoterless CmR and gfp
           (Dunn et al., 2006)

pYLW20  Δhmp allele, R6Kγ and ColE1 replication origins, RP4 oriT, ermR, kanR
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**Primers**

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*Abbreviations used: CamR, chloramphenicol resistance; ErmR, erythromycin resistance; KanR, kanamycin resistance; kb, kilobase; gfp, green fluorescent protein gene; rfp, red fluorescent protein gene.

†Sequences in underlined italics indicate the restriction-enzyme recognition sites introduced into primers (XbaI, TCTAGA; SacI, GAGCTC; AvrII, CCTAGG; StuI, AGGCCT).
Fig. S1.
Fig. S2.
Fig. S3.
Fig. S4.
Fig. S5.
Fig. S6.
SUPPLEMENTAL DATA

Legends for supplementary figures

**Fig. S1.** NO exerts bacteriostatic effects on the growth of *V. fischeri*. Wild type (WT) and the Δhmp mutant were grown in minimal-salts medium plus GlcNAc, either in the presence or absence of an NO generator (100 μM DEA-NONOate). OD_{600} (A) and viable colony forming units (CFU) of the cultures (B) were monitored over time.

**Fig. S2.** Growth under anoxic conditions without NO challenge. Wild-type (WT) and four mutant strains were grown in minimal-salts medium plus GlcNAc. Growth was measured as OD_{600}.

**Fig. S3.** NO inhibition of oxygen consumption. The fractional inhibition of oxygen consumption of wild-type (open squares) and Δhmp (filled squares) strains was calculated from the steady-state oxygen consumption rate immediately after NO addition, relative to the rate immediately before addition. The NO donor, Proli-NONOate, was added at several concentrations. The data were fit to the Hill equation, from which an estimate of the NO concentration required for half-maximal inhibition was derived. The experiment was repeated three times with similar results, and one representative data set is shown here.

**Fig. S4.** The minimal infection dose (ID_{50}) of wild-type (WT) and Δhmp strains. Newly hatched juvenile squids were exposed to between approximately 500 and 3000 CFU (per ml) of either WT (circles) or Δhmp (squares), and the percentage of animals colonized was determined after 24 hrs. Colonization efficiency was estimated as the inoculation concentration at which 50% of the animals became colonized (dashed lines). The R^2 values of both regressions (solid lines) were > 0.8.

**Fig. S5.** Colonization after a mixed inoculation with wild-type (WT) and ΔnsrR strains. Newly hatched juvenile squids were exposed to a mixture of WT and ΔnsrR at a ratio of
approximately 1:1 (total dose: 3000 CFU/ml). The relative competitive index (RCI) was determined 24 hrs post-inoculation for each animal (circles). Dominance of the wild type over the mutant is indicated by an RCI value >1. The experiment was repeated three times with similar results. The mean value and 1 SEM are indicated. One representative data set is shown here. Filled circle: light organ was mono-colonized by one strain; open circle: light organ was colonized by both strains.

**Fig. S6.** Activation of the *V. fischeri hmp* promoter by exogenous NO. Wild-type (WT) cells carrying the *hmp* promoter driving *gfp* were exposed to different concentrations of the NO generator DEA-NONOate in FSW. Cell density \( \text{OD}_{600} \) and GFP fluorescence level were monitored over time. The experiment was repeated twice with similar results. One representative data set is shown here.
References for supporting materials


