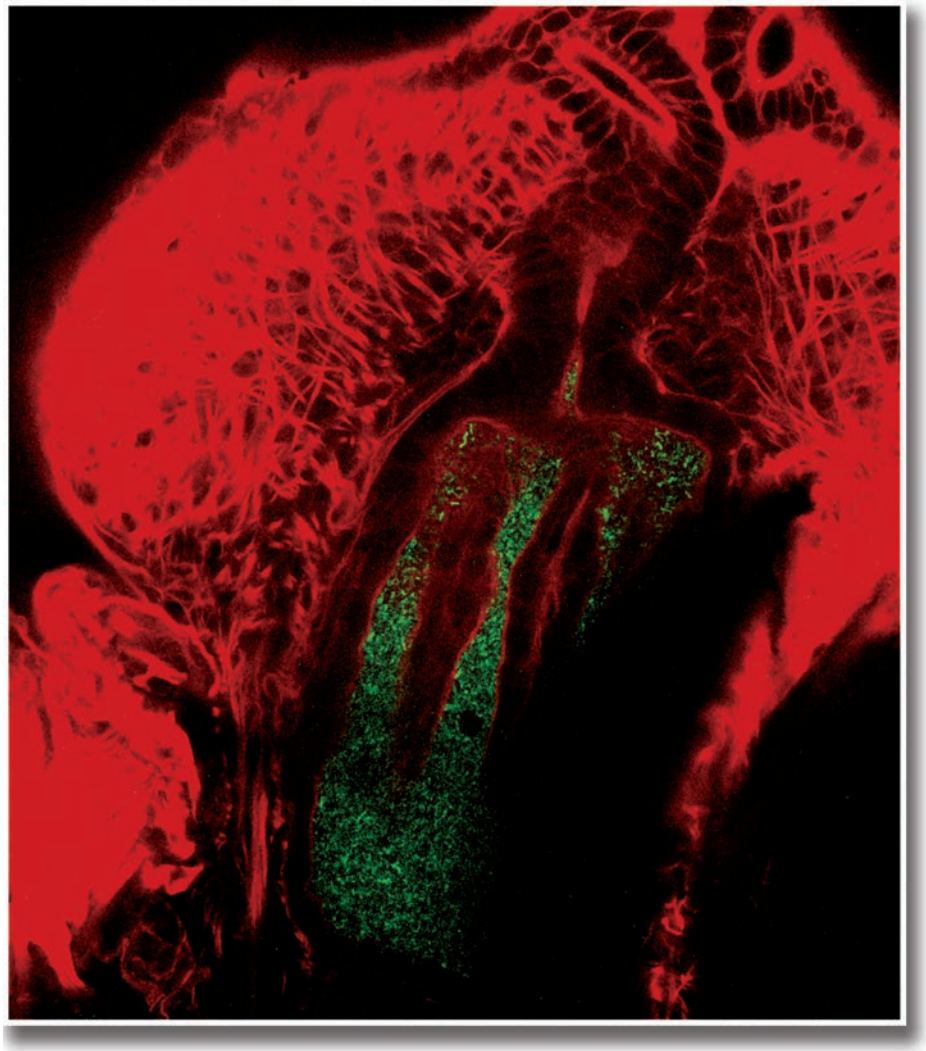
VOLUME 78 | NUMBER 4 | NOVEMBER 2010 | ISSN 0950-382X | www.mol-micro.com

molecular microbiology



On the cover:
Nitric oxide affects initiation of the squid–*Vibrio* symbiosis

Outer membrane insertion of the trimeric autotransporter YadA by BamA

Metallopeptidase required for cell shape and virulence in *Helicobacter*

The COP9 signalosome and *A. nidulans* development

WILEY-BLACKWELL

Molecular Microbiology (2010) 78(4), 903–915 ■
doi:10.1111/j.1365-2958.2010.07376.x
First published online 29 September 2010

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

Yanling Wang,[1] Anne K. Dunn,[2] Jacqueline Wilneff,[1] Margaret J. McFall-Ngai,[1] Stephen Spiro[3] and Edward G. Ruby[1]*

[1]*Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, WI, USA.*
[2]*Department of Botany and Microbiology, University of Oklahoma, Norman, OK, USA.*
[3]*Department of Molecular and Cell Biology, University of Texas at Dallas, Dallas, TX, USA.*

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 Δ*hmp* 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 Δ*hmp* strain fails to form normal-sized 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.

Accepted 25 August, 2010. *For correspondence. E-mail egruby@wisc.edu; Tel. (+1) 608 262 5911; Fax (+1) 608 262 8418.

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 O_2 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*-nitrosoglutathione, 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 flavorubredoxin NorV (FlRd) 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 NorVW 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 NorVW (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 (qRT-PCR), 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

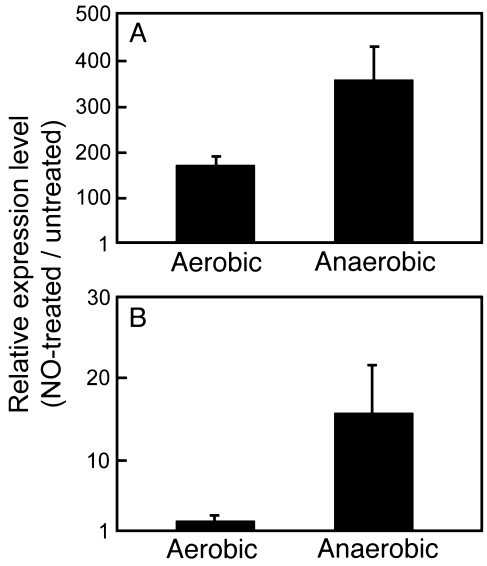


Fig. 1. Expression of *V. fischeri hmp* responds to NO under both oxic and anoxic conditions, while *norV* responds only when oxygen is absent. Wild-type cells were grown in minimal-salts medium plus GlcNAc, either in the presence or in the absence of O_2. The relative expression levels (NO-treated/untreated) of either *hmp* (A) or *norV* (B) transcripts were determined by qRT-PCR. Data points are the means (± 1 SEM) calculated from three biological replicate experiments.

NsrR upstream of *hmp* (Rodionov *et al.*, 2005). Thus, we hypothesized that the *V. fischeri* NsrR is an NO-responsive regulator of *hmp* expression. To test this hypothesis, we constructed a deletion mutant of *nsrR* (Δ*nsrR*) by allelic exchange, and used qRT-PCR to measure the relative expression levels of *hmp* under different conditions. Under normal growth conditions, *hmp* was constitutively expressed at a higher level (230- to 250-fold) in the Δ*nsrR* mutant, irrespective of the presence or absence of oxygen (Table 1). This observation strongly supports the role of NsrR as a negative regulator when NO is absent. When *nsrR* was deleted, *hmp* expression was constitutively high, and no longer responded to NO; that is, the *hmp* expression levels in NO-treated and untreated Δ*nsrR* cells were within a factor of 2 of each other (Table 1). Thus, NsrR appears to be the major NO-responsive regulator for *hmp* transcription under both aerobic and anaerobic growth conditions.

Table 1. NsrR is the major NO-responsive negative regulator of *hmp*.

	Relative *hmp* expression[a]	
Comparison	Aerobic	Anaerobic
Δ*nsrR*/WT	231 ± 33	250 ± 44
Δ*nsrR*+NO/WT+NO[b]	1.0 ± 0.2	1.6 ± 0.2
Δ*nsrR*+NO/Δ*nsrR*	0.9 ± 0.1	2.3 ± 0.3

a. The ratio of expression levels of *hmp* transcripts in each pair of strains being compared as measured by qRT-PCR.
b. Cultures were exposed to NO by adding either 80 μM (aerobic conditions) or 5 μM (anaerobic conditions) DEA-NONOate.
WT; wild type.

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 (Δ*nsrR*, Δ*hmp*, Δ*norV* and Δ*hmp-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 (OD_{600} 0.2–0.4) resulted in an immediate growth arrest only in the Δ*hmp* and Δ*hmp-norV* strains (Fig. 2A). After 2 h, both these strains had recovered and begun 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* Δ*norV* 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 Δ*nsrR* 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 Δ*nsrR* 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 Δ*hmp* and Δ*norV* strains were arrested for 2 h after the challenge, and growth of the double mutant (Δ*hmp-norV*) was even more severely inhibited by NO addition (Fig. 2C). The delayed recovery of the three mutants suggests that,

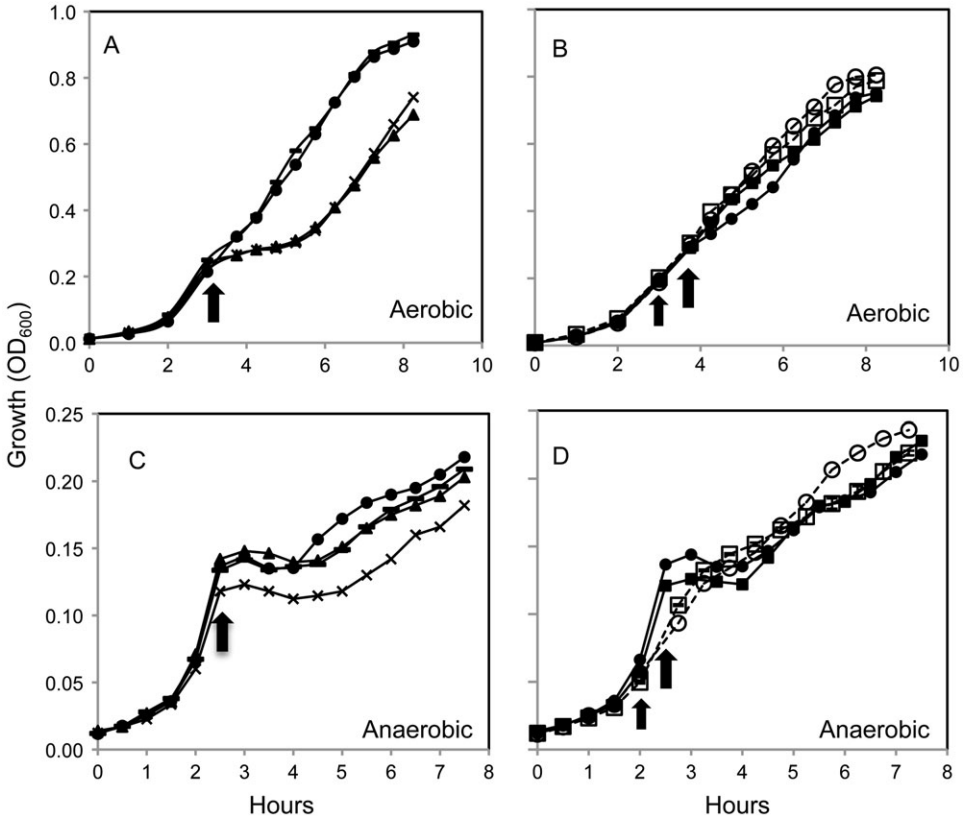


Fig. 2. Hmp and NorV confer protection from NO inhibition on growth, depending on the oxygen availability. Growth in minimal-salts medium plus GlcNAc was monitored by OD_{600}. Aerobic cultures (A and B) were challenged with 100 μM of the NO donor DEA-NONOate (thick arrow), when growth reached early log phase. To pre-adapt the cells (B), a pre-treatment of 40 μM DEA-NONOate was added (thin arrow) 45 min before the challenge. Similarly, anaerobic cultures (C and D) were challenged with 40 μM DEA-NONOate (thick arrow). To pre-adapt the cells (D), 5 μM DEA-NONOate was added (thin arrow) 45 min before the challenge. Growth, either without (filled symbols) or with (open symbols) pre-treatment, was monitored for the wild-type (circle), Δ*hmp* (triangle), Δ*norV* (dash line), Δ*hmp-norV* (cross) and Δ*nsrR* (square) strains. Experiments were repeated three times with similar results. One representative experiment is shown here. Data points are the means calculated from the three technical replicates of that experiment.

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

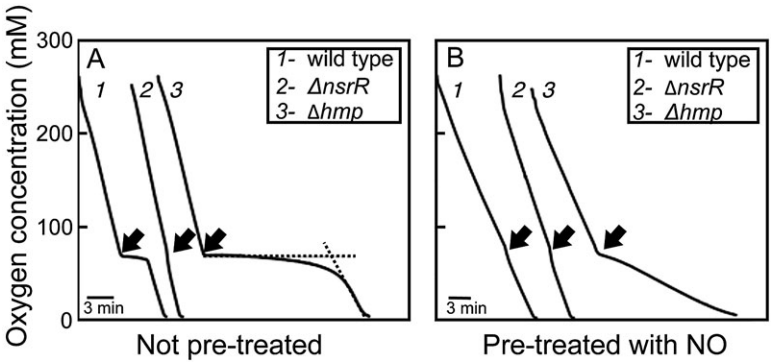


Fig. 3. *V. fischeri* Hmp protects aerobic respiration from NO inhibition. Measurements of oxygen consumption by wild type (trace 1), Δ*nsrR* (trace 2) and Δ*hmp* (trace 3) were made before and after addition (arrow) of the NO generator Proli-NONOate (arrow). The measurements were performed on cells either without (A) or with (B) pre-treatment with 80 μM DEA-NONOate. The dashed lines in trace 3 of (A) define how the period of inhibition was derived. The experiment was repeated three times with similar results. One representative experiment is shown here.

(Fig. 3A); however, the addition of NO immediately inhibited oxygen uptake by wild-type and Δ*hmp* strains (traces 1 and 3, Fig. 3A). In contrast, respiration by the Δ*nsrR* 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 Δ*hmp* and Δ*hmp-norV* strains was fivefold longer than that of the wild-type and Δ*norV* strains (Table 2), further implicating Hmp as the major contributor to NO removal under these conditions. Of note, when either wild-type or Δ*hmp* cells carried pYLW29, 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 Δ*norV* 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 Δ*hmp* and Δ*hmp-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).

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

Strain	Period of inhibition (min)[a]	
	Without pre-treatment	With NO pre-treatment
WT	3.1 ± 0.5	< 0.5
Δ*hmp*	14.9 ± 0.5	Partial inhibition[b]
Δ*norV*	3.1 ± 0.1	< 0.5
Δ*hmp-norV*	15.3 ± 0.1	Partial inhibition
Δ*nsrR*	< 0.5	< 0.5
WT+pVSV105	3.8 ± 0.1	ND
WT+pYLW29	< 0.5	ND
Δ*hmp*+pVSV105	16.3 ± 1.3	ND
Δ*hmp*+pYLW29	< 0.5	ND

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.

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 Δ*norV*, Δ*hmp* and Δ*hmp-norV* strains respectively (Table 3), suggesting a lower colonization efficiency. In addition, the Δ*hmp-norV* and Δ*hmp* strains consistently took longer to initiate symbiotic bioluminescence and colonization compared with either the wild-type or Δ*norV* 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 Δ*hmp* strains by determining the inoculum dose that resulted in colonization of 50% of the animals (ID_{50}). In this assay, about 670 cfu ml^{-1} of wild type were required to colonize half of a cohort of squids, while the ID_{50} of Δ*hmp* was approximately twofold higher (Fig. S4). Interestingly, the Δ*nsrR* 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 Δ*hmp* or Δ*hmp-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 Δ*nsrR* strain did not show

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

Strain	Single-strain colonization[a]		Competitive colonization as RCI[b] (WT/mutant)	
	Percentage of squids becoming luminous	Hours until colonization detected	No treatment	+ NOS inhibitor[c]
WT	50 ± 15	9.3 ± 0.1	–	–
Δ*norV*	40 ± 5	**9.7 ± 0.1**	1.1 ± 0.2	ND
Δ*hmp*	**22 ± 5**	**10.2 ± 0.2**	**2.6 ± 0.5**	0.9 ± 0.1
Δ*hmp-norV*	**17 ± 3**	**12.0 ± 1.2**	**3.4 ± 0.3**	1.0 ± 0.1
Δ*nsrR*	60 ± 20	9.5 ± 0.2	1.1 ± 0.1	ND

a. More effective colonization is indicated by a higher percentage of squid 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.

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 Δ*hmp* or Δ*hmp-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 Δ*hmp* 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 Δ*nsrR* strains. The appearance of fluorescence in these strains was monitored by a microplate 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 Δ*nsrR* 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 Δ*nsrR* 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 Δ*nsrR* cells, regardless of the test conditions (Fig. 4). In aggregates formed by the wild-type and Δ*nsrR* 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.

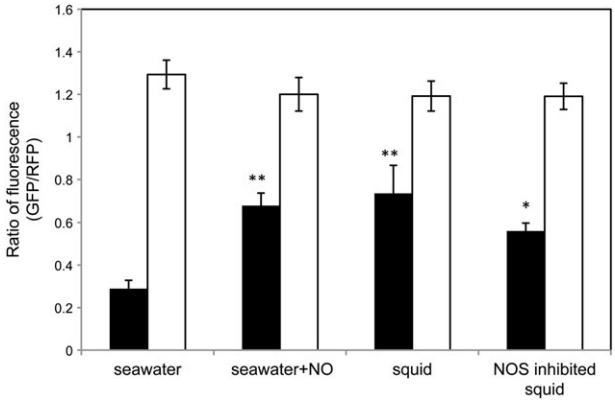


Fig. 4. The *hmp* promoter responds to host-derived NO during the aggregation stage of the symbiosis. Cells of wild type (black bars) or Δ*nsrR* (white bars), carrying the *hmp* promoter-reporter plasmid pYLW45, were added to seawater, or used to inoculate newly hatched squids. To induce the *hmp* promoter in the seawater experiment, 80 μM DEA-NONOate was added to seawater. To lower the intrinsic level of NO production in the mucus, some squids were pre-treated with 100 μM 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 (± 1 SEM) calculated from three biological replicates.

Hmp protects against NO in the mucus and affects the size of aggregates

Because *V. fischeri* detects NO in the mucus (Fig. 4), and high levels of NO limit the size of its aggregates (Davidson *et al*., 2004), we wondered whether removal of NO by Hmp contributes to the ability of *V. fischeri* to form normal-sized aggregates. It seemed reasonable to predict that the Δ*nsrR* mutant would form a larger aggregate than wild type, while an aggregate formed by the Δ*hmp* mutant would be smaller than that of wild type. When we observed the area of the host's mantle cavity in which the symbiont aggregates (Fig. 5A), we found that the Δ*nsrR* strain had formed large and tight aggregates, which were easily detected as bright green patches, 2–3.5 h after inoculation (Fig. 5B). In contrast, the Δ*hmp* strain formed much smaller aggregates of only a few cells. Over the next few hours, the size of wild-type and Δ*hmp* aggregates increased, although the former remained generally larger (data not shown).

Pre-treating juvenile squids with the NOS inhibitor SMTC for 2–3 h before inoculation ameliorated these differences between the strains (Fig. 5B). Specifically, Δ*hmp* now formed larger aggregates, further suggesting that ambient NO levels somehow limit aggregation. In contrast, Δ*nsrR* cells appeared blind to NO levels in the mucus, forming aggregates of similar size and appearance in both non-treated and SMTC-treated squids (Fig. 5B). Intriguingly, when wild type and one of the two mutants were differentially marked with either constitutively expressed *gfp* (pVSV102) or constitutively expressed *rfp* (pVSV208), and co-inoculated at a 1:1 ratio, wild type did not appear to dominate Δ*hmp* in the aggregation, nor did Δ*nsrR* dominate over wild type (data not shown). One possibility is that the strain with the higher level of Hmp effectively removed the ambient NO, compensating for the other strain thereby masking the difference seen in single-strain colonization experiments. Taken together, these data support the conclusion that the capacity to remove NO by Hmp controls the size of aggregates in the early stages of the symbiosis.

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

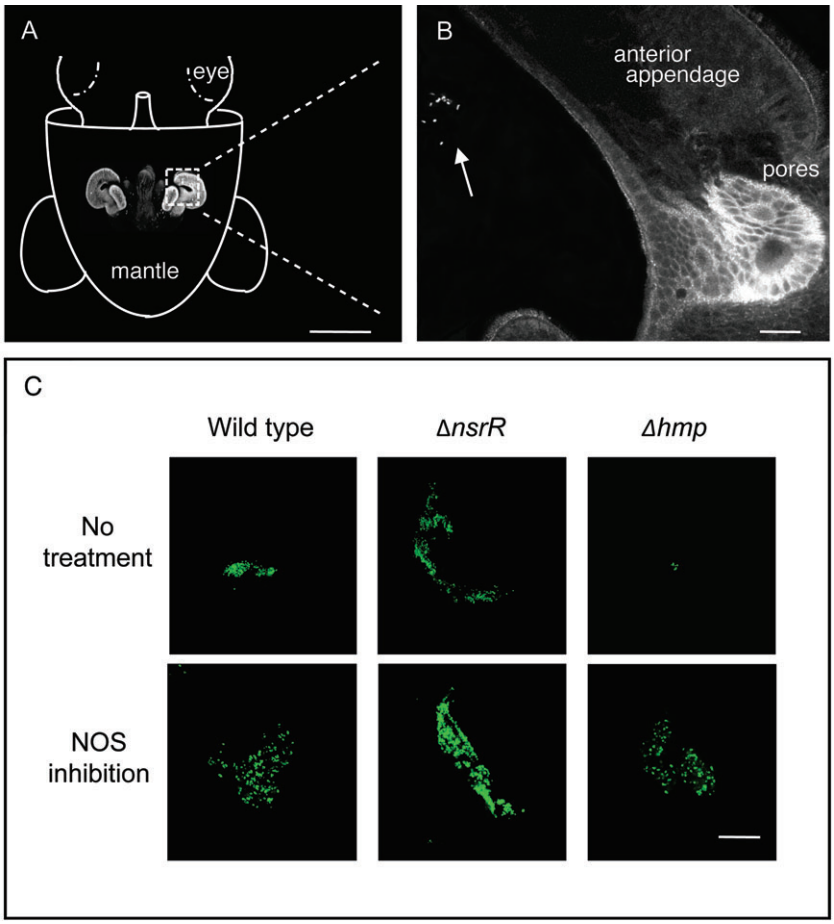


Fig. 5. The size of aggregates formed by *V. fischeri* during squid colonization correlates with the potential of the bacteria to remove NO.
A. Orientation figure indicating the position of the juvenile's nascent light organ. A confocal image of the light organ is placed on the cartoon in its approximate position within the mantle cavity (scale bar = 300 μm).
B. Enlarged confocal micrograph of the region of the light organ located within the dotted square of (A). An aggregate of *V. fischeri* cells is indicated by the arrow (scale bar = 10 μm).
C. The bacterial aggregates formed in host-derived mucus by wild-type, Δ*nsrR* or Δ*hmp* strains carrying GFP-encoded pVSV102, and observed using confocal microscopy (at 400×) between 2.5 and 4 h after inoculation (scale bar = 20 μm). To lower the intrinsic level of NO production in the mucus, some squids were pre-treated with the nitric-oxide synthesis inhibitor SMTC for 2–3 h before inoculation with *V. fischeri*. The experiments were repeated three times with similar results.

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 Δ*hmp* strain was only partially inhibited by NO after pre-treatment, oxygen consumption by a Δ*hmp-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 Δ*nsrR* strain appeared to be more resistant to NO challenge under culture conditions, we were surprised to find that the Δ*nsrR* strain did not out-compete wild-type bacteria in a competitive colonization of juvenile squid. One possible explanation is that the Δ*nsrR* 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 (O_2^-) (Bang *et al*., 2006; Gilberthorpe *et al*., 2007). Thus, an Hmp-over-producing strain like Δ*nsrR* might be more susceptible to oxidative killing, either directly by O_2^- or its derivative hydrogen peroxide (H_2O_2), or indirectly by providing H_2O_2 for halide peroxidase-mediated synthesis of highly toxic hypochlorous acid (Small and McFall-Ngai, 1999). Thus, although the *V. fischeri* Δ*nsrR* strain has an increased resistance to host-derived NO stress, it may be exposed to a more severe oxidative killing by H_2O_2 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 HNOX 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 NorVW. 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% K_2HPO_4, 50 ml of 1 M Tris-HCl buffer (pH 7.5) and 449 ml of tap water. Ten-millimolar *N*-acetyl-D-glucosamine (GlcNAc) (MP Biomedicals) was added as the sole carbon and nitrogen source. To achieve anoxic conditions, the headspace of culture tubes was sequentially evacuated and flushed 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 (NheI). 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

(pYLW45) was made by cloning approximately 300 bp of DNA upstream of the Hmp start codon into pVSV209 (Table S1) at the SalI–AvrII 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 (Stabb 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 (OD_{600}) 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 OD_{600} of approximately 0.3, and 10 ml of culture was transferred to each of two flasks. To one flask, DEA-NONOate (in 10 mM NaOH) was added to a final concentration of 80 μM, while the other was treated with a corresponding volume of 10 mM NaOH as a control. Samples were allowed to grow at 28°C for 30 min with shaking. Twenty millilitres of RNAprotect Bacteria Reagent (Qiagen) was added to both cultures, which were incubated for 5 min at room temperature. Cells were then pelleted by centrifugation at 8000 *g* for 30 min at 4°C, and frozen at −80°C before further processing. Total RNA was prepared using the RNeasy Mini Kit (Qiagen). Residual DNA was removed by using the RNase-free DNase Set (Qiagen), with both on- and off-column digestion steps. cDNA was synthesized by AMV reverse transcriptase (Promega) using 3 μg of purified RNA from each sample. qRT-PCR was performed on an iCycler iQ™ real-time PCR machine (Bio-Rad), with 400 ng of cDNA/reaction in the iQ™ SyBR Green Supermix (Bio-Rad), with an annealing/extension temperature of 59°C. Primers were designed to target an internal fragment (~120–150 bp) of *hmp, norV*, or a normalizing control gene (DNA polymerase I, *VF_0074*). PCR efficiency was similar for all the primer sets. The relative expression levels were calculated using the ΔΔCt method (http://pathmicro.med.sc.edu/pcr/realtime-home.htm). Samples from anaerobic cultures were processed following the same procedures as above, except 5 μM DEA-NONOate was added 45 min before fixation with RNAprotect Bacteria Reagent (Qiagen).

Growth during the NO challenge

Three millilitres of each culture of *V. fischeri* cells was grown to an OD_{600} of approximately 0.8 in LBS medium by shaking at 28°C. Twenty microlitres was subcultured into 20 ml of mineral-salts medium containing GlcNAc in a 125 ml Erlenmeyer flask. Aerobic growth was monitored by measuring the OD_{600} of the cultures every 30 min. When the culture reached early exponential phase (OD_{600} ~ 0.3), DEA-NONOate was added to a final concentration of 100 μM to produce the NO challenge. For those cultures receiving an aerobic NO pretreatment, 40 μM DEA-NONOate was added 45 min before the NO challenge.

For *V. fischeri* cultures grown under anoxic conditions, 40 μl of the pre-culture was subcultured into 4 ml of minimal-salts medium plus GlcNAc, degassed and sealed in an 11 ml Pyrex culture tube. Every 30 min, the OD_{600} of the culture was monitored on a Genesys 20 Spectrophotometer (Thermo Scientific) without the need for subsampling. Because all the strains were more susceptible to NO under anoxic conditions (data not shown), DEA-NONOate was added as a challenge at a final concentration of only 40 μM during the early-mid-log phase (OD_{600} = 0.1–0.15) of culture. Similarly, for the pretreatment, only 5 μM DEA-NONOate was added, 45 min before the challenge.

NO inhibition of oxygen consumption

A fresh culture (3 ml) of each *V. fischeri* strain was grown in LBS medium to an OD_{600} 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 (OD_{600} ~ 0.3) were harvested at 6000 r.p.m. for 10 min at 4°C The pellet was washed and resupended 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 $CaCl_2$, $MgCl_2$, NaH_2PO_4 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 $I = 1 - (O_2$ consumption rate after NO addition/rate before addition). I was plotted against NO concentration and, using Kaleidagraph (Synergy Software), the data were fit to the Hill equation: $I = [NO]^n/(K_i)^n + [NO]^n$, where K_i 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 (ID_{50}), 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 logit of percent of animals colonized, and applied the log-logistic model to estimate the ID_{50} values of the wild-type and Δ*hmp* 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* (pVSV102) or *rfp* (pVSV208) 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 Δ*nsrR* strains (~10^8 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 (OD_{600}).

Either wild-type or Δ*nsrR* cells, each carrying pYLW45, were used to inoculate between 5 and 10 newly hatched juvenile squids at 10^6 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% $MgCl_2$ 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 (10^6 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 10^6 cfu (per ml) of *V. fischeri* strains that were marked with constitutively expressed *gfp* (pVSV102) or *rfp* (pVSV208) 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 10^6 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

Arai, H., Hayashi, M., Kuroi, A., Ishii, M., and Igarashi, Y. (2005) Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of *Pseudomonas aeruginosa*. *J Bacteriol* **187:** 3960–3968.

Bang, I.-S., Liu, L., Vazquez-Torres, A., Crouch, M.-L., Stamler, J.S., and Fang, F.C. (2006) Maintenance of nitric oxide and redox homeostasis by the *Salmonella* flavohemoglobin Hmp. *J Biol Chem* **281:** 28039–28047.

Bartnikas, T.B., Wang, Y., Bobo, T., Veselov, A., Scholes, C.P., and Shapleigh, J.P. (2002) Characterization of a member of the NnrR regulon in *Rhodobacter sphaeroides* 2.4.3 encoding a haem-copper protein. *Microbiology* **148:** 825–833.

Baudouin, E., Pieuchot, L., Engler, G., Pauly, N., and Puppo, A. (2006) Nitric oxide Is formed in *Medicago truncatula–Sinorhizobium meliloti* functional nodules. *Mol Plant Microbe Interact* **19:** 970–975.

Boccara, M., Mills, C.E., Zeier, J., Anzi, C., Lamb, C., Poole, R.K., and Delledonne, M. (2005) Flavohaemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host. *Plant J* **43:** 226–237.

Boettcher, K.J., and Ruby, E.G. (1990) Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. *J Bacteriol* **172:** 3701–3706.

Borisov, V.B., Forte, E., Konstantinov, A.A., Poole, R.K., Sarti, P., and Giuffr, A. (2004) Interaction of the bacterial terminal oxidase cytochrome *bd* with nitric oxide. *FEBS Lett* **576:** 201–204.

Bose, J.L., Kim, U., Bartkowski, W., Gunsalus, R.P., Overley, A.M., Lyell, N.L., *et al*. (2007) Bioluminescence in *Vibrio fischeri* is controlled by the redox-responsive regulator ArcA. *Mol Microbiol* **65:** 538–553.

Cary, S.P.L., Winger, J.A., Derbyshire, E.R., and Marletta, M.A. (2006) Nitric oxide signaling: no longer on or off. *Trends Biochem Sci* **31:** 231–239.

D'Autreaux, B., Tucker, N.P., Dixon, R., and Spiro, S. (2005) A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature* **437:** 769–772.

Davidson, S.K., Koropatnick, T.A., Kossmehl, R., Sycuro, L., and McFall-Ngai, M.J. (2004) NO means 'yes' in the squid–vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell Microbiol* **6:** 1139–1151.

Dunn, A.K., and Stabb, E.V. (2008) Genetic analysis of trimethylamine N-oxide reductases in the light organ symbiont *Vibrio fischeri* ES114. *J Bacteriol* **190:** 5814–5823.

Dunn, A.K., Karr, E.A., Wang, Y., Barron, A.R., Ruby, E.G., and Stabb, E.V. (2010) The alternative oxidase (AOX) gene in *Vibrio fischeri* is controlled by NsrR and upregulated in response to nitric oxide. *Mol Microbiol* **77:** 44–55.

Fang, F.C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* **2:** 820–832.

Gardner, A.M., Helmick, R.A., and Gardner, P.R. (2002) Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. *J Biol Chem* **277:** 8172–8177.

Gilberthorpe, N.J., Lee, M.E., Stevanin, T.M., Read, R.C., and Poole, R.K. (2007) NsrR: a key regulator circumventing *Salmonella enterica* serovar Typhimurium oxidative and nitrosative stress *in vitro* and in IFN-{gamma}-stimulated J774.2 macrophages. *Microbiology* **153:** 1756–1771.

Graf, J., and Ruby, E.G. (1998) Host-derived amino acids support the proliferation of symbiotic bacteria. *Proc Natl Acad Sci USA* **95:** 1818–1822.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001) Molecular analysis of commensal host–microbial relationships in the intestine. *Science* **291:** 881–884.

Hutchings, M.I., Mandhana, N., and Spiro, S. (2002) The NorR Protein of *Escherichia coli* activates expression of the flavorubredoxin gene *norV* in response to reactive nitrogen species. *J Bacteriol* **184:** 4640–4643.

Iyer, L., Anantharaman, V., and Aravind, L. (2003) Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins. *BMC Genomics* **4:** 5.

Koropatnick, T.A., Engle, J.T., Apicella, M.A., Stabb, E.V., Goldman, W.E., and McFall-Ngai, M.J. (2004) Microbial factor-mediated development in a host–bacterial mutualism. *Science* **306:** 1186–1188.

Lee, K., and Ruby, E.G. (1994) Effects of the squid host on the abundance and distribution of symbiontic *Vibrio fischeri* in nature. *Appl Environ Microbiol* **60:** 1565–1571.

McCann, J., Stabb, E.V., Millikan, D.S., and Ruby, E.G. (2003) Population dynamics of *Vibrio fischeri* during infection of *Euprymna scolopes*. *Appl Environ Microbiol* **69:** 5928–5934.

Mathieu, C., Moreau, S., Frendo, P., Puppo, A., and Davies, M.J. (1998) Direct detection of radicals in intact soybean nodules: presence of nitric oxide–leghemoglobin complexes. *Free Radic Biol Med* **24:** 1242–1249.

Meilhoc, E., Cam, Y., Skapski A., and Bruand, C. (2010) The response to nitric oxide of the nitrogen-fixing symbiont *Sinorhizobium meliloti*. *Mol Plant Microbe Interact* **23:** 748–759.

Miller, J.H. (1992) *A Short Course in Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mills, P.C., Rowley, G., Spiro, S., Hinton, J.C.D., and Richardson, D.J. (2008) A combination of cytochrome *c* nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar Typhimurium against killing by NO in anoxic environments. *Microbiology* **154:** 1218–1228.

Nyholm, S.V., and McFall-Ngai, M.J. (2004) The winnowing: establishing the squid–*Vibrio* symbiosis. *Nat Rev Microbiol* **2:** 632–642.

Nyholm, S.V., Stabb, E.V., Ruby, E.G., and McFall-Ngai, M.J.

(2000) Establishment of an animal–bacterial association: recruiting symbiotic vibrios from the environment. *Proc Natl Acad Sci USA* **97:** 10231–10235.

Poock, S.R., Leach, E.R., Moir, J.W.B., Cole, J.A., and Richardson, D.J. (2002) Respiratory detoxification of nitric oxide by the cytochrome *c* nitrite reductase of *Escherichia coli*. *J Biol Chem* **277:** 23664–23669.

Poole, R.K., and Hughes, M.N. (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Mol Microbiol* **36:** 775–783.

Rawls, J.F., Samuel, B.S., and Gordon, J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc Natl Acad Sci USA* **101:** 4596–4601.

Richardson, A.R., Dunman, P.M., and Fang, F.C. (2006) The nitrosative stress response of *Staphylococcus aureus* is required for resistance to innate immunity. *Mol Microbiol* **61:** 927–939.

Rodionov, D.A., Dubchak, I.L., Arkin, A.P., Alm, E.J., and Gelfand, M.S. (2005) Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comput Biol* **1:** e55.

Ruby, E.G., and Asato, L.M. (1993) Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Arch Microbiol* **159:** 160–167.

Ruby, E.G., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., *et al*. (2005) Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc Natl Acad Sci USA* **102:** 3004–3009.

Small-Howard, A.L. (2004) A halide peroxidase and reactive oxygen species in the mutulistic light organ symbiosis between the sepilolid squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri*. PhD thesis. Faculty of the Graduate School, University of Southern California, Los Angeles.

Small, A.L., and McFall-Ngai, M.J. (1999) Halide peroxidase in tissues that interact with bacteria in the host squid *Euprymna scolopes*. *J Cell Biochem* **72:** 445–457.

Spiro, S. (2007) Regulators of bacterial responses to nitric oxide. *FEMS Microbiol Rev* **31:** 193–211.

Stabb, E.V., and Ruby, E.G. (2002) RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. *Methods Enzymol* **358:** 413–426.

Stevanin, T.M., Ioannidis, N., Mills, C.E., Kim, S.O., Hughes, M.N., and Poole, R.K. (2000) Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes *bc* or *bd*, from nitric oxide. *J Biol Chem* **275:** 35868–35875.

Stevanin, T.M., Poole, R.K., Demoncheaux, E.A.G., and Read, R.C. (2002) Flavohemoglobin Hmp protects *Salmonella enterica* serovar Typhimurium from nitric oxide-related killing by human macrophages. *Infect Immun* **70:** 4399–4405.

Stewart, V. (2003) Nitrate- and nitrite-responsive sensors NarX and NarQ of proteobacteria. *Biochem Soc Trans* **31:** 1–10.

Tsou, A.M., Cai, T., Liu, Z., Zhu, J., and Kulkarni, R.V. (2009) Regulatory targets of quorum sensing in *Vibrio cholerae*: evidence for two distinct HapR-binding motifs. *Nucleic Acids Res* **37:** 2747–2756.

Tucker, N.P., Le Brun, N.E., Dixon, R., and Hutchings, M.I. (2010) There's NO stopping NsrR, a global regulator of the bacterial NO stress response. *Trends Microbiol* **18:** 149–156.

Visick, K.L., and Ruby, E.G. (1998) The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. *J Bacteriol* **180:** 2087–2092.

Wang, Y., Dufour, Y.S., Carlson, H.K., Donohue, T.J., Marletta, M.A., and Ruby, E.G. (2010) H-NOX-mediated nitric oxide sensing modulates symbiotic colonization by *Vibrio fischeri*. *Proc Natl Acad Sci USA* **107:** 8375–8380.

Ward, W.W. (2005) Biochemical and physical properties of green fluorescent protein. In *Green Fluorescent Protein*, 2nd edn. Martin Chalfie, S.R.K. (ed.). New York: John Wiley & Sons, pp. 39–65.

Weis, V., Small, A., and McFall-Ngai, M. (1996) A peroxidase related to the mammalian antimicrobial protein myeloperoxidase in the Euprymna–Vibrio mutualism. *Proc Natl Acad Sci USA* **93:** 13683–13688.

Wier, A.M., Nyholm, S.V., Mandel, M.J., Massengo-Tiassé, R.P., Schaefer, A.L., Koroleva, I., *et al*. (2010) Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *Proc Natl Acad Sci USA* **107:** 2259–2264.

Wollenberg, M.S., and Ruby, E.G. (2009) Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* from two different host populations. *Appl Environ Microbiol* **75:** 193–202.

Supporting information

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

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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

Strain, plasmid or primer sequence	Relevant characteristics*	Source or reference
Bacterial strains		
Escherichia coli		
DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k-, m_k+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	(Hanahan, 1983)
DH5-T1^R	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A (confers resistance to phage T1)	Invitrogen Inc.
DH5λpir	DH5α lysogenized with lambda pir	(Dunn *et al.*, 2005)
Vibrio fischeri		
AKD711	ES114ΔnsrR	(Dunn *et al.,* 2010)
ES114	Wild-type isolate from *E. scolopes* light organ	(Boettcher & Ruby, 1990)
YLW9	ES114 carrying pVSV102, Kan^R	this study
YLW8	ES114 carrying pVSV208, Cam^R	this study
YLW62	ES114ΔnorV carrying pVSV208, Cam^R	this study
YLW61	ES114ΔnorV carrying pVSV102, Kan^R	this study
YLW60	ES114Δhmp carrying pYLW29, Cam^R	this study
YLW59	ES114Δhmp carrying pVSV105, Cam^R	this study
YLW57	ES114Δhmp carrying pVSV208, Cam^R	this study
YLW56	ES114Δhmp carrying pVSV102, Kan^R	this study
YLW55	ES114ΔnsrR carrying pLYW45	this study

YLW52	ES114ΔnsrR carrying pVSV208,Cm^R	this study
YLW51	ES114ΔnsrR carrying pVSV102, Kan^R	this study
YLW50	ES114ΔnsrR carrying pVSV209,Kan^R and promoterless Cm^R	this study
YLW48	ES114 carrying pYLW45, Kan^R and promoterless Cm^R	this study
YLW42	ES114 carrying pYLW29, Cam^R	this study
YLW40	ES114 carrying pVSV209, Kan^R and promoterless Cm^R	this study
YLW38	ES114ΔnorV	this study
YLW25	ES114 carrying pVSV105, Cam^R	this study
YLW20	ES114Δhmp	this study
YLW117	ES114Δhmp-norV carrying pVSV102, Kan^R	this study
YLW116	ES114Δhmp-norV carrying pVSV208, Cm^R	this study
YLW113	ES114Δhmp-norV	this study

Plasmids

pAKD711	*Δ*nsrR allele, R6Kγ and ColE1 replication origins, RP4 *oriT*, *ermR*, *kanR*	(Dunn *et al.*, 2010)
pCR-BluntII TOPO	TOPO PCR-cloning vector, Kan^R	Invitrogen Inc.
pEVS104	$oriV_{R6K\gamma}$, $oriT_{RP4,}$ RP4-derived conjugative helper plasmid, Kan^R	(Stabb, 2002)
pVSV102	$oriV_{R6K\gamma}$, $oriT_{RP4,}oriV_{pES213}$, *Dsgfp*-tagged, Kan^R,	(Dunn *et al.*, 2006)
pVSV105	$oriV_{R6K\gamma}$, $oriT_{RP4,}oriV_{pES213}$, complementation vector, Cam^R	(Dunn *et al.*, 2006)
pVSV208	$oriV_{R6K\gamma}$, $oriT_{RP4,}oriV_{pES213}$, *rfp*-tagged, Cam^R,	(Dunn *et al.*, 2006)
pVSV209	$oriV_{R6K\gamma}$, $oriT_{RP4,}oriV_{pES213}$, KanR-constitutively expressed *rfp*, transcriptional terminators-(AvrII, SalI, StuI)-promoterless CmR and *gfp*	(Dunn *et al.*, 2006)
pYLW20	*Δ*hmp allele, R6Kγ and ColE1 replication origins, RP4 *oriT*, *ermR*, *kanR*	this study

pYLW27	Δ*norV* allele, R6Kγ and ColE1 replication origins, RP4 *oriT*, *ermR*, *kanR*	this study
pYLW29	pComhmp, *hmp*+ complementing fragment cloned into pVSV105	this study
pYLW45	pPhmp::*gfp*, a 300bp *hmp* promoter region cloned into pVSV209 to drive expression of promoterless *gfp*	this study

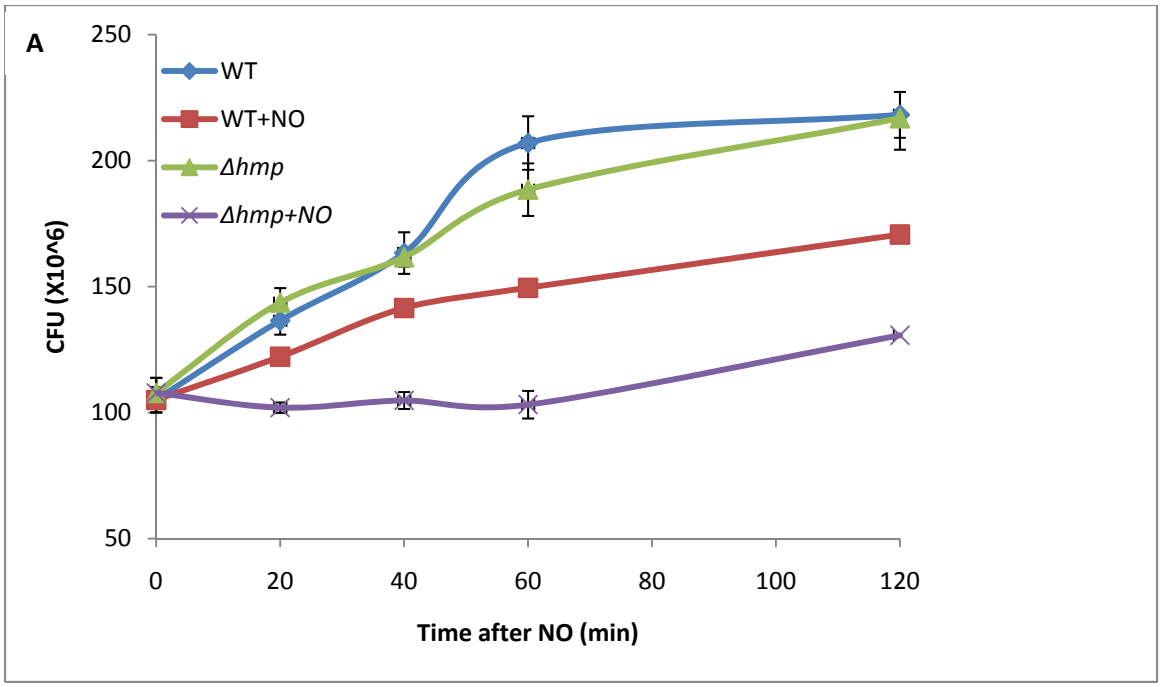
Primers[†]

hmpcomp-for	5'-AGT CGT AC*T CTA GA*C TCG AAT GAC ATC ACC AA-3'	this study
hmpcomp-rev	5'-GCT AGA TC*G AGC TC*T GAG TTT CAT CAA CTC GAA-3'	this study
hmppro-for	5'-TGA CGC TA*C CTA GG*C GAA AGA GGT GCT TTC AT-3'	this study
hmppro-rev	5'-CAT GGT AC*A GGC CT*C CCA AAT TTA TCT TCA ACT AAC TC-3'	this study
VF0074qrt-for	5'- CGG TAT GAG TGC GTT TGG TCT TGC -3'	this study
VF0074qrt-rev	5'- CTG CAT CAC TCC TGG GTA ACG CTC -3'	this study
hmpqrt-for	5'- TGA AGT GCT ATT ACC AGC GGT TG -3'	this study
hmpqrt-rev	5'- AAG AGT GGC AAG TAA GTG ACC AC -3'	this study
norVqrt-for	5'- TCT TAC GCC ATT CAG TGC TTT GG -3'	this study
norVqrt-rev	5'- CTC GCC ATA CAC AAC CGT GAG -3'	this study

---

*Abbreviations used: Cam^R, chloramphenicol resistance; Erm^R, erythromycin resistance; Kan^R, 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).


A
250
200
150
100
50
CFU (X10^6)
WT
WT+NO
Δhmp
Δhmp+NO
0
20
40
60
80
100
120
Time after NO (min)

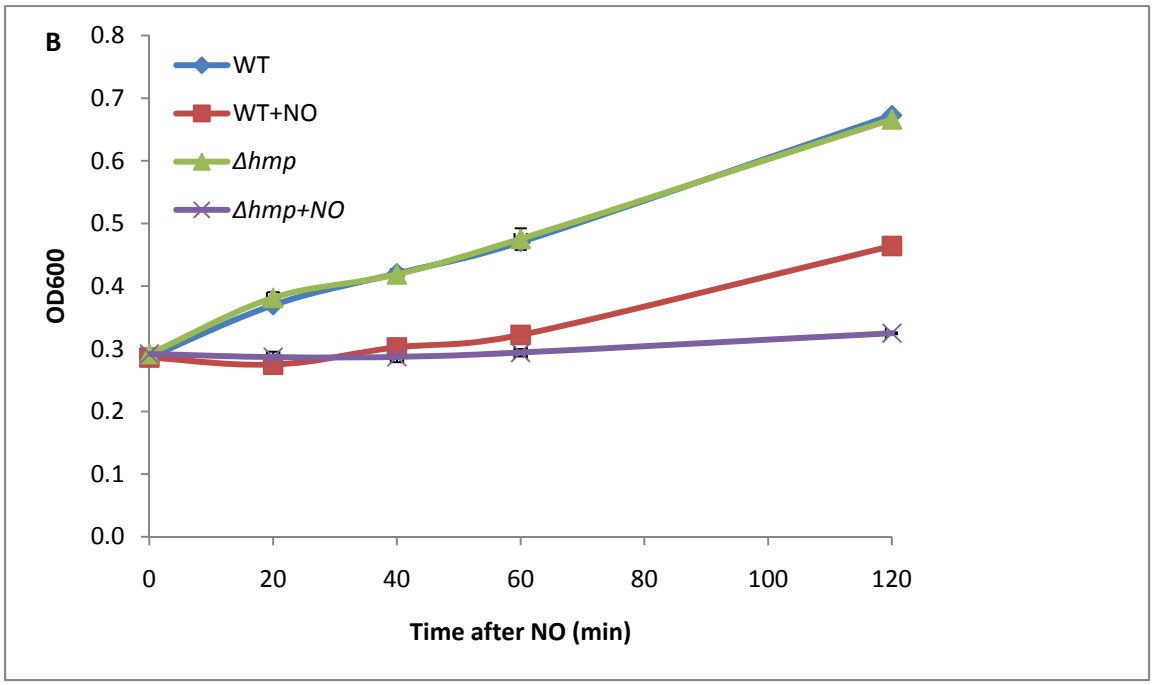

B
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD600
WT
WT+NO
Δhmp
Δhmp+NO
0
20
40
60
80
100
120
Time after NO (min)

Fig. S1.

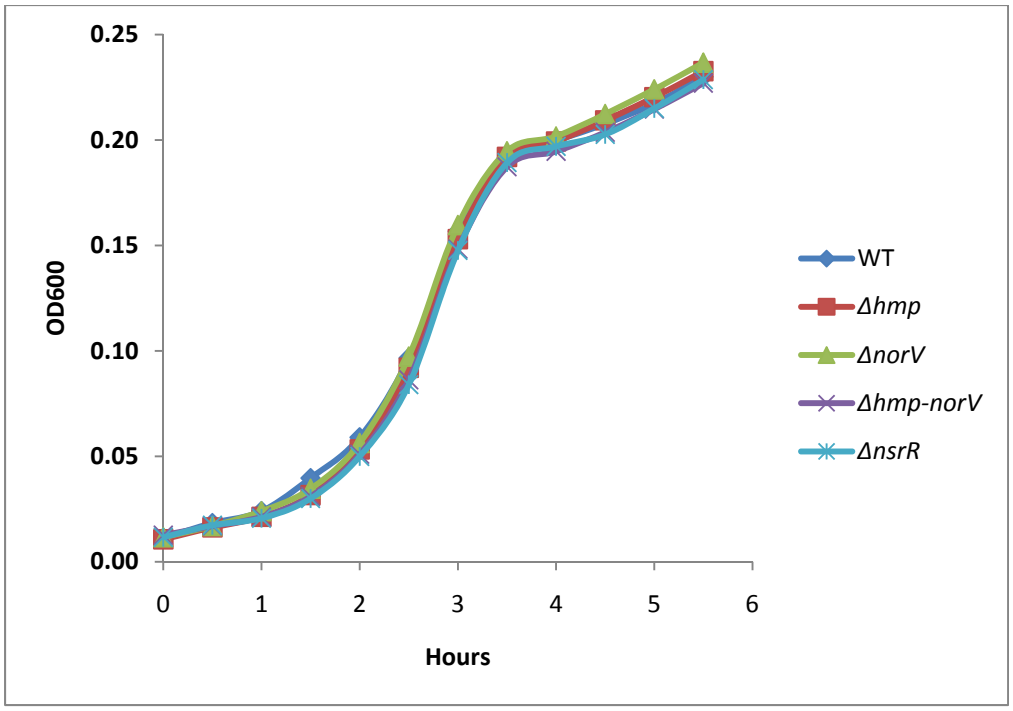

0.25
0.20
0.15
0.10
0.05
0.00
OD600
0
1
2
3
4
5
6
Hours
WT
Δhmp
ΔnorV
Δhmp-norV
ΔnsrR

Fig. S2.

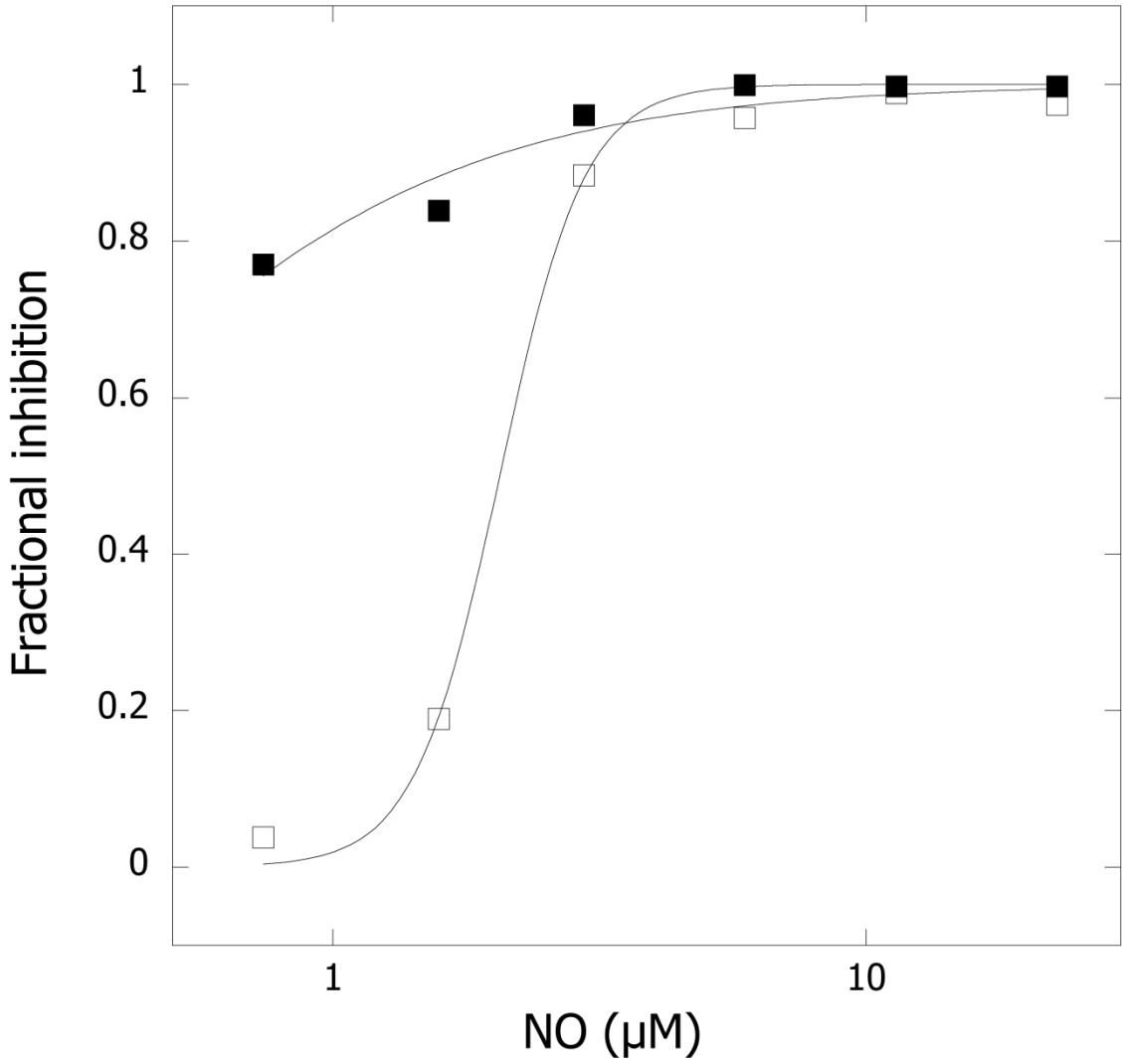


Fig. S3.

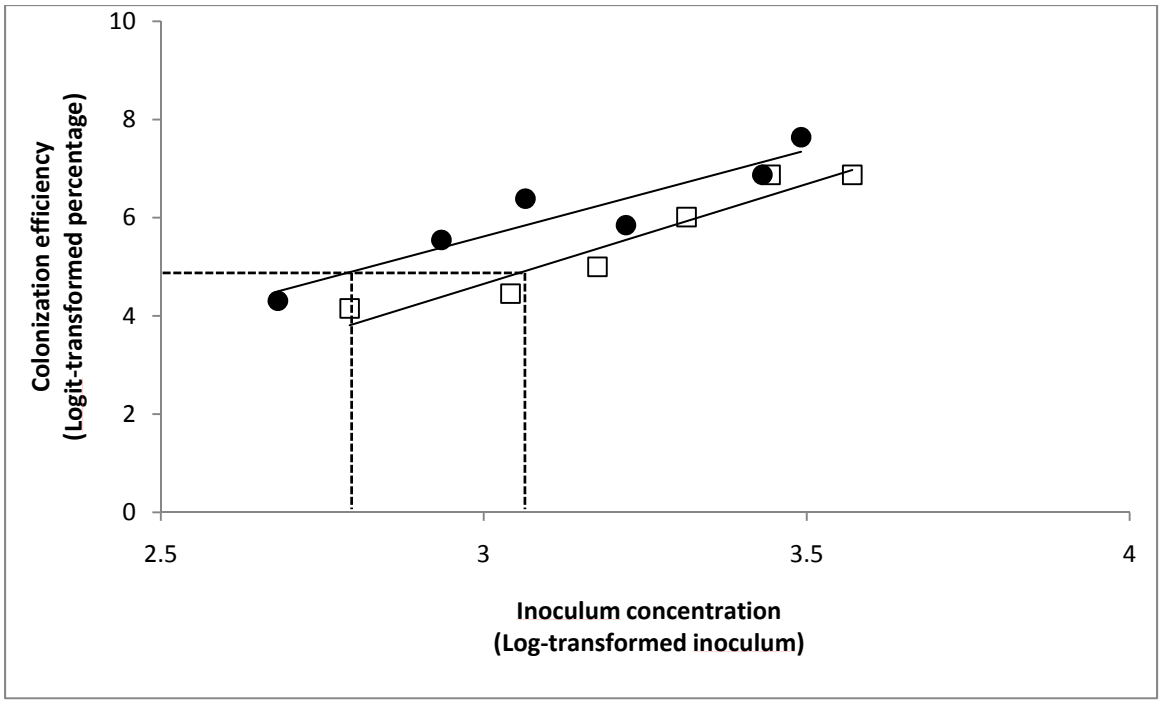

10
8
6
4
2
0
2.5
3
3.5
4
Colonization efficiency
(Logit-transformed percentage)
Inoculum concentration
(Log-transformed inoculum)

Fig. S4.

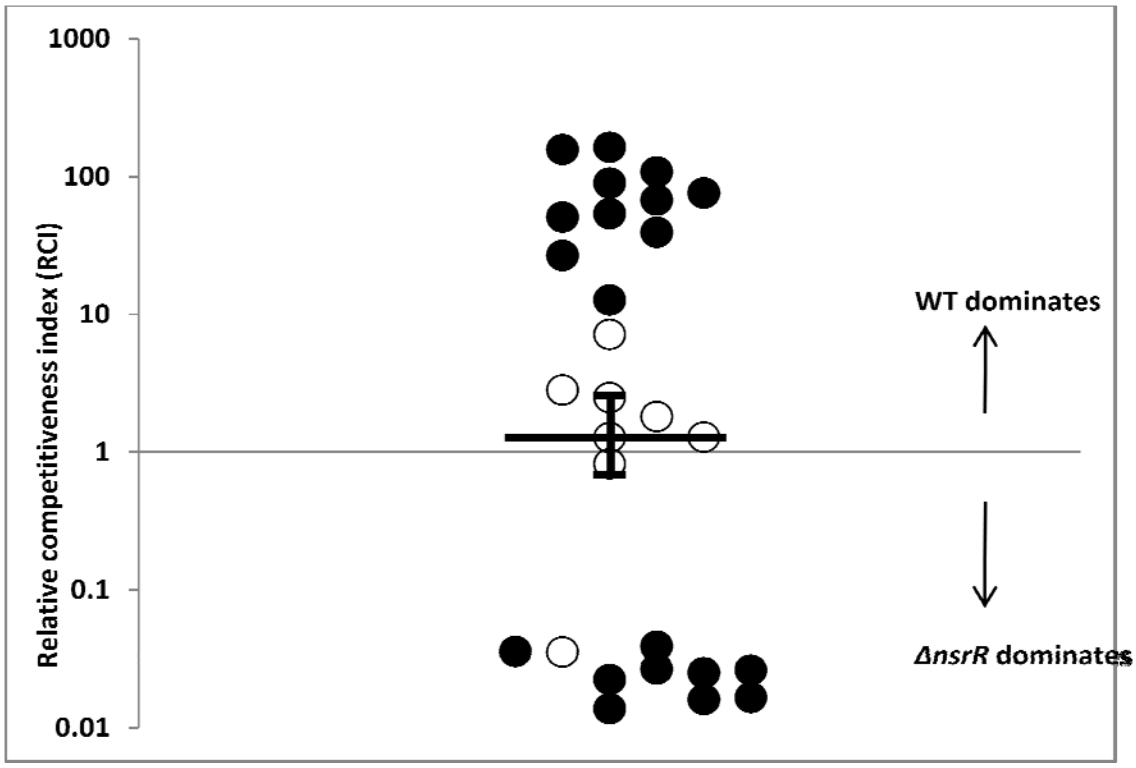

1000
100
10
1
0.1
0.01
Relative competitiveness index (RCI)
WT dominates
ΔnsrR dominates

Fig. S5.

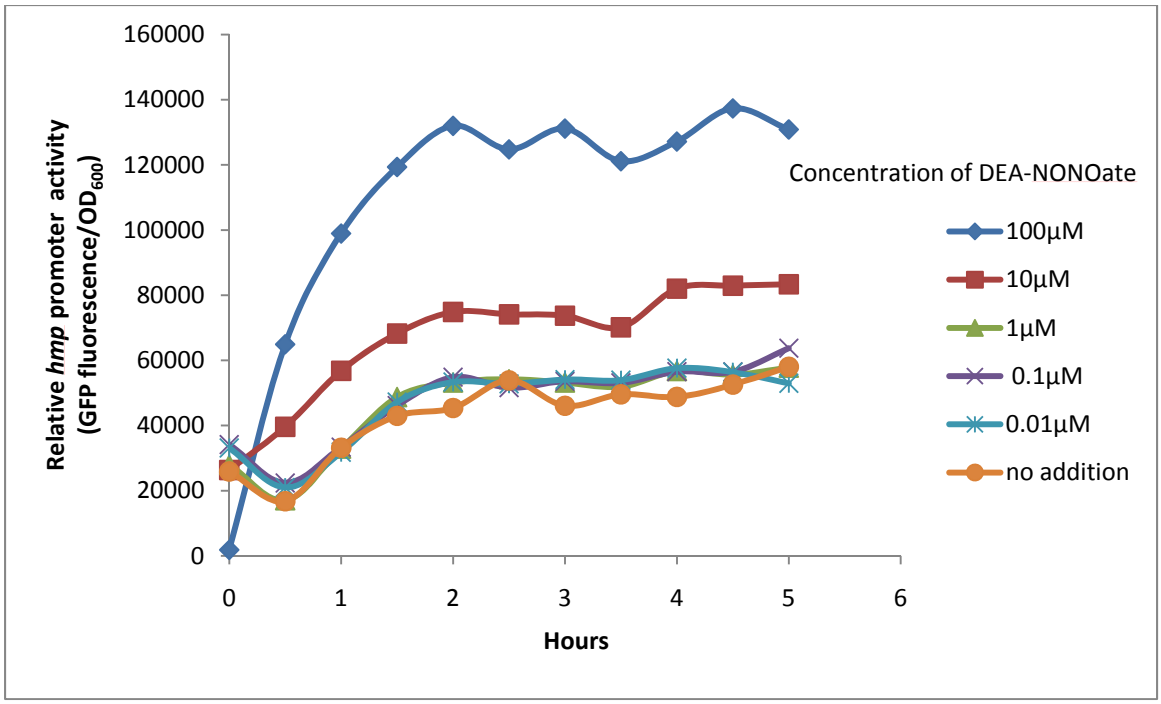

160000
140000
120000
100000
80000
60000
40000
20000
0
0
1
2
3
4
5
6
Hours
Relative hmp promoter activity (GFP fluorescence/OD600)
Concentration of DEA-NONOate
100µM
10µM
1µM
0.1µM
0.01µM
no addition

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 (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

Boettcher, K. J. & E. G. Ruby, (1990) Depressed light emission by symbiotic Vibrio fischeri of the sepiolid squid Euprymna scolopes. *J. Bacteriol.* **172**: 3701-3706.

Dunn, A. K., E. A. Karr, Y. Wang, A. R. Batton, E. G. Ruby & E. V. Stabb, (2010) The alternative oxidase (AOX) gene in Vibrio fischeriis controlled by NsrR and upregulated in response to nitric oxide. *Mol Microbiol.* **77:** 44-55.

Dunn, A. K., D. S. Millikan, D. M. Adin, J. L. Bose & E. V. Stabb, (2006) New rfp- and pES213-Derived Tools for Analyzing Symbiotic Vibrio fischeri Reveal Patterns of Infection and lux Expression In Situ. *Appl. Environ. Microbiol.* **72**: 802-810.

Dunn, A. K., M. O. Martin & E. V. Stabb, (2005) Characterization of pES213, a small mobilizable plasmid from Vibrio fischeri. *Plasmid* **54**: 114-134.

Hanahan, D., (1983) Studies on transformation of Escherichia coli with plasmids. *J Mol Biol*. **166**: 557-580.

Stabb, E. V., and E. G. Ruby, (2002) RP4-based plasmids for conjugation between Escherichia coli and members of the Vibrionaceae. *Methods Enzymol.* **358**: 413-426.