Microreview

The roles of NO in microbial symbioses

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Summary

Because of its unique chemical properties, nitric oxide (NO) is a pluripotent signalling and effector molecule that is implicated in a variety of biological roles. Although NO is known to function in host innate immunity against pathogen invasion, its possible roles in microbial symbioses with animal and plant hosts remain relatively less well defined. In this review, we discuss the mechanisms by which bacteria sense and/or detoxify NO. We then focus specifically on its roles in microbial symbioses of diverse eukaryotic hosts. Using the squid-vibrio light-organ symbiosis as a well-characterized example, we discuss the ways in which NO serves as a signal, antioxidant and specificity determinant in this model symbiosis. Because beneficial microbial associations are older and much more prevalent than pathogenic ones, it seems likely that the former may be evolutionary precursors of the latter. Thus, knowledge of the roles played by NO in mutualisms will provide insights into its function in disease interactions as well.

Nitric oxide (NO): its biochemical properties and biological generation

Nitric oxide is a small gaseous, free-radical molecule that, because of its unique chemical properties, is a pluripotent signalling and effector molecule. NO is implicated in a variety of biological roles including as part of the antimicrobial arsenal against invading pathogens (Fang, 2004), an intracellular signalling mediator in the cardiovascular system (Cannon, 1998), and a cytoprotectant against UV stress (Crane et al., 2010) or oxidative stress (Gusarov and Nudler, 2005). Because NO readily undergoes reactions with oxygen or superoxide anion present in the local environment, secondary reactive nitrogen species (RNS), such as peroxynitrite (ONOO−) and dinitrogen trioxide (N2O3) can be generated (Reiter, 2006). RNS are potent oxidizing or nitrosating species that modify various biomolecular structures, and initiate a series of physiological responses in the cell (Reiter, 2006). Further, NO itself reacts with iron-containing biomolecules like haem, iron-sulfur clusters and metal cofactors, resulting in conformational changes or inactivation of the proteins (Fang, 2004). Thus, with its high solubility in water and lipids, and reactivity with diverse biomolecules at physiological pH, NO has a range of chemical properties that allow it to participate in various biological processes.

NO sensing mechanisms in bacteria

Since 1985, NO has been recognized as a multifunctional player in the immune system (Bogdan, 2001). Its functions involve antimicrobial activity, anti-tumour activity, tissue-damaging effects, and modulation of the production and function of cytokines and growth factors. Together with NADPH oxidase-mediated reactive oxygen species (ROS) produced during the phagocyte respiratory burst, NO creates a variety of secondary RNS of even higher toxicity (Fang, 2004). When high concentrations of ROS and RNS work synergistically to attack the proteins, lipids and DNA of invading pathogens, they effectively kill or reduce their replication. Thus, the ability to sense NO may be critical for the microorganisms to prepare for...
their defences against these host-derived antimicrobial compounds.

Microorganisms, including many pathogens, use various transcriptional regulators to sense NO and mount cellular responses. This topic has been extensively reviewed (Spiro, 2007; 2008; Tucker et al., 2010) and will not be discussed here. Recently, bioinformatic analyses of sequenced microbial genomes have revealed that the haem NO/Oxygen binding (H-NOX) protein is widely present in bacteria (Iyer et al., 2003), adding a new member to the family of bacterial NO sensors. In mammals, NO at low (i.e. nanomolar) concentrations exerts most of its signalling effects by binding to the H-NOX domain of the soluble guanylate cyclase (Boon et al., 2005; Cary et al., 2006). This interaction results in conformational changes in the protein, and triggers guanylate cyclase activity, which catalyses the production of 3′, 5′-cyclic GMP, an important second-messenger molecule that engages in various physiological processes including vasodilation, immunomodulation and platelet disaggregation (Blaise et al., 2005). Interestingly, bacterial H-NOX can be a stand-alone small protein, or be fused to either a methyl-accepting chemotaxis receptor or the signal-sensing domains of a histidine kinase. When the hnoX gene encodes a stand-alone protein, it is generally co-transcribed with the gene encoding a histidine kinase or a ORF encoding a GGDEF protein (i.e. diguanylate cyclase, the enzyme catalysing the production of c-di-GMP). GGDEF proteins are associated with the modulation of diverse cellular responses (Iyer et al., 2003). Such a genetic organization leads to the compelling hypothesis that bacterial H-NOX proteins function by sensing an environmental NO signal, and then initiating two-component signal transduction or regulating levels of c-di-GMP.

Surprisingly, in contrast to the extensive knowledge on the molecular basis of H-NOX/NO interaction (Boon et al., 2005; Cary et al., 2006), the biological function of bacterial H-NOX has only recently begun to be elucidated. In Shewanella oneidensis MR-1, binding of NO to H-NOX inhibits the autophosphorylation activity of its cognate histidine kinase (Price et al., 2007). Because NO is formed during anaerobic nitrate respiration, the H-NOX/NO and histidine kinase are believed to work together to function as a novel two-component signalling transduction pathway in S. oneidensis. Unfortunately, no further study has been reported on the genes that are differentially regulated as a result of this H-NOX/NO interaction. More recently, using the squid-vibrio light-organ symbiosis as a model system (see below), Wang et al. reported that the H-NOX of Vibrio fischeri senses host-derived NO and modulates symbiotic colonization (Wang et al., 2010a). H-NOX/NO sensing was also found to regulate c-di-GMP metabolism and biofilm formation in the pathogen Legionella pneumophila. In this latter study, binding of NO to the L. pneumophila H-NOX inhibited the diguanylate cyclase activity of a GGDEF-EAL protein, preventing c-di-GMP accumulation and hyper-biofilm formation (Carlson et al., 2010). NO signalling and its effects on c-di-GMP turnover and biofilm dispersal have been reported previously in the opportunistic pathogen Pseudomonas aeruginosa (Barraud et al., 2009). However, both the nature of the NO sensor, and the mechanism by which sensing leads to changes in c-di-GMP levels, still remain unclear. Because microbes are present in many other diverse NO-containing environmental niches, it is likely that novel biological functions of H-NOX-dependent NO sensing remain to be discovered.

NO detoxification in bacteria

Because NO is used by the host innate immune system to counter microbial infection, and is endogenously produced as an intermediate during denitrification, microorganisms have evolved mechanisms to cope with it. With the advent of genome-wide studies using DNA microarrays, the global genetic responses to NO (Hyduke et al., 2007) or other RNS (Mukhopadhyay et al., 2004) in E. coli have been identified. For instance, Hyduke et al. demonstrated that flavohemoglobin (Hmp), flavobredoxin and its redox partner (NorVW), and the iron-sulfur cluster repair module (IscRSU) are the major NO defence systems. Hmp is one of the best-studied NO-defence mechanisms this far, and has been shown to be a virulence factor in Salmonella and other pathogens (Bang et al., 2006). In summary: (i) the expression of hmp gene is highly inducible by NO and nitrosating agents via its key regulator NsrR (Mukhopadhyay et al., 2004; Hyduke et al., 2007; Wang et al., 2010a), (ii) purified Hmp binds NO avidly in vitro (Poole and Hughes, 2000), (iii) in the presence of oxygen, Hmp converts NO to nitrate through NO denitrosylase or dioxygenase activities, while it reduces NO to nitrous oxide under microaerophilic or anaerobic conditions (Poole and Hughes, 2000) and (iv) an hmp mutant is hypersensitive to NO and nitrosative stress, and is arrested in aerobic respiration when NO is present (Stevanin et al., 2000). Interestingly, an Hmp homologue is found in many microorganisms with different life styles: e.g. enterobacteria (Hyduke et al., 2007), bacterial pathogens (Bang et al., 2006; Richardson et al., 2006) and bacterial symbionts (Meilhoc et al., 2010; Wang et al., 2010b). This diversity suggests that NO stress is often encountered by bacteria as they grow and colonize very distinct niches.

In the absence of oxygen, the major mechanism for removing NO involves reduction by the combined action of flavobredoxin (NorV) and its associated NADH: flavobredoxin oxidoreductase (NorW) (Gardner et al., 2010).
genic enteric bacteria have and NorVW work together to protect the pathogen in the oxygen-limited environment of the gut. Indeed, Nrf serovar Typhimurium against NO killing under enterica detoxifiers (Poock et al., 2002). Because many pathogenic enteric bacteria have nrf genes, it is possible that Nrf plays roles in dealing with the NO challenge occurring in the oxygen-limited environment of the gut. Indeed, Nrf and NorVW work together to protect the pathogen S. enterica serovar Typhimurium against NO killing under anoxic conditions (Mills et al., 2008). In addition, given that iron-sulfur clusters readily react with NO, and that enzymes containing them play critical physiological roles, it is not surprising that genes encoding complexes (e.g. IscRSrSCA) that can play a role in repairing these clusters are also responsive to NO induction. While they do not directly consume NO, the repair complexes confer protection to anaerobically grown E. coli against NO (Justino et al., 2005; Hyduke et al., 2007). Therefore, the presence of NO sensors, detoxifying pathways and enzyme-repair modules allows microorganisms to sense environmental NO, mount appropriate responses and cope with this challenging molecule.

**Host–microbe signalling in beneficial symbioses**

Almost all plants and animals have symbiotic associations with microorganisms for part or all of their life cycle. The presence of these associations exerts an enormous influence on the hosts' growth, development and adaptation to the environment. Given the pivotal roles that the natural microbiota play in maintaining a healthy state, it is necessary to understand the factors that create stable associations between the host and its many microbial partners.

Experimentally accessible model systems have proved to be invaluable tools for understanding signalling within host–microbe associations. The rapid development and application of molecular genetics and biochemistry make it increasingly possible to decipher the 'molecular conversations' between the partners as they establish and maintain their long-term associations (Ruby, 2008). For example, Nod factor, the signalling molecule produced by nitrogen-fixing rhizobia, induces transcriptional and developmental changes in the roots of leguminous plants, thereby allowing bacterial invasion (Mitra et al., 2004). Similarly, during the initiation of the squid light-organ association, the bacterial symbiont releases a peptidoglycan fragment called 'tracheal cytotoxin' (TCT) that acts in synergy with lipopolysaccharide (LPS) to trigger tissue development in the host (Koropatnick et al., 2004). No doubt, further developments in molecular technology, such as high-throughput genome sequencing and the application of RNAi, will advance the knowledge of the specificity and biochemical communication that occur in such persistent, stable host–microbe interactions.

Beneficial and pathogenic symbioses are often viewed as fundamentally different manifestations of host–microbe interactions. However, recent research has suggested that these two types of associations share common molecular mechanisms underlying the way they function with their hosts (Hentschel et al., 2000). As a result, a new exciting paradigm has emerged: bacterial signalling molecules (such as TCT) and their corresponding host response pathways, while originally discovered as microbrial toxins and immunological reactions to pathogens are, in fact, critical to the communication between the host and its normal microbiota (Koropatnick et al., 2004; Rakoff-Nahoum et al., 2004; Rawls et al., 2004).

**The roles of NO in beneficial microbial symbioses with plants and animals**

There has been much work published on NO production in pathogenic microbial infections; in contrast, only recently has its role in beneficial symbioses been recognized. For instance, in the leguminous plant-rhizobia symbioses, NO can be detected in the root nodules, where nitrogen fixation by the bacterial symbiont takes place (Baudouin et al., 2006). However, the source of this NO, and the cells that produce it, remain unclear. In the past, NO was believed to be produced only by the plant's nitrite reductase and a NOS-like enzyme (Baudouin et al., 2007). More recent studies suggest that bacterial nitrate and nitrite reduction also contribute to NO generation in the nodules (Meakin et al., 2007). Because it has been detected during conditions of hypoxia brought on by drought, flooding and pathogen infection, NO is generally regarded as a plant stress signal (Dordas et al., 2003; Ferrarini et al., 2008). In addition, given its reactivity with haem groups, NO binds readily with leghaemoglobin (Meakin et al., 2007), the important protective buffer against the destructive effect of oxygen on nitrogenase. As a result, NO-mediated nitrosylhaemoglobin formation competitively inhibits leghaemoglobin from binding oxygen, thereby decreasing nitrogenase activity and nitrogen fixation. This mechanism explains why NO is a potent inhibitor of nitrogen fixation (Trinchant and Rigaud, 1982). Accordingly, studies of a mutant of the plant Lotus japonicus, which produces less NO inside nodules, demonstrate increased nitrogen fixation activity and nodulation (Tominaga et al., 2009). Interestingly, a recent transcriptomic study of the responses of Sinorhizobium meliloti to NO has revealed that 100 genes are upregulated in response to NO, and most of these are predicted to be regulated by one of the two-component systems.
FixLJ or NnrR. Not surprisingly, among the most inducible genes is hmp (Meilhoc et al., 2010). An hmp mutant of S. meliloti not only displays a growth defect in culture upon NO challenge, but also shows decreased nitrogen fixation in planta. These data are the first indication of bacterial NO responses in a plant-microbial symbiosis (Meilhoc et al., 2010), and they suggest that an NO detoxification system (e.g. Hmp) may contribute to ameliorating the inhibitory effects of NO on the vital symbiotic process of nitrogen fixation.

In a transcriptional analysis of the host plant, Medicago truncatula, the expression of plant genes was determined in response to NO under conditions of either pathogenic or beneficial microbial colonization (Ferrarini et al., 2008). Interestingly, NO-responsive genes were found to behave differently depending, not only on the plant tissue examined, but also on the type of bacterial interaction occurring. For instance, only 10 NO-related genes were significantly upregulated during both pathogenic and beneficial interactions. However, the expression of an additional 47 NO-related genes, mainly related to signal transduction and regulation of glutaredoxin synthesis, while changed in the presence of NO, had an opposite response pattern in pathogenesis compared with mutualism. These findings suggest that NO participates in signal transduction in both types of plant-microbe interactions; however, the differential regulation of the target region of genes may lead to very different outcomes for the bacterial interaction. Similar observations were described for another model leguminous plant, L. japonicus (Nagata et al., 2008). In this study, the host also responded differentially to its beneficial symbiont relative to a pathogen. NO was produced as a plant defence molecule in both associations. However, when the beneficial symbiont was inoculated, the host expressed the class-1 haemoglobin gene, presumably to effectively modulate the increased NO levels; in contrast, exposure to the pathogen did not induce this host gene, resulting in the continuous production of NO and subsequent defence responses. Thus, leghaemoglobin should be regarded as an important modulator of NO levels in the initial stages of plant-rhizobia interactions. In summary, NO appears to be a two-edged sword in plant associations with rhizobial symbionts: it serves as a defensive and signalling molecule in the early stages of the symbiosis but, at the same, reduces nitrogenase activity by reacting with leghaemoglobin.

Besides being active in nitrogen-fixing plants, NO is also detected in diverse microbial symbioses with animal hosts ranging from insects, to nematodes, to marine invertebrates. For example, immunohistochemical localization has indicated that NO is primarily synthesized by the calcium/calmodulin-dependent isoform of NOS, and functions during embryological development of aphids. This NOS was localized to bacteriocytes, the insect cells that house the bacterial endosymbiont Buchnera aphidicola. It is speculated that that aphid-derived NO is involved in both host-defensive responses and symbiotic specificity (Ganassi et al., 2005). Another common insect-associated bacterium is Wolbachia, the endosymbiont of Dirofilaria immitis, a filarial nematode implicated in human pulmonary dirofilariasis. Interestingly, when the nematode infects a mouse, the major surface protein of the endosymbiont induces both iNOS expression and NO production, as well as pro-inflammatory cytokine accumulation in the infected vertebrate host (Morchón et al., 2007). Thus, besides nematode-specific molecules (Tezuka et al., 2002), Wolbachia surface components can also induce pro-inflammatory responses.

Corals are symbiotic assemblages consisting of the cnidarian host and its intracellular dinoflagellates. In this ecologically important association, these algae provide the host with photosynthetically derived nutrients, and promote calcification (Trapido-Rosenthal et al., 2005). However, environmental stresses (e.g. heat and UV illumination) cause coral bleaching, the loss of symbiotic algae, which results in significantly depressed coral growth and increased susceptibility to diseases and mortality (Trapido-Rosenthal et al., 2005). The levels of NO found in host tissues strongly correlate with the extent of coral bleaching, leading to its hypothetical role as a stress signal that mediates the breakdown of the symbiosis (Perez and Weis, 2006). As a result, the source of this NO, as well as how it is linked to coral bleaching, have become an important focus in the study of coral biology. Staining of NO with the sensitive and specific fluorescent probe diaminofluorescein (DAF-2DA), and measurement of NOS activity, show that NO can be produced by both the host coral and the symbiont during bleaching (Trapido-Rosenthal et al., 2005; Perez and Weis, 2006; Bouchard and Yamasaki, 2008; Safavi-Hemami et al., 2010). To understand the molecular and cellular basis of bleaching, the changes in gene expression were determined in a Caribbean coral (Desalvo et al., 2008). This medium-scale transcriptome study showed that, in response to thermal stress, host-derived NO may contribute to a decrease in metabolism. That is, because NO specifically inhibits mitochondrial NADH-ubiquinone reductase, it will also indirectly disturb ATP generation (Ribó et al., 2001). In addition, NO may decrease fatty acid biosynthesis and disrupt amino acid metabolism in coral tissues. Finally, NO, together with stress-induced mitochondrial ROS generation, creates reactive RNS that are toxic to the algal symbiont, resulting in a reduced level of photosynthesis (Desalvo et al., 2008). Together these effects work in synergy to disrupt intracellular Ca$^{2+}$ homeostasis, which in turn can produce a subsequent breakdown of the actin cytoskeleton and host cell death.

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Hundreds of species of symbiotic bacteria live inside and on the surfaces of humans and other vertebrates as well, and they have a profound influence on our NO metabolism and disease susceptibility. For instance, because sweat contains nitrate in appreciable amounts, and symbiotic bacteria on the skin contribute to the reduction of nitrate to nitrite, microbial acidification of the skin surface can further promote nitrite conversion to NO, providing a primary defence against infections by pathogenic fungi and bacteria (Weller et al., 1996). Thus, there have been attempts to design artificial NO-generating devices that can be applied to the treatment of infected wounds (Jones et al., 2010).

**NO in the squid-vibrio symbiosis: signal, antioxidant and specificity determinant**

The light-organ association between the bioluminescent Gram-negative marine bacterium *V. fischeri* and the Hawaiian bobtail squid *Euprymna scolopes* has been studied for more than 20 years as a natural, experimentally tractable, symbiosis. Studies of the roles of NO in this beneficial host–microbe association (Davidson et al., 2004; Wang et al., 2010a,b; Altura et al., 2011) have made it perhaps the best-characterized symbiosis system in terms of the source of NO production, the effects of NO on both partners and the responses that NO initiates in the symbiont.

During the initiation of symbiosis, *V. fischeri* cells aggregate in mucus that is secreted by a superficial, ciliated, host epithelium in response to peptidoglycan present in the surrounding seawater (Nyholm and McFall-Ngai, 2004) (Fig. 1). Once aggregated, symbionts then migrate towards the pores of the light organ, through the epithelium-lined ducts/antechamber, finally arriving at the deep crypts, where they form a persistent extracellular association with the host. Staining with specific antibodies and DAF-2DA have shown that NOS, and its product NO, are active in the early stages of the symbiosis (Davidson et al., 2004). Briefly, NOS and NO are both detected in vesicles in the secreted mucus, where *V. fischeri* cells aggregate (Fig. 2A). The NO contained in these vesicles controls the aggregate size and probably also contributes to specificity determination: treatment with an NO scavenger allows the normally non-symbiotic *Vibrio parahaemolyticus* to form hyperaggregates in the mucus (Davidson et al., 2004). A stronger level of NO staining occurs in the epithelium-lined duct/antechamber region, along which *V. fischeri* cells pass before reaching their final destination (Fig. 2B). Successful colonization of the deep crypts irreversibly attenuates the NOS and NO levels in these regions (Fig. 2C), suggesting that the host has specific mechanisms to sense and respond to the presence of the correct symbiont. Taken together, these observations immediately pose some interesting questions: (i) why is NO produced at different positions and concentrations in the symbiosis? (ii) How does the bacterial symbiont respond to NO? and (iii) what is the signal that tells the host squid to reduce NO production once the mature colonization is established?

As described above, *V. fischeri hnoX* encodes an NO-binding protein with a picomolar affinity (Wang et al., 2010a). H-NOX apparently acts as an NO sensor involved in signal transduction and regulation of symbiosis-related genes during colonization of the squid light organ. Given that host-derived NO is present during the early stages of the symbiosis, one would predict that deletion of this NO sensor would make the resultant mutant strain (Δ*hnoX*) defective in host colonization. However, the mutant is actually 10-fold more effective in initiating colonization than the wild-type parent, and 16-fold more competitive in the first 24 h in a dual-colonization assay. A whole-genome transcriptome study revealed 20 genes that are repressed in an NO- and H-NOX-dependent manner (Wang et al., 2010a). Ten of these genes, including those encoding the ability to use haemin as an iron source, have a predicted Fur-box in their promoter region, thus linking NO/H-NOX sensing with regulation of iron utilization in *V. fischeri*. Accordingly, NO-pretreated wild-type cells do not grow as fast as the mutant when haemin is the sole iron source, probably because of its repression of haemin utilization. Not surprisingly, the competitive dominance of
the mutant during squid colonization can be reversed by supplementation of the surrounding seawater either with excess haemin or with an inhibitor of NO production, suggesting that the mutant’s advantage in colonizing the iron-limited tissues of the light-organ (Graf and Ruby, 2000) is at least partially due to its greater ability to obtain and grow on haemin (Wang et al., 2010a), which is probably the form of iron provided by the squid host (A. Dunn, pers. comm.), as it is for certain pathogens (Anzaldi and Skaar, 2010).

Interestingly, the operon responsible for the transport of ROS-reactive cystine is also downregulated by NO/H-NOX signalling. Conversely, stress-response genes such as glutathione-S-transferase and the sigma-E regulon are induced (Fig. 2A). Given that NO and ROS are present in the duct/antechamber region of the light organ (Visick and Ruby, 1998; Small and McFall-Ngai, 1999), and that nascent symbionts are exposed to NO-containing vesicles even before they enter the light organ (Davidson et al., 2004), it was hypothesized that NO/H-NOX signalling may prepare the bacterial symbiont to cope with subsequent oxidative stress when migrating through the duct/antechamber region. In agreement with this hypothesis, when pre-grown on haemin as sole iron source, NO-pretreated \( \Delta hnoX \) mutant cells survived \( \text{H}_2\text{O}_2 \) treatment significantly less well than their wild-type parent. One explanation is that damage inflicted by the Fenton reaction is facilitated by the elevated intracellular iron concentration in the mutant. Indeed, the NO-pretreated \( \Delta hnoX \) cells are more susceptible to streptonigrin (Y. Wang and E. Ruby, unpubl. data), whose bacteriocidal activity correlates with the intracellular iron levels (Yeowell and White, 1982). More interestingly, unlike the synergistic killing effect reported in \( E. coli \) (Woodmansee and Imlay, 2003), the simultaneous presence of NO and \( \text{H}_2\text{O}_2 \) dramatically increased the survival of \( V. fischeri \) cells over \( \text{H}_2\text{O}_2 \) treatment alone. This protection may result from a cytoplasmic accumulation of NADH because of an NO-mediated inhibition of respiration (Y. Wang and E. Ruby, unpubl. data). Because both the wild type and the \( \Delta hnoX \) mutant were rescued to a similar degree, this NO-mediated cytoprotection appears to be NO/H-NOX-independent. Therefore, in contrast to those cases where NO works in synergy with ROS to prevent pathogen invasion, NO in the vibrio-squid symbiosis not only may serve a signalling function to prepare the bacteria for subsequent ROS exposure, but also function directly as a potential antioxidant to promote the symbiont’s survival under oxidative stress (Fig. 2).

To cope with NO stress, the symbionts must be equipped with effective NO detoxification mechanisms. Indeed, \( V. fischeri \) encodes several such genes, including \( hmp, \text{norVW} \) and the \( nrf \) operon, as well as their cognate regulators (Rodionov et al., 2005). As in \( E. coli \), the \( hmp \) gene of \( V. fischeri \) is controlled by its negative regulator NsrR, through an inactivation of this protein’s NO-sensitive Fe-S cluster cofactor (Wang et al., 2010b). The expression of \( V. fischeri hmp \) is highly NO-inducible, mainly through the inactivation of NsrR. Interestingly, pretreatment of the wild-type strain with a low concentration

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**Fig. 2.** The roles of NO at different stages of the initiation of the squid-vibrio symbiosis.

A. As \( V. fischeri \) cells aggregate in host mucus, they first contact host-derived NO (red), which initiates a series of responses (dotted lines indicate hypothesized events)

B. While migrating down the duct, the cells encounter higher NO levels.

C. Once in the crypts, surface molecules from the symbionts initiate developmental signalling and morphogenesis in the host by downregulating nitric-oxide synthase (NOS) activity (see text for details).
of NO makes it resistant to a subsequent high-dose NO challenge. When *V. fischeri* mutant strains that are defective in one or more of NO detoxification systems are tested in squid colonization, only the Δhmp shows any defects; however, this mutant can be rescued in the presence of a NOS inhibitor, suggesting the importance of aerobic NO detoxification for a successful initiation of symbiosis. Further experiments indicated that: (i) the hmp promoter is activated via NsrR-mediated NO sensing during aggregation and (ii) high levels of hmp expression lead to larger than normal aggregates (Wang et al., 2010b). Thus, it appears that exposure to NO-containing vesicles during the aggregation stage serves as a pretreatment during which the bacteria induce the expression of hmp, the major NO detoxification system of *V. fischeri*, to cope with a subsequent host-derived NO stress.

The *V. fischeri* genome also encodes the unusual alternative oxidase (AOX), a respiratory oxidase mainly found in eukaryotes and a few bacterial species. Like the hmp gene, aox also belongs to the NO-responsive NsrR regulon. Interestingly, NO partially inhibits cytochrome bd-type quinol oxidase, CydAB-dependent, NADH oxidation, while having no effect on AOX-mediated NADH oxidation. This study was the first report of a physiological function for any bacterial AOX (Dunn et al., 2010). During host colonization, a Δaox mutant does not have any detectable defects, while a Δhmp-aox behaves like a Δhmp, suggesting that Hmp-mediated NO detoxification may mask the AOX function (Y. Wang and E. Ruby, unpubl. data). It is tempting to propose that the pre-exposure to NO occurring during aggregation may also induce aox expression. Such induction would, in turn, enable *V. fischeri* cells to continue aerobic respiration despite the presence of host-derived NO stress and, thereby, provide an alternative route of NO resistance (Spiro, 2010).

Nitric oxide production by the juvenile squid responds to the presence of symbiotic *V. fischeri* in the light organ (Altura et al., 2011). Briefly, LPS and TCT released by the symbionts work in synergy to strongly attenuate NOS/NO activity (Fig. 2C); in contrast, bacterial mutants that either are defective in TCT release or have an altered LPS fail to do so. Moreover, experiments in which juvenile squid were treated with either a NOS inhibitor or an NO donor (Altura et al., 2011) demonstrated that changes in NO-production alone can mediate the apoptosis and tissue morphogenesis typical of symbiosis-induced development (Koropatnick et al., 2004). Thus, while in certain pathogenic host–microbe interactions, LPS and TCT work together to increase NOS/NO (Flak et al., 2000), in the beneficial squid-vibrio symbiosis, these microbial products exert an opposite effect on NO production by the host. Thus, this work reveals another case of context-specific host responses to bacteria-associated surface molecules. Taken together, the studies presented above demonstrate that in the squid-vibrio light organ, NO not only acts as a signal molecule that prepares symbiont cells to better survive oxidative and NO stress, but also participates in host tissue development.

In conclusion, NO has an increasingly recognized repertoire of biological functions: as a signal effector, a defence molecule, and an antioxidant in diverse microbial symbioses with plant and animals. In contrast to its important role as an antimicrobial molecule during pathogen invasion, NO in beneficial associations may serve as an important ‘pre-adaptation’ signal that induces critical stress responses in appropriate bacteria. Thus, co-evolution of both partners seems to confer upon a symbiont the capacity to exploit host-derived signals to increase its colonization proficiency. In the future, using emerging experimental strategies and technological approaches, novel functions for NO in other microbial symbioses are likely to be discovered. In this way, we will not only broaden our understanding of the roles of this small molecule in symbiosis, but also continue to use a comparative approach to provide insights into the role of NO in pathogenic microbe–host interactions as well.

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