Cellular Microbiology (2011) doi:10.1111/j.1462-5822.2011.01576.x

Microreview

The roles of NO in microbial symbioses

Yanling Wang and Edward G. Ruby*
Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, 1550 Linden Dr., Madison, WI 53706, USA.

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 (N_2O_3) 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.

Nitric oxide is typically produced enzymatically by NO synthase (NOS) (Palmer *et al.*, 1988). In the presence of oxygen, NOS catalyses the oxidation of L-arginine to NO and L-citrulline. Studies in mammals have linked NOS-derived NO with a number of normal biological processes, such as the control of blood pressure and nerve cell transmission. Interestingly, over the past 10 years, bacterial proteins homologous to eukaryotic NOS have been identified and characterized, mainly in Gram-positive microbes and archaea. The biological roles of bacterial NOS have just begun to be appreciated, and point to an association of NO with cytoprotection against oxidative stress, UV stress and the biosynthesis of toxin (Crane *et al.*, 2010).

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

Received 11 November, 2010; revised 10 January, 2011; accepted 13 January, 2011. *For correspondence. E-mail egruby@wisc.edu; Tel. (+608) 262 5911; Fax (+608) 262 8418.

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), flavorubredoxin and its redox partner (NorVW), and the iron-sulfur cluster repair module (IscRSUA) 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 flavorubredoxin (NorV) and its associated NADH: flavorubredoxin oxidoreductase (NorW) (Gardner *et al*.,

2002). It is believed that the O_2-dependent Hmp and the O_2-sensitive NorVW work in concert to detoxify NO throughout the physiological range of oxygen concentrations a cell may encounter (Gardner *et al*., 2003). In addition, the periplasmic cytochrome-c nitrite reductase (NrfA) of *E. coli* catalyses NO reduction both *in vitro* and *in vivo*, adding another member to the family of anaerobic NO 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. IscRSCA) 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 microbial 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 nitrosylleghemoglobln 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 regulon 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 (Riobó *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.

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, symbiont cells 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 parahemolyticus* 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

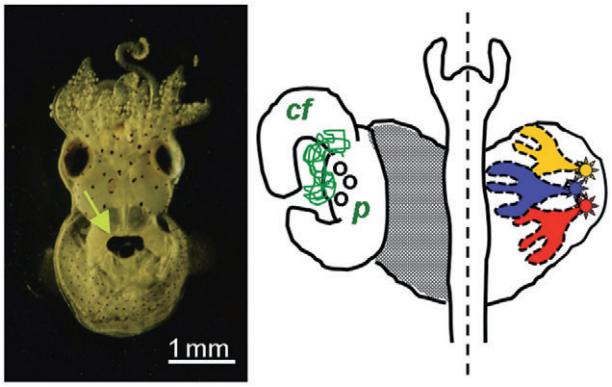

Fig. 1. The symbiotic light organ of the squid *E. scolopes*. Left, a ventral view of a juvenile animal revealing the nascent light organ in the centre of the animal's mantle (body) cavity (arrow). Right, a diagram of the bisected organ (dashed line) to show the surface (left half) and interior (right half). The surface of the hatchling organ has transparent, superficial ciliated fields (*cf*) on its lateral faces that facilitate the harvesting of environmental *V. fischeri* cells into the region of the three pores (*p*, below and right of the circular pores) on each side of the organ. *V. fischeri* cells from the environment aggregate in the mucus (green) associated with the cilia The symbionts then enter the pores, travel down ducts and take up residence in the three independent blind crypt spaces (yellow, blue, red) on either side of the organ. During this entire process, the symbionts interact with host-derived NO (see text for details).

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

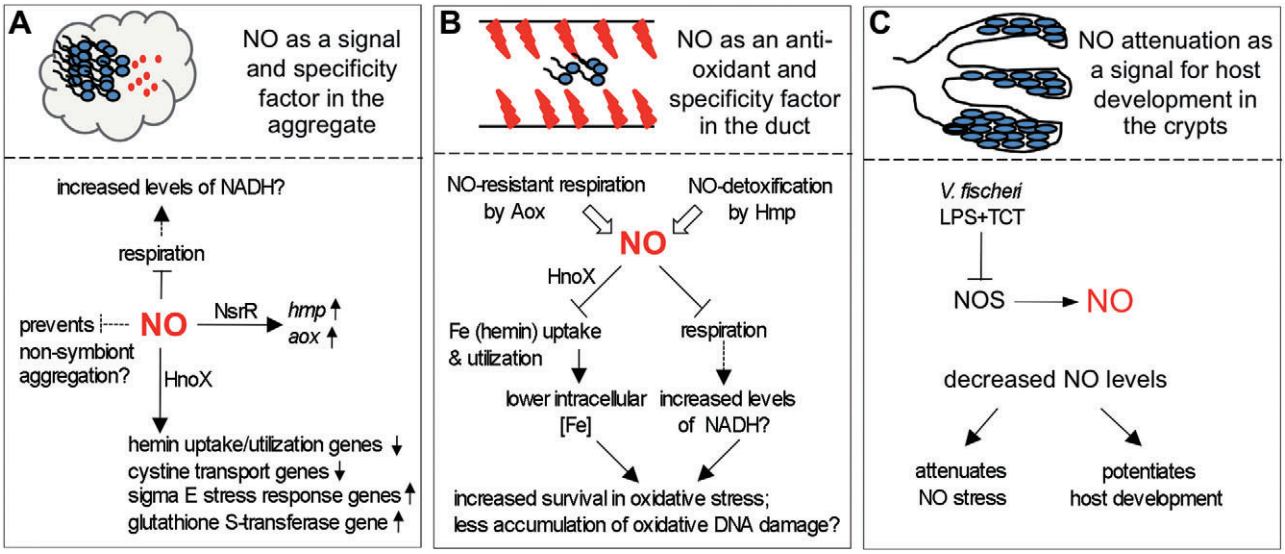

Fig. 2. The roles of NO at different stages of the initiation of the squid-vibrio symbiosis.
A. As *V. fischeri* cells aggregate in host mucus, they first contact host-derived NO (red), which initiates a series of responses (dotted lines indicate hypothesized events).
B. While migrating down the duct, 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).

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-tranferase 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 Δ*hnoX* mutant cells survived H_2O_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 Δ*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 H_2O_2 dramatically increased the survival of *V. fischeri* cells over H_2O_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 Δ*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*, *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

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 Δ*hmp* 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 pre-treatment 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 Δ*aox* mutant does not have any detectable defects, while a Δ*hmp-aox* behaves like a Δ*hmp*, 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.

Acknowledgements

The authors thank M. Altura, A. Dunn and M. McFall-Ngai for critical advice, unpublished data and images. Funding support came from NSF Grant IOS-0817232 (M.M-N and E.G.R.) and NIH Grant R01 RR 12294 (E.G.R and M.M-N.).

References

Altura, M.A., Stabb, E.V., Goldman, W., Apicella, M., and McFall-Ngai, M.J. (2011) Attenuation of host NO production by MAMPs potentiates development of the host in the squid-vibrio symbiosis. *Cell Microbiol* (in press).

Anzaldi, L.L., and Skaar, E.P. (2010) Overcoming the heme paradox: heme toxicity and tolerance in bacterial pathogens. *Infect Immun* **78:** 4977–4989.

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.

Barraud, N., Shlehech, D., Klebensberger, J., Webb, J.S., Hassett, D.J., Rice, S.A., and Kjelleberg, S. (2009) Nitric oxide signaling in *Pseudomonas aerogina* biofilms mediates phosphodieterase activity, deceased cyclic-di-GMP lelves and enchanced dispersal. *J Bacteriol* **191:** 7333–7342.

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.

Baudouin, E., Pauly, N., and Puppo, A. (2007) Nitric oxide in nitrogen-fixing symbiosis. In *Nitric Oxide in Plant Growth, Development and Stress Physiology*. Lamattina, L., and Polacco, J. (eds). Berlin: Springer, pp. 173–186.

Blaise, G.A., Gauvin, D., Gangal, M., and Authier, S. (2005) Nitric oxide, cell signaling and cell death. *Toxicology* **208:** 177–192.

Bogdan, C. (2001) Nitric oxide and the immune response. *Nat Immunol* **2:** 907–916.

Boon, E.M., Huang, S.L., and Marletta, M.A. (2005) A molecular basis for NO selectivity in soluble guanylate cylase. *Nat Chem Biol* **1:** 53–59.

Bouchard, J.N., and Yamasaki, H. (2008) Heat stress stimulates nitric oxide production in *Symbiodinium microadriaticum*: a possible linkage between nitric oxide and the coral bleaching phenomenon. *Plant Cell Physiol* **49:** 641–652.

Cannon, R.O., III (1998) Role of nitric oxide in cardiovascular disease: focus on the endothelium. *Clin Chem* **44:** 1809–1819.

Carlson, H.K., Vance, R.E., and Marletta, M.A. (2010) H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Mol Microbiol* **77:** 930–942.

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

Crane, B.R., Sudhamsu, J., and Patel, B.A. (2010) Bacterial nitric oxide synthases. *Annu Rev Biochem* **79:** 445–470.

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.

Desalvo, M.K., Voolstra, C.R., Sunagawa, S., Schwarz, J.A., Stillman, J.H., Coffroth, M.A., *et al*. (2008) Differential gene expression during thermal stress and bleaching in the Caribbean coral *Montastraea faveolata*. *Mol Ecol* **17:** 3952–3971.

Dordas, C., Hasinoff, B.B., Igamberdiev, A.U., Manac'h, N., Rivoal, J., and Hill, R.D. (2003) Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. *Plant J* **35:** 763–770.

Dunn, A.K., Karr, E.A., Wang, Y., Batton, 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.

Ferrarini, A., Stefano, M.D., Baudouin, E., Pucciariello, C., Polverari, A., Puppo, A., and Delledonne, M. (2008) Expression of *Medicago truncatula* genes responsive to nitric oxide in pathogenic and symbiotic conditions. *Mol Plant Microbe Interact* **21:** 781–790.

Flak, T.A., Heiss, L.N., Engle, J.T., and Goldman, W.E. (2000) Synergistic epithelial responses to endotoxin and a naturally occurring muramyl peptide. *Infect Immun* **68:** 1235–1242.

Ganassi, S., Tagliazucchi, D., and Mola, L. (2005) Occurrence of nitric oxide synthase in *Megoura viciae* Buckton (Homoptera, Aphididae): a histochemical and immunohistochemical localisation. *Eur J Histochem* **49:** 385–393.

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.

Gardner, A.M., Gessner, C.R., and Gardner, P.R. (2003) Regulation of the nitric oxide reduction operon (*norRVW*) in *Escherichia coli*: role of NorR and sigma 54 in the nitric-oxide stress response. *J Biol Chem* **278:** 10081–10086.

Graf, J., and Ruby, E.G. (2000) Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. *Mol Microbiol* **37:** 168–179.

Gusarov, I., and Nudler, E. (2005) NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. *Proc Natl Acad Sci USA* **102:** 13855–13860.

Hentschel, U., Steinert, M., and Hacker, J. (2000) Common molecular mechanisms of symbiosis and pathogenesis. *Trends Microbiol* **8:** 226–231.

Hyduke, D.R., Jarboe, L.R., Tran, L.M., Chou, K.J.Y., and Liao, J.C. (2007) Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in *Escherichia coli*. *Proc Natl Acad Sci USA* **104:** 8484–8489.

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.

Jones, M., Ganopolsky, J., Labbé, A., Wahl, C., and Prakash, S. (2010) Antimicrobial properties of nitric oxide and its application in antimicrobial formulations and medical devices. *Appl Microbiol Biotechnol* **88:** 401–407.

Justino, M.C., Vicente, J.B., Teixeira, M., and Saraiva, L.M. (2005) New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. *J Biol Chem* **280:** 2636–2643.

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.

Meakin, G.E., Bueno, E., Jepson, B., Bedmar, E.J., Richardson, D.J., and Delgado, M.J. (2007) The contribution of bacteroidal nitrate and nitrite reduction to the formation of nitrosylleghaemoglobin complexes in soybean root nodules. *Microbiology* **153:** 411–419.

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.

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.

Mitra, R.M., Shaw, S.L., and Long, S.R. (2004) Six nonnodulating plant mutants defective for Nod factor-induced transcriptional changes associated with the legume-rhizobia symbiosis. *Proc Natl Acad Sci USA* **101:** 10217–10222.

Morchón, R., Bazzocchi, C., López-Belmonte, J., Martín-Pacho, J.R., Kramer, L.H., Grandi, G., and Simón, F. (2007) iNOs expression is stimulated by the major surface protein (rWSP) from *Wolbachia* bacterial endosymbiont of *Dirofilaria immitis* following subcutaneous injection in mice. *Parasitol Int* **56:** 71–75.

Mukhopadhyay, P., Zheng, M., Bedzyk, L.A., LaRossa, R.A., and Storz, G. (2004) Prominent roles of the NorR and Fur regulators in the *Escherichia coli* transcriptional response to reactive nitrogen species. *Proc Natl Acad Sci USA* **101:** 745–750.

Nagata, M., Murakami, E., Shimoda, Y., Shimoda-Sasakura, F., Kucho, K., Suzuki, A., *et al*. (2008) Expression of a class 1 hemoglobin gene and production of nitric oxide in response to symbiotic and pathogenic bacteria in *Lotus japonicus*. *Mol Plant Microbe Interact* **21:** 1175–1183.

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

Palmer, R.M.J., Ashton, D.S., and Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333:** 664–666.

Perez, S., and Weis, V. (2006) Nitric oxide and cnidarian bleaching: an eviction notice mediates breakdown of a symbiosis. *J Exp Biol* **209:** 2804–2810.

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.

Price, M.S., Chao, L.Y., and Marletta, M.A. (2007) *Shewanella oneidensis* MR-1 H-NOX regulation of a histidine kinase by nitric oxide. *Biochemistry* **46:** 13677–13683.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* **118:** 229–241.

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.

Reiter, T.A. (2006) NO* chemistry: a diversity of targets in the cell. *Redox Rep* **11:** 194–206.

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.

Riobó, N.A., Clementi, E., Melani, M., Boveris, A., Cadenas, E., Moncada, S., and Poderoso, J.J. (2001) Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. *Biochem J* **359:** 139–145.

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. (2008) Symbiotic conversations are revealed under genetic interrogation. *Nat Rev Microbiol* **6:** 752–762.

Safavi-Hemami, H., Young, N.D., Doyle, J., Llewellyn, L., and Klueter, A. (2010) Characterisation of nitric oxide synthase in three cnidarian-dinoflagellate symbioses. *PLoS ONE* **5:** e10379.

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.

Spiro, S. (2008) Metalloregulatory proteins and nitric oxide signalling in bacteria. *Biochem Soc Trans* **36:** 1160–1164.

Spiro, S. (2010) An alternative route to nitric oxide resistance. *Mol Microbiol* **77:** 6–10.

Stevanin, T., Ioannidis, N., Mills, C., Kim, S., Hughes, M., and Poole, R. (2000) Flavohemoglobin *hmp* affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes bo' or bd, from nitric oxide. *J Biol Chem* **275:** 35868–35875.

Tezuka, H., Imai, S., Tsukidate, S., and Fujita, K. (2002) A *Dirofilaria immitis* polyprotein up-regulates nitric oxide production. *Infect Immun* **70:** 5283–5286.

Tominaga, A., Nagata, M., Futsuki, K., Abe, H., Uchiumi, T., Abe, M., *et al*. (2009) Enhanced nodulation and nitrogen fixation in the abscisic acid low-sensitive mutant enhanced nitrogen fixation1 of *Lotus japonicus*. *Plant Physiol* **151:** 1965–1976.

Trapido-Rosenthal, H., Zielke, S., Owen, R., Buxton, L., Boeing, B., Bhagooli, R., and Archer, J. (2005) Increased zooxanthellae nitric oxide synthase activity is associated with coral bleaching. *Biol Bull* **208:** 3–6.

Trinchant, J.-C., and Rigaud, J. (1982) Nitrite and nitric oxide as inhibitors of nitrogenase from soybean bacteroids. *Appl Environ Microbiol* **44:** 1385–1388.

Tucker, N.P., LeBrun, N., Dixon, R., and Hutchings, M. (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 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. (2010a) H-NOX–mediated nitric oxide sensing modulates symbiotic colonization by *Vibrio fischeri*. *Proc Natl Acad Sci USA* **107:** 8375–8380.

Wang, Y., Dunn, A.K., Wilneff, J., McFall-Ngai, M.J., Spiro, S., and Ruby, E.G. (2010b) *Vibrio fischeri* flavohaemoglobin protects against nitric oxide during initiation of the squid–*Vibrio* symbiosis. *Mol Microbiol* **78:** 903–915.

Weller, R., Pattullo, S., Smith, L., Golden, M., Ormerod, A., and Benjamin, N. (1996) Nitric oxide is generated on the skin surface by reduction of sweat nitrate. *J Investig Dermatol* **107:** 327–331.

Woodmansee, A.N., and Imlay, J. (2003) A mechanism by which nitric oxide accelerates the rate of oxidative DNA damage in *Escherichia. coli*. *Mol Microbiol* **49:** 11–22.

Yeowell, H.N., and White, J.R. (1982) Iron requirement in the bactericidal mechanism of streptonigrin. *Antimicrob Agents Chemother* **22:** 961–968.