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NITRIC OXIDE REGULATION OF H-NOX SIGNALING PATHWAYS IN BACTERIA

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Abstract

Nitric oxide (NO) is a freely diffusible, radical gas that has now been established as an integral signaling molecule in eukaryotes and bacteria. It has been demonstrated that NO signaling is initiated upon ligation to the heme iron of an H-NOX domain in mammals and in some bacteria. Bacterial H-NOX proteins have been found to interact with enzymes that participate in signaling pathways and regulate bacterial processes such as quorum sensing, biofilm formation and symbiosis. Here, we review the biochemical characterization of these signaling pathways, and where available, describe how NO ligation to H-NOX specifically regulates the activity of these pathways and their associated bacterial phenotypes.

Graphical Abstract

BACKGROUND

Nitric oxide (NO) is a highly diffusible gas molecule that is soluble in water and lipids. NO has been shown to interact with an array of biomolecules at physiological pH, and has

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consequently been shown to be involved in many biological processes in both bacteria and eukaryotes.^{1,2} The biological effects of NO are concentration-dependent. In mammals, at low concentrations (sub-micromolar), NO plays an integral role in regulating physiological processes such as smooth muscle relaxation, vasodilation and neurotransmission.^{3,4} In eukaryotes⁵ and some bacteria,⁶ NO is synthesized by the enzyme nitric oxide synthase (NOS) via the oxidation of L-arginine to NO and L-citrulline. Subsequently, in eukaryotes, NO binds to the H-NOX (heme-nitric oxide/oxygen binding) domain of the enzyme soluble guanylate cyclase (sGC). The cyclase domain of sGC then becomes active and catalytically converts GTP into cyclic GMP (c-GMP). The production of c-GMP regulates downstream signaling events, such as those mentioned above.⁷

At high concentrations, NO is a toxic gas produced by eukaryotes to fight tumors and bacterial infections.^{2,8–10} The concentrations of NO used to kill invading pathogens also damage host cells, thus eukaryotes are able to respond to NO present at concentrations above that needed to activate sGC.^{9–12} From the bacterial perspective, in addition to NO exposure during infection, bacteria are also exposed to high amounts of NO during denitrification, a process in which bacteria respire nitrate or nitrite under oxygen-limiting conditions.¹³ Because bacteria experience high concentrations of NO during detoxification and denitrification, many NO-responsive proteins have been characterized, including FNR-like transcription factors (fumarate and nitrate regulatory proteins),¹⁴ the NO-responsive transcriptional activator NorR (regulator of NO reductase),¹⁵ and the NO-sensitive repressor NsrR (repressor of nitrosative stress).¹⁶ Bacteria typically detoxify high concentrations of NO using NO-binding enzymes such as flavohemoglobins, flavorubredoxin nitric oxide reductases, respiratory nitric oxide reductases, and cytochrome c nitrite reductases, each of which converts NO into less toxic molecules such as ammonia, nitrate, and nitrous oxide.^{17–21}

Interestingly, recent data indicate that bacteria also respond to low concentrations of NO to elicit physiological responses other than those involved in NO elimination. The details of these signaling pathways are not fully elucidated, but one sensitive NO sensor has been described in bacteria. Namely, like eukaryotes, bacteria code for H-NOX domains.

The heme domain of the eukaryotic NO sensor sGC is a member of a family of hemoproteins termed H-NOX. H-NOX domains are encoded in many bacterial genomes, including some pathogens.^{22–24} Bacterial H-NOX domains share 15–40% sequence identity with mammalian sGC H-NOX domains.²⁵ H-NOX proteins encoded by facultative anaerobes, like mammalian sGCs, bind NO and carbon monoxide (CO),^{23,25} whereas H-NOX proteins from obligate anaerobes bind NO, CO, and also molecular oxygen.^{22,23} In fact, recent structural studies have suggested that H-NOX proteins from obligate anaerobes may function as oxygen sensing proteins.²⁶ All H-NOX proteins, however, are histidine-ligated protoporphyrin IX hemoproteins that bind their gaseous ligands at a ferrous iron center, and all exhibit slow NO dissociation kinetics with an assumed diffusion-limited association rate constant of $\sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$.^{27,28} Therefore, H-NOX proteins have approximately picomolar affinity for NO,²⁹ which is consistent with their roles as selective NO sensors in both mammals (sGC) and bacteria (isolated H-NOX domains). For more

information on the ligand binding properties of H-NOX proteins, several reviews are available.^{24,30–32}

Within bacterial genomes, *hnoX* genes code for stand-alone proteins found in the same putative operons as signaling proteins such as two-component signaling histidine kinases and diguanylate cyclases.³³ The most common arrangement is for *hnoX* to be in the same putative operon as an orphan two-component signaling histidine kinase (without a cognate response regulator in the same operon).²⁹ The histidine kinase and diguanylate cyclase proteins found adjacent to H-NOX domains typically do not contain a sensory domain. Consequently, it is hypothesized that H-NOX functions as a NO sensor and regulates the downstream signaling activities of these proteins *in trans*. The focus of this review will be to elaborate on how bacterial H-NOX proteins regulate their respective signaling pathways in a NO dependent manner, with a focus on the reported enzymology of H-NOX-regulated proteins (Figure 1).

NITRIC OXIDE REGULATION OF H-NOX/ HACE SIGNALING PATHWAYS

In some bacteria an *hnoX* gene is predicted to be encoded in the same operon with genes that code for diguanylate cyclase and/or phosphodiesterase proteins, recently collectively termed HaCEs (H-NOX-associated cyclic-di-GMP processing enzymes). These proteins are comprised of diguanylate cyclase and/or phosphodiesterase domains, which are identified from conserved GGDEF and EXL or HD-GYP amino acid motifs, respectively. Diguanylate cyclase domains synthesize cyclic-di-GMP (c-di-GMP) by cyclizing two molecules of GTP at a GGDEF-motif-containing active site. Phosphodiesterase domains have either an EXL or HD-GYP motif in their active site and degrade c-di-GMP into the linear molecule pGpG.³⁴ HD-GYP domains typically further cleave pGpG into two molecules of GMP.³⁵ Cyclic-di-GMP is of significance, as it is the major bacterial secondary messenger molecule that regulates the lifestyle switch between planktonic and biofilm phenotypes; it also regulates other important bacterial processes such as virulence and motility.³⁶

Biofilms occur when bacteria accumulate (frequently attached to a surface) in moist environments within a self-secreted exopolymeric material.³⁷ Biofilms are the leading cause of nosocomial infections^{38,39} and are extremely difficult to treat; cells in a biofilm exhibit up to 1,000-fold higher tolerance to immune defenses and antibiotics.^{40,41} Interestingly, in recent years, low non-toxic concentrations of NO have been shown to mediate biofilm dispersal.⁴²

In some bacteria, the molecular mechanism for NO-regulated biofilm formation has been demonstrated to be dependent on H-NOX/HaCE signaling. Since the *hnoX* gene in certain bacteria is present in the same operon as genes for HaCE proteins, as expected, it has been shown that NO ligation to H-NOX can regulate HaCE activity, contributing to the regulation of intracellular concentrations of cyclic-di-GMP, and ultimately leading to regulation of biofilm formation. Currently, a few of these H-NOX/HaCE systems have been biochemically characterized, including those from the bacteria *Legionella pneumophila*, *Shewanella woodyi* and more recently, *Agrobacterium vitis* (Figure 2).

Legionella pneumophila

In *L. pneumophila*, there are two H-NOX-encoding genes that are involved in regulating two different signaling pathways. One H-NOX gene (*hnoX1*) is adjacent to a di-domain GGDEF-EAL (*LpgHaCE*) protein, while the other H-NOX gene is adjacent to a histidine kinase and a CheY-like response regulator.⁴³ In a recent study it was shown that the EAL domain of *LpgHaCE* is inactive; the protein has only diguanylate cyclase activity. Furthermore, in the absence of NO, the Fe(II) unligated form of *LpgH-NOX1* does not affect the cyclase activity of *LpgHaCE*. When bound to NO, however, *LpgH-NOX1* was shown to inhibit *LpgHaCE* activity. The *LpgHaCE* used in this study, however, was only ~50% pure and demonstrated very low diguanylate cyclase activity; the authors were not able to detect measurable production of c-di-GMP until 24 h post incubation with GTP.⁴³ As a result, the kinetic parameters of *LpgHaCE* were not assessed. Therefore, it is unknown which kinetic parameters of *LpgHaCE* are regulated by Fe(II)-NO *LpgH-NOX1*. This would be interesting to determine as it would provide a more detailed understanding of how NO specifically regulates *LpgHaCE* activity and possibly biofilm formation in this bacterium.

Based on their biochemical results, the authors hypothesized that exposure to NO should decrease the intracellular c-di-GMP concentrations in this bacterium by regulating the activity of *LpgHaCE* via ligation to *LpgH-NOX1*, leading to a decrease in biofilm formation upon exposure to NO. Indeed, they found that genetic deletion of *LpgH-NOX1* led to a hyperbiofilm phenotype, consistent with *LpgH-NOX1* inhibition of *LpgHaCE* activity. The authors assumed that the media in this study contained low nanomolar concentrations of NO generated from the decomposition of nitrate. The NO concentration was not determined, however. Furthermore, no biofilm studies were conducted in the absence of nitrate, thus the effect of this presumed NO cannot be conclusively determined.

Shewanella woodyi

S. woodyi encode only one H-NOX gene adjacent to a di-domain GGDEF-EAL (*SwHaCE*) protein. Our laboratory has shown that *SwHaCE* has both diguanylate cyclase and phosphodiesterase activities.⁴⁴ Using purified proteins we demonstrated that *SwHaCE* has basal diguanylate cyclase activity and relatively efficient phosphodiesterase activity in the absence of *SwH-NOX*. In the presence of the Fe(II) unligated form of the *SwH-NOX*, however, the diguanylate cyclase activity increases ~10 fold, while the phosphodiesterase activity remains unchanged, which therefore enables this protein to function primarily as a diguanylate cyclase when complexed to ferrous H-NOX. In the presence of the Fe(II)-NO ligated form of *SwH-NOX*, however, the diguanylate cyclase activity returns to basal levels but the phosphodiesterase domain hydrolyzes c-di-GMP 1.5 times faster than it did in the presence of the Fe(II) unligated form of *SwH-NOX* or in the absence of H-NOX.⁴⁴ Further analysis of the phosphodiesterase domain revealed that in the presence of Fe(II)-NO ligated *SwH-NOX*, the catalytic efficiency of this domain increased 13-fold as both the k_{cat} and K_M parameters were enhanced. Therefore, *SwHaCE* activity is regulated in an NO dependent manner via ligation to *SwH-NOX*, where Fe(II) *SwH-NOX* increases *SwHaCE* diguanylate cyclase activity, while leaving the phosphodiesterase activity unchanged, and the presence of Fe(II)-NO *SwH-NOX* does the opposite, it decreases *SwHaCE* diguanylate cyclase activity, and enhances *SwHaCE* phosphodiesterase activity.⁴⁴

In a subsequent study, the interaction between *S*wH-NOX and *S*wHaCE was investigated using NMR Chemical Shift Perturbation assays. It was found that ~11 *S*wH-NOX amino acids undergo a chemical shift upon exposure to *S*wHaCE. Of these 11 residues, the 5 that yield the greatest shifts are co-located in the N-terminal helices of *S*wH-NOX, suggesting a potential binding surface on *S*wH-NOX for *S*wHaCE.⁴⁵ To further investigate this hypothesis, three *S*wH-NOX point mutants E16K, E20K, and F17A were made and binding interactions with *S*wHaCE were assessed using fluorescence quenching assays. An increase in quenching of the intrinsic fluorescence of *S*wHaCE was observed upon titration with the Fe(II)-NO bound form of wild-type *S*wH-NOX, but not upon titration with any one of the mutants, suggesting a loss of binding between *S*wH-NOX and *S*wHaCE, and confirming that the N-terminal helical region of *S*wH-NOX is involved in binding to *S*wHaCE.⁴⁵

Interestingly, this same region of the H-NOX domain of mammalian sGC has been implicated in regulation of its cyclase activity. Direct inter-domain interactions between the PAS domain/H-NOX domain heme-associated helix (α F) and the cyclase domain in sGC were mapped with Hydrogen/deuterium exchange mass spectrometry,⁴⁶ and NO binding to sGC was shown to regulate these interactions and ultimately cyclase domain activity.⁴⁷ This suggests an evolutionarily conserved mechanism for NO/H-NOX regulation of enzyme activity.

The biochemical results reported above predict that NO/H-NOX should down-regulate *S. woodyi* biofilm formation in the presence of NO. We found that exposure to nanomolar concentrations of NO indeed causes a decrease in *S. woodyi* biofilm formation. Further, we found decreased biofilm in a mutant strain in which the *hnoX* gene was deleted, as well as no NO phenotype. These biofilm results are consistent with *S*wH-NOX being the NO sensor responsible for regulating biofilm as well as the enzymology described above, as both the absence of *S*wH-NOX, as well as NO-bound *S*wH-NOX, result in low *S*wHaCE cyclase activity, which would lead to decreased intracellular c-di-GMP concentrations and a subsequent decrease in biofilm formation.

In addition, NO-bound *S*wH-NOX also leads to efficient c-di-GMP hydrolysis, which presumably could result in a dramatic decrease in intracellular cyclic-di-GMP and explain the decrease in *S. woodyi* biofilm upon exposure to NO.

Agrobacterium vitis

Recently, our lab has also worked on the characterization of an H-NOX signaling pathway in *A. vitis*. As previously seen in *L. pneumophila* and *S. woodyi*, *A. vitis* H-NOX is encoded in the same operon as a gene that codes for a di-domain GGDEF-EAL (*Av*HaCE). Like *S*wHaCE, using purified proteins, we have shown that *Av*HaCE has both diguanylate cyclase and phosphodiesterase activities, but in the absence of *Av*H-NOX the phosphodiesterase domain is more catalytically active with a k_{cat}/K_m value that is ~12 times more efficient than the diguanylate cyclase domain (Nesbitt et al, unpublished data). Moreover, preliminary biofilm data also reveals that the genetic deletion of *Av*H-NOX or exposure to nanomolar concentrations of NO results in a decrease in biofilm formation (Nesbitt et al, unpublished data). These findings are consistent with our results in *S. woodyi*; since the *Av*H-NOX/HaCE is homologous to the *S*wH-NOX signaling pathway, we

hypothesize that NO also regulates the *Av*H-NOX/HaCE signaling network in a similar manner.

NO/H-NOX-ASSOCIATED TWO-COMPONENT SIGNALING INVOLVED IN C-DI-GMP SIGNALING NETWORKS

Two-component signaling systems (also referred to as His-Asp phosphorelay signaling systems) are utilized by both Gram-negative and Gram-positive bacteria to detect and respond to various environmental stimuli such as nutrient availability, pH, osmolarity and host factors.⁴⁸ Simple two-component signaling systems are comprised of two proteins, a sensor histidine kinase and a response regulator protein. The sensor histidine kinase functions to detect (via its sensory domain) and respond to environmental stimuli using ATP as a phosphodonor for the autophosphorylation of a conserved histidine residue. Hereafter, the signal is transmitted downstream via phosphotransfer from the histidine residue to a conserved aspartic acid residue in the receiver domain of a cognate response regulator protein, a process that is catalyzed by the latter protein.²⁹ Phosphorylation of the response regulator activates its output domain, which then elicits the appropriate cellular response.⁴⁹

Many two-component signaling systems deviate from the simple architecture described above. These alternate systems include hybrid histidine kinases²⁹ and the replacement of the histidine kinase sensory domain by an accessory protein, thus resulting in a three-component signaling system.^{50–52} In the hybrid systems, typically, the histidine kinase has histidine phosphotransfer and catalytic domains, as well as a receiver domain present within the same polypeptide.⁴⁹ In response to a stimulus, the hybrid kinase autophosphorylates its conserved histidine residue and subsequently transfers the phosphate intramolecularly to its receiver domain. Hereafter the phosphate is transferred to a histidine-containing phosphotransfer protein (Hpt) which then transfers the phosphate to the appropriate response regulators.⁴⁹

In the three-component signaling systems, an accessory protein functions to directly detect the environmental stimuli and convey this information to a histidine kinase via a protein-protein interaction.^{50,51} H-NOX genes are most commonly encoded for in the same operon as two-component signaling histidine kinases that lack sensory domains. Consequently, H-NOX can function to detect NO and thus regulate these histidine kinase-signaling pathways in a NO dependent fashion. These histidine kinases are often called HaHKs (H-NOX-associated histidine kinases) and this portion of the review will focus on how NO has been shown to regulate H-NOX-associated three-component signaling systems in various bacteria (Figure 3).

Shewanella oneidensis

The first H-NOX-associated two-component signaling pathway characterized was from the bacterium *S. oneidensis*.⁵³ In the first study, the authors found that in the absence of *SoH*-NOX, *SoHaHK* displays *in vitro* histidine kinase activity with ATP-dependent autophosphorylation in a time-dependent manner. In the presence of Fe(II) unligated *SoH*-NOX, *SoHaHK* activity is not affected. In the presence of Fe(III), Fe(II)-NO, and Fe(II)-CO *SoH*-NOX, however, *SoHaHK* activity decreases in a *SoH*-NOX concentration dependent

manner.⁵³ The authors found 50% inhibition of kinase activity in the presence of 5–10 μM of Fe(II)-NO *SoH*-NOX, and the highest degree of inhibition occurred with 100 μM (100-fold excess) of Fe(II)-NO or Fe(II)-CO *SoH*-NOX.⁵³

Structural studies on *SoH*-NOX have revealed the molecular mechanism of NO-induced *SoH*-NOX activation.⁵⁴ Using X-ray crystallography experiments, it was found that in the Fe(II) unligated state, the heme is highly distorted due to van der Waals interaction between proline 116 and pyrrole A which results in the displacement of pyrroles A and D from planarity. Additionally, the presence of an ordered water molecule in the proximal heme pocket facilitates a hydrogen bonding network with H103, H99, P116 and I118. This network not only maintains the conformation of the αF helix (which contains the P116 residue), but is essential for preserving the basal conformational state of the protein.⁵⁴ In the Fe(II)-NO bound state, consistent with spectroscopic studies, NO was found to bind as a five-coordinate adduct. Hereafter, the iron-histidine bond is cleaved due to a conformational change in the αF helix which leads to the heme-ligating histidine (H103) moving $\sim 8.5 \text{ \AA}$ away from the heme iron, and the P116 residue moving away from the heme, which subsequently results in the relaxation of the heme towards planarity. Further, a new backbone hydrogen bonding network between the residues D100 and L104 forms which stabilizes the new conformation and the out position of the αF helix and H103 residue respectively. These findings support the hypotheses that NO binding induces heme flattening and subsequent activation of H-NOX proteins.⁵⁴

Additional support for the hypothesis of NO activation was also demonstrated when the authors mimicked the transient six coordinate Fe(II)-NO bound state using Manganese (II) protoporphyrin IX. As expected, no significant conformational changes were observed and the iron-histidine (H103) bond remained intact, revealing that conformational activation of NO bound *SoH*-NOX results from cleavage of the iron-histidine bond.⁵⁴ Together, these findings not only collectively reveal the conformational changes that *SoH*-NOX endures upon NO binding, but also disclose the molecular mechanism of NO-H-NOX activation and subsequent regulation of the H-NOX associated signaling pathway in *Shewanella oneidensis* and possibly in other bacteria.

Events downstream of *SoH*-NOX/HaHK have also been determined. It has been demonstrated that *SoHaHK* engages in phosphotransfer with at least three cognate response regulators, an EAL-domain containing protein, a degenerate HD-GYP-domain containing protein, and an HTH-domain containing protein.⁵⁵ Because NO-bound *SoH*-NOX inhibits *SoHaHK* autophosphorylation⁵³ and phosphotransfer activity (*vide supra*),⁵⁵ a model was generated predicting that phosphate flux through the whole pathway should be down regulated in the presence of NO, which would subsequently lead to an increase in cyclic-di-GMP levels inside the bacterium (due to inhibition of the EAL-response regulator in the absence of phosphorylation) and an increase in biofilm formation.⁵⁵ Interestingly, the authors only tested their model under anaerobic conditions, but they did find that under these conditions, *S. oneidensis* exhibits increased biofilm formation in the presence of NO. It has been well-documented that *S. oneidensis* does not form robust biofilms in the absence of oxygen, however, instead *S. oneidensis* rapidly disperses from biofilm in response to decreasing oxygen levels.⁵⁶ Therefore, it is difficult to interpret the biofilm data in this

study, and it is possible that the apparent increase in biofilm formation in the presence of NO was a caveat of an extremely low cell density (due to biofilm dispersion). Indeed, in our laboratory, when grown under aerobic conditions, we find that NO causes a decrease in *S. oneidensis* biofilm formation. (Arora et al, manuscript in preparation) Nevertheless, the results indicate that NO signaling in this bacterium is very complex.

To further investigate this proposed model, the authors also assessed the role of the HTH-domain containing response regulator protein in the *S. oneidensis* NO signaling pathway. Gene expression profiling experiments using an *S. oneidensis* mutant containing a deletion of the HTH-domain containing response regulator revealed that 7 genes are upregulated, 6 of which are contained in the NO signaling pathway (the *hno* genes).⁵⁷ These genes are present in 3 operons that are at two separate loci in the *S. oneidensis* chromosome. The *hnoX* and *hahK* are in an isolated operon under the control of one promoter termed *hnoXp*, and the other two operons share a bidirectional promoter and are termed *hnoCp* (includes the *eal* and *hth* domain containing response regulator genes) and *hnoDp* (includes the *hd-gyp* domain containing response regulator genes). Using GFP reporter assays, the authors found that the HTH-domain containing response regulator protein interacts with all three promoters, and that only in the HTH deletion mutant strain could measurable levels of GFP fluorescence be detected, whereas in the wild-type and *hnoX* and *hahK* deletion strains, no GFP fluorescence could be detected. Thus the authors conclude the HTH is a gene repressor for all three operons.⁵⁷

In additional experiments, the gene transcription regulation mechanism by this response regulator was found to be direct interaction with the *hnoX* and *hnoC/D* promoters, as the HTH binding site overlaps with the transcription start site for all three promoters, and could interfere with transcription of the associated operons. Furthermore, from phosphorylation and size exclusion experiments, the HTH domain-containing response regulator was found to bind all three promoters in a tetrameric state (apparent size of ~175 kDa) when unphosphorylated. Upon phosphorylation, however, the subunits dissociate into monomers (apparent size of ~45 kDa) and enable promoter accessibility for gene transcription.⁵⁷ These findings enabled the authors to generate a model predicting that unphosphorylated HTH domain-containing response regulator inhibits gene transcription and subsequently inhibits phosphate flux through the signaling pathway,⁵⁵ while the phosphorylated HTH domain-containing response regulator, on the other hand, enables gene transcription and enhances phosphate flux through the signaling pathway.⁵⁷ These changes in phosphate flux not only have implications for *S. oneidensis* biofilm formation, but further reveal the complexity that underlies the regulation of the NO/H-NOX signaling pathway in *Shewanella oneidensis*.

Vibrio cholerae

An H-NOX-associated two-component signaling network similar to that of *S. oneidensis* has been identified in *V. cholerae*.⁵⁵ Biochemical characterization of this two-component signaling pathway indicates that, as observed in *S. oneidensis*, VcHaHK engages in phosphotransfer with homologous EAL-domain and degenerate HD-GYP-domain containing response regulator proteins, but *V. cholerae* does not encode the homologous HTH-domain containing response regulator.⁵⁵

New evidence suggests that VcH-NOX may function as both an NO and a redox sensor.⁵⁸ The effect of heme-free (apo VcH-NOX) and heme-bound VcH-NOX on VcHaHK autophosphorylation activity was assessed using purified proteins. It was found that VcHaHK activity is inhibited by heme-bound Fe(III), Fe(II)-NO and apo VcH-NOX, with the heme-bound forms being the most effective inhibitors *in vitro*.⁵⁸ Moreover, the authors investigated the mechanism of heme-independent (apo VcH-NOX) inhibition of VcHaHK autophosphorylation activity and observed that only the cysteine oxidized form of apo VcH-NOX could inhibit VcHaHK activity, and that this inhibition was due to a stable interaction with VcHaHK.⁵⁸ Since VcH-NOX may have dual functionality, it would be interesting to determine if both functions can regulate biofilm formation and pathogenicity in *Vibrio cholerae*. Furthermore, this paper raises questions about whether H-NOX proteins in general can also function as redox sensors. Interestingly, it has also been proposed that *Nostoc punctiforme* H-NOX may function as a redox sensor, although through oxidation of the heme, not cysteine amino acids.⁵⁹

Pseudoalteromonas atlantica

NO has also been shown to regulate an H-NOX-associated two-component signaling pathway in the bacterium *P. atlantica*.²⁹ In the absence of PaH-NOX, purified PaHaHK displays autophosphorylation activity in an ATP- and time-dependent manner. Further, PaHaHK activity is inhibited by all forms of PaH-NOX [Fe(III), Fe(II), Fe(II)-NO and Fe(II)-CO]. The greatest degree of PaHaHK inhibition, however, occurs with the Fe(II)-NO bound form of PaH-NOX (present in only a two-fold excess of PaHaHK). Furthermore, the inhibition of PaHaHK activity by NO bound PaH-NOX was shown to be dose-dependent.²⁹ Additionally, a cognate response regulator (homologous to the HD-GYP domain in the *S. oneidensis* H-NOX/HaHK pathway) was identified via a bioinformatics screen. By homology, the *P. atlantica* H-NOX signaling pathway also contains the EAL and HTH-domain containing response regulators,⁵⁵ but only the HD-GYP protein has been biochemically characterized in this system. It was further shown that the HD-GYP response regulator exhibits autophosphatase activity towards the generic substrate 3-O-methylfluorescein phosphate (OMFP), which is essential in understanding how response regulator proteins can mediate the duration of a stimulus response.⁶⁰ Whether this autophosphatase activity is explicitly regulated in an NO dependent manner is currently unknown. But based on the biochemical data, we can hypothesize that inhibition of PaHaHK via NO bound PaH-NOX would decrease phosphate flux to the response regulator and subsequently regulate its autophosphatase activity.

NO/H-NOX-ASSOCIATED TWO-COMPONENT SIGNALING INVOLVED IN QUORUM SENSING NETWORKS

In addition to regulating canonical histidine kinases, H-NOX proteins have also been shown to regulate the activity of hybrid histidine kinases, and therefore the phosphorelay of associated signaling pathways. In particular, H-NOX-associated hybrid histidine kinase signaling pathways have been implicated in quorum sensing networks (Figure 4).

Vibrio harveyi

The first biochemical characterization of an H-NOX-associated hybrid histidine kinase two-component signaling system is from the bacterium *V. harveyi*.⁶¹ *V. harveyi* is known to participate in quorum sensing (QS), a phenomenon in which gene expression is regulated in bacterial communities, and affects behaviors such as virulence, biofilm formation, bioluminescence and antibiotic resistance in response to the detection of secreted molecules called autoinducers.⁶¹ Autoinducers are small molecules that accumulate in the environment as a function of bacterial cell density. At a critical population size, the autoinducer threshold limit is attained, and a signal transduction cascade is initiated that leads to community-wide changes in gene expression and group behaviors.⁶² *V. harveyi* has been used as a model organism for studying QS because at high cell density (high autoinducer concentration), bioluminescence is promoted.⁶²

Interestingly, low concentrations of NO have been shown to mediate bacterial community behaviors.³⁴ The *hnoX* gene in *V. harveyi* is coded for in the same operon as a hybrid histidine kinase with high similarity to the kinase domain of LuxQ, a kinase involved in the *V. harveyi* QS network. Therefore, our laboratory hypothesized that NO/H-NOX may affect community behaviors by directly participating in QS through this hybrid kinase. Our lab investigated this hypothesis in *V. harveyi* and found that nanomolar concentrations of NO induce bioluminescence, thus suggesting an autoinducer-like role for NO.

The molecular mechanism of this phenomenon was determined by biochemical and genetic characterization of the H-NOX-associated two-component signaling pathway. The H-NOX-associated quorum sensing kinase (HqsK) was shown to exhibit time-dependent, stable autophosphorylation activity in the absence and presence of Fe(II) *VhH*-NOX. In the presence of Fe(III) and Fe(II)-CO bound *VhH*-NOX, HqsK activity was inhibited. The greatest degree of inhibition, however, was seen in the presence of Fe(II)-NO *VhH*-NOX in a concentration-dependent manner.⁶¹ These findings suggest that NO participates in QS by regulating the activity of *VhHqsK* and influencing phosphate flux through LuxU/LuxO, leading to LuxR regulation, which results in regulation of bioluminescence, among other QS-regulated phenotypes. Indeed, transfer of phosphate from *VhHqsK* to LuxU was demonstrated *in vitro* and occurred in a time dependent manner.⁶¹

To further test the outlined hypothesis above, an *hnoX* gene deletion mutant was produced and it was found that in the absence of NO, this mutant displayed delayed bioluminescence in comparison to wild-type *V. harveyi*. In the presence of NO, no change in bioluminescence was observed in the *hnoX* gene deletion mutant. Plasmid complementation of the *hnoX* gene, however, restored bioluminescence comparable to that of wild-type *V. harveyi*. These findings further substantiate the role of NO as an autoinducer-like molecule, that acts through the *VhH*-NOX/HqsK signaling network.⁶¹

In a follow up study, the effect of NO signaling on flagella production and biofilm formation in *Vibrio harveyi* was assessed.⁶³ Biofilm experiments revealed that low NO concentrations (50 nM) enhance *V. harveyi* biofilm formation, but higher concentrations of NO (100 nM and 200 nM) decrease biofilm formation. Deletion of the *hnoX* gene yielded an overall decrease in biofilm formation, and further addition of cell free media containing the other

autoinducers did not induce the observed 50 nM NO biofilm phenotype. Only plasmid complementation of the *hnoX* gene restored the observed increased biofilm phenotype and is suggestive of NO/H-NOX regulation of biofilm formation in *V. harveyi*.⁶³

iTRAQ proteomics experiments were used to further substantiate the biofilm analysis, and it was found that the proteome pattern at 50 nM NO was different from the observed patterns at 100 nM and 200 nM, as many proteins, including all 5 of the flagellin proteins, displayed decreased expression at 50 nM NO, but normal expression at 100 nM and 200 nM NO. Moreover, another protein, CheY, displayed a similar NO-dependent pattern in the proteome,⁶³ and was of interest as this protein is known to interact with flagellar motor proteins, modify flagellar behavior,⁶⁴ and when overexpressed in *E. coli*, mediate clockwise flagellar rotation^{65–67} and reduce bacteria motility. Taken together, these experimental findings reveal that NO/H-NOX not only regulates bioluminescence, but influences flagellar protein expression and biofilm formation, and is therefore suggestive of all three processes being NO-QS-regulated phenotypes in *V. harveyi*.

Vibrio parahaemolyticus

To determine if NO participation in QS is a general phenomenon, a homologous H-NOX-associated two-component signaling system was identified in, and characterized from, the bacterial species *V. parahaemolyticus*. *VpHqsK*, like *VhHqsK*, displays ATP-dependent autophosphorylation activity in a time-dependent manner in the absence of *VpH-NOX*. (Ueno et al, manuscript in preparation) *VpHqsK* was also shown to transfer phosphate to the *VpLuxU* protein, thus revealing how this pathway participates in QS. To ensure that the phosphotransfer between *VpHqsK* and *LuxU* was due to kinetic preference, the *VpHqsK* kinase domain and internal receiver domain were separately cloned, expressed and simultaneously incubated with *LuxU* as well as two other *VpHpt* proteins. From this experiment it was shown that only *LuxU* was able to engage in phosphotransfer with *VpHqsK* domains within fifteen minutes of incubation. These data indicate that the *VpHqsK/LuxU* interaction is not due to crosstalk but rather is a consequence of *LuxU* and *HqsK* being a cognate pair. (Ueno et al, manuscript in preparation)

VpHqsK activity was also assessed in the presence of *VpH-NOX*. *VpHqsK* autophosphorylation activity was inhibited in the presence of Fe(III), Fe(II), Fe(II)-NO and Fe(II)-CO forms of *VpH-NOX*. The greatest degree of inhibition, however, was seen in the presence of Fe(II)-NO bound *VpH-NOX*, where the autophosphorylation also decreased in a *VpH-NOX* concentration dependent manner. (Ueno et al, manuscript in preparation) Finally, the expression of the master QS regulators *OpaR* and *AphA* were investigated in the presence of NO, and it was found that *OpaR* gene expression increased as a function of increasing NO concentrations. *AphA* gene expression, however, remained relatively constant, but did increase at 200 μ M concentration of NO donor. These findings are consistent with the hypothesis that NO can participate in and influence QS, and thus reveal that both *VpH-NOX/HqsK* (Ueno et al, manuscript in preparation) and *VhH-NOX/HqsK*⁶¹ are NO-responsive quorum sensing signaling pathways that are likely regulated in a similar manner.

NO/H-NOX-ASSOCIATED TWO-COMPONENT SIGNALING TO REGULATE SYMBIOTIC RELATIONSHIPS

In addition to being involved in regulating various two-component signaling systems and c-di-GMP signaling networks, NO has also been shown to be involved in many microbial symbiotic relationships with various hosts including insects, nematodes and marine invertebrates (Figure 5).¹

Vibrio fischeri

One such example is the symbiotic relationship between the bacterium *V. fischeri* and the Hawaiian bobtail squid *Euprymna scolopes*.⁶⁸ Using transcriptional profiling with wild-type and an *hnoX*-insertion mutant *V. fischeri* strain, it was found that iron-uptake genes of *V. fischeri* are regulated in an NO-dependent manner via ligation to V_H-NOX. In particular, of 20 genes that were down-regulated in the presence of NO (exposed for 30 minutes), 10 of these genes were found to have promoter sequences similar to the Fur (ferric iron uptake regulator) binding site in *E. coli*.⁶⁹ Further investigation revealed that 8 of the 10 genes are involved in iron acquisition or use, and their expression is dependent on the Fur regulon in the presence of NO, as no changes in gene expression were observed in the *hnoX*-insertion mutant strain.

The effect of NO on *V. fischeri* grown in iron depleted minimal media supplemented with hemin was then assessed and it was concluded that NO suppresses *V. fischeri* hemin uptake, as both wild-type and *hnoX*-deletion mutant strains demonstrated delayed initial growth in the presence of NO. Hereafter, the wild-type strain grew at a slower rate than the mutant. Genetic *in trans* complementation of the *hnoX* gene, however, restored the observed wild-type growth phenotype, and in the absence of NO, both wild-type and the mutant strains utilize hemin as an iron source.

Based on the previous experimental findings, the authors hypothesized that V_H-NOX functions as a sensor for host-derived NO and its absence may lead to defects in symbiosis. Consequently, the role of *VfhnoX* gene in symbiotic colonization was investigated, and it was found that the *VfhnoX* gene deletion mutant strain displayed faster initiation of colonization than wild-type *Vibrio fischeri*, as juveniles colonized with the mutants achieved bioluminescence sooner.⁶⁸ This observed competitive advantage was reversed by genetic complementation of the *VfhnoX* gene, and further experiments revealed that the mutant is better able to acquire iron during colonization, but in the presence of seawater supplemented

¹The abbreviations used are: NO, nitric oxide; CO, carbon monoxide; H-NOX, heme-nitric oxide/oxygen-binding protein; HaCE, H-NOX-associated cyclic-di-GMP processing enzyme; HaHK, H-NOX-associated histidine kinase; HqsK, H-NOX-associated quorum sensing kinase; ATP, adenosine-5'-triphosphate; GTP, guanosine-5'-triphosphate; GMP, guanosine-5'-monophosphate; c-GMP or cyclic GMP, guanosine-3',5'-cyclic monophosphate; c-di-GMP or cyclic-di-GMP, bis-(3'-5')-cyclic dimeric guanosine monophosphate; pGpG, 5'-phosphoguanlyl-(3'-5')-guanosine; GGDEF, conserved amino acids in the catalytic sites of diguanylate cyclases (the domain containing these conserved residues is often termed a GGDEF domain); EAL, conserved amino acids in the catalytic site of some phosphodiesterases (the domain containing these conserved residues is often termed an EAL domain); HD-GYP, conserved amino acids in the catalytic site of some phosphodiesterases (the domain containing these conserved residues is often termed an HD-GYP domain); HTH, helix-turn-helix domain, common in transcription factors; NOS, nitric oxide synthase; Hpt, Histidine-containing phosphotransfer protein; sGC, soluble guanylate cyclase; PAS, PER-ARNT-SIM domain containing protein; Fur, ferric iron uptake regulator; OMFP, 3-O-methylfluorescein phosphate; QS, quorum sensing; NMR, nuclear magnetic resonance spectroscopy; iTRAQ, isobaric tags for relative and absolute quantification; TSM, *Trichodesmium erythraeum* spent medium.

with excess hemin (0.2 μ M) or free iron (10 μ M), the mutant becomes outcompeted by the wild-type strain. This observed phenotype was also seen in experiments conducted with the addition of *S*-methyl-L-thiocitrulline (SMTC), a general NOS inhibitor.⁶⁸

Taken together, these experimental findings collectively suggest that NO/ *V*H-NOX mediates *V. fischeri* and *Euprymna scolopes* symbiosis by suppressing rapid growth and hemin uptake of the bacterium, which may be necessary to exert temporal control of bioluminescence and subsequent counter-illumination of the Hawaiian bobtail squid *Euprymna scolopes*. At the molecular level, however, this regulation is still presently unclear, as no published biochemical data on how NO explicitly regulates the *V*H-NOX/HaHK pathway is currently available. Since, however, the *V*H-NOX/HaHK is similar in architecture to other H-NOX/HaHK systems, we predict that it is regulated in a similar NO-dependent manner.²⁴

Silicibacter* sp. strain *Trich4B

NO has also been shown to regulate an H-NOX/HaHK signaling network in the bacterium *Silicibacter* sp. strain *Trich4B*.⁵ Interestingly, this bacterium most closely resembles the mammalian sGC signaling pathway, as both NOS and H-NOX proteins are encoded in the genome of this bacterium.⁵ Full length *Sil*NOS expression in an active form was unsuccessful so the oxygenase domain (*Sil*NOSox) was expressed and it was found to produce NO in the presence of oxygen.

The *S. Trich4B* H-NOX/HaHK network is comprised of *Sil*H-NOX, *Sil*HaHK (a hybrid histidine kinase), *Sil*Hpt, and a response regulator diguanylate cyclase protein, *Sil*DGC. Using purified proteins the authors demonstrated that in the absence of *Sil*H-NOX, *Sil*HaHK displays autophosphorylation activity in a time-dependent manner. Additionally, *Sil*HaHK engages in phosphorelay with *Sil*Hpt. Phosphotransfer profiling experiments were independently conducted with different orphan response regulators and only the incubation of *Sil*HaHK and *Sil*Hpt with the *Sil*DGC response regulator resulted in a loss of phosphorylation from both *Sil*HaHK and *Sil*Hpt proteins within 1 minute, while all other response regulators yielded a slow loss of phosphorylation from *Sil*HaHK and *Sil*Hpt (within 15 to 30 minutes). These findings indicate that *Sil*HaHK, *Sil*Hpt, and the *Sil*DGC response regulator are cognate pairs. Further biochemical investigation revealed that *Sil*DGC activity decreases by approximately 40% in the presence of *Sil*HaHK/*Sil*Hpt and ATP. This change in *Sil*DGC activity, however, was not observed when either the *Sil*HaHK D386A mutant (unable to transfer phosphate to *Sil*Hpt) was present in, or *Sil*Hpt or ATP was absent from, the reaction mixture.⁵ To understand how NO regulates this signaling pathway, *Sil*HaHK autophosphorylation activity was assessed in the presence of *Sil*H-NOX. Fe(II) and Fe(II)-CO *Sil*H-NOX do not affect *Sil*HaHK activity. *Sil*HaHK autophosphorylation activity, however, is inhibited in the presence of Fe(II)-NO *Sil*H-NOX, thus indicating that the *Sil*H-NOX/HaHK pathway may be regulated in a NO dependent manner.⁵

Hereafter, the authors investigated the effect of NO on biofilm formation in *S. Trich4B* under anaerobic conditions, and found that exogenous NO increases biofilm formation in a concentration dependent manner.⁵ This result is consistent with the observed biochemical

data as NO/*SilH*-NOX inhibits *SilH*HaHK, which decreases phosphate flow to *SilH*Hpt and *SilH*DGC, and therefore enables c-di-GMP production and biofilm formation to occur in this bacterium.⁵

Silicibacter sp. strain *Trich4B* is typically found in association with the alga *Trichodesmium erythraeum*. Consequently, the effect of *T. erythraeum* on *Silicibacter* sp. strain *Trich4B* NO formation was assessed, and it was found that only *T. erythraeum* spent medium (TSM) increased *Silicibacter* sp. strain *Trich4B* NO production (by ~7-fold). Neither a small molecule fraction of the spent medium nor proteinase K treated spent medium increased NO production, as they yielded similar NO production phenotypes as the no spent medium fraction condition.⁵ Finally, upon investigating if *T. erythraeum*, via TSM, could regulate *SilNOS* gene expression and biofilm formation in *Silicibacter* sp. strain *Trich4B*, the authors found that TSM increases both *SilNOS* mRNA levels and *Silicibacter* sp. strain *Trich4B* biofilm formation in a concentration-dependent manner.⁵

Collectively, these experimental findings suggest that *Silicibacter* sp. strain *Trich4B* symbiosis with *T. erythraeum* mediates biofilm formation in this bacterium, as *T. erythraeum* upregulates *SilNOS* gene expression, which ultimately leads to NO-induced biofilm formation in *Silicibacter* sp. strain *Trich4B* and symbiotic colonization with *T. erythraeum*. It should be noted, however, that TSM treated biofilms were grown under aerobic conditions while exogenous NO treated biofilms were grown anaerobically; and although a biofilm phenotype was observed for the latter biofilm conditions, it would still be interesting to demonstrate whether exogenous NO has the same predicted biofilm effect as *SilNOS* produced NO under aerobic conditions. Furthermore, it would be interesting to assess the biofilm formation capabilities and symbiotic competence of a *SilHnoX* gene deletion mutant as these findings could provide more insight as to how NO explicitly regulates these two processes in *Silicibacter* sp. strain *Trich4B*.

PERSPECTIVES AND CONCLUSIONS

In this review, we have presented a summary of the documented roles for NO in bacterial signaling networks through ligation to an H-NOX domain. From the studies reviewed in this paper, it has been shown that the H-NOX proteins are predicted to have approximately picomolar affinity for NO and H-NOX-associated signaling pathways are governed at nanomolar concentrations of NO.²⁴ Biochemically, the various H-NOX signaling pathways have been shown to be involved in the regulation of processes including biofilm formation, quorum sensing, and regulation of symbiosis, in an NO dependent manner; the overall biological significance of NO signaling in bacteria is still currently being addressed, however.

In the instance of *V. harveyi*, for example, NO has been shown to participate in QS and regulate bioluminescence, biofilm formation, and flagellar motility.^{61,63} As NO functions analogously to an autoinducer, it follows that *V. harveyi* might be the source of NO, as QS bacteria typically synthesize and release autoinducers to their external environment.⁷⁰ *V. harveyi*, however, does not encode for a NOS protein and is thus not expected to produce NO. This raises the question of the source of the NO. Nitrate respiration under anaerobic

conditions (via nitrate reductase) is a possible source of NO.⁷¹ *V. harveyi* is predominantly found at the surface in marine environments where oxygen content is high⁷² and nitrate reductase expression levels are low.⁷¹ Nonetheless, it is certainly possible that NO signaling is triggered upon a drop in local oxygen concentration. Interestingly, *V. harveyi* has been found to be pathogenic to various marine organisms such as shrimp,⁷³ pearl oysters, fish, seahorses and lobsters,⁷⁴ and has also been shown to be involved in a mutualistic symbiotic relationship with the hydrozoan *Aglaophenia octodonta*.⁷⁵ Consequently, as observed with *V. fischeri* and its eukaryotic host *Euprymna scolopes*, it is possible that the aforementioned eukaryotes could function as a source of NO, which could then ligate to VhH-NOX and enable these host- *V. harveyi* relationships, as QS has been shown to be implicated in mediating *V. harveyi* pathogenicity.⁷⁶ Additionally, since *V. harveyi* also displays bioluminescence during these host-bacteria relationships, it is very likely, especially in the case of symbiosis, that bioluminescence may serve as a useful light source for the *V. harveyi* symbiont *Aglaophenia octodonta*.⁷⁵ Further studies, however, need to be conducted to determine if this is a physiological function of *V. harveyi* bioluminescence, after which, the effect of NO on regulating this bioluminescence and subsequent symbiotic relationship (as was observed in *Silicibacter Trich4B* and *T. erythraeum*) between *V. harveyi* and *Aglaophenia octodonta* should be further assessed.

NO has been shown to mediate biofilm formation in many bacteria.⁷⁷ Interestingly, however, in some bacteria, the NO/H-NOX pathway appears to induce biofilm formation, while in other bacteria it reduces biofilm formation. This could easily be due to differences in the lifestyle and environmental niche of the various bacteria. It is interesting to note, however, that in many of the cases where NO/H-NOX was shown to increase biofilm formation, the biofilm experiments were carried out under anaerobic conditions. It is yet to be determined if NO/H-NOX truly differentially regulates biofilm formation in various bacteria or if differences in oxygen concentrations are skewing the data. Nonetheless, the role of NO/H-NOX in biofilm formation is of clinical relevance because pathogenic bacteria such as *L. pneumophila*⁷⁸ and *V. cholerae*^{79,80} utilize biofilms to either enhance their infectivity or facilitate their persistence and they code for NO/H-NOX signaling pathways. In future studies it would be interesting to determine if these NO/H-NOX signaling pathways play a role in mediating bacterial infectivity.

Furthermore, although H-NOX has been shown to be the primary NO sensor in some bacteria, there are many bacteria, including most pathogens, that lack an H-NOX domain but are still NO responsive. Multiple NO responsive regulatory proteins have been identified and characterized, but their responsive pathways are primarily involved in NO detoxification,^{81,82} rather than regulating various bacterial processes in an NO dependent manner. As a result, the identity of the putative primary NO sensor and its subsequent responsive signaling pathways are all still unknown in these bacteria, and the molecular mechanisms by which NO regulates these signaling pathways are poorly understood. This area of research, however, is currently expanding as further studies are being conducted in the hope of elucidating the primary NO sensor and the NO responsive pathways in these bacteria. These studies will be of significance as identifying the putative NO sensor protein will not only reveal how NO signaling may function in these bacteria, but will also identify

responsive pathways which could then be utilized as novel drug targets for those bacteria that are pathogenic to humans in the future.

Finally, although this review has focused on H-NOX detection of low (sub-micromolar) NO concentrations, it is important to note that bacteria respond to a wide range of NO concentrations. Generally speaking, from the current data, it seems that bacteria detect higher concentrations of NO to initiate NO elimination pathways^{17–21} and lower concentrations to regulate motility and group behaviors.^{43,44,55,61} Recent studies have shown, however, that in some bacteria (as is the case in *Paracoccus denitrificans*) NO detoxification proteins have nanomolar affinities for NO, enabling NO detoxification systems to be active at sub-micromolar concentrations of NO.⁸³ Conversely, higher concentrations of NO have been shown to affect biofilm formation.^{32,77} It is possible that the activation of NO detoxification pathways at low concentrations is not exclusively for NO elimination, and/or that biofilm regulation at high NO levels is a defense mechanism. Most likely there is a continuum between detoxification and non-toxic signaling responses that depend on the bacteria or environment. These findings hint at the complexity of NO signaling in bacteria, and how much remains to be discovered.

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Figure 1. NO/H-NOX signaling pathways regulates bacterial processes such as biofilm formation, quorum sensing, and symbiosis.

Figure 2.

NO regulates HaCE activity through ligation to H-NOX. **(A)** *LpgHaCE* is only functional as a diguanylate cyclase, and its activity remains unchanged in both the presence and absence of Fe(II) *LpgH-NOX1*, but in the presence of Fe(II)-NO bound *LpgH-NOX1*, *LpgHaCE* diguanylate cyclase activity decreases **(B)** *SwHaCE* has basal diguanylate cyclase and relatively efficient phosphodiesterase activity in the absence of *SwH-NOX*. In the presence of Fe(II) *SwH-NOX*, *SwHaCE* diguanylate cyclase activity becomes upregulated while the phosphodiesterase activity remains unchanged. In the presence of Fe(II)-NO bound *SwH-NOX*, however, *SwHaCE* phosphodiesterase activity becomes upregulated, while the diguanylate cyclase activity returns to basal levels.

Figure 3.

NO regulates HaHK autophosphorylation activity via ligation to H-NOX in *Shewanella oneidensis* (A) *Vibrio cholerae* (B) and *Pseudoalteromonas atlantica* (C). (A) *SoHaHK* autophosphorylation activity remains unchanged in the presence and absence of Fe(II) *SoH-NOX*. *SoHaHK* activity, however, is inhibited in the presence of Fe(III), Fe(II)-NO and Fe(II)-CO bound forms of *SoH-NOX*, with Fe(II)-NO and Fe(II)-CO yielding the greatest inhibition effect. (B) *VcHaHK* activity remains unchanged in the absence of *VcH-NOX*. In the presence of the Fe(III), Fe(II)-NO, and apo forms of *VcH-NOX*, *VcHaHK* activity is inhibited, with the Fe(III) and Fe(II)-NO bound forms yielding the highest degree of inhibition. (C) *PaHaHK* displays autophosphorylation activity in the absence of *PaH-NOX*. Inhibition of *PaHaHK* activity is observed in the presence of all forms of *PaH-NOX*. The greatest degree of inhibition occurs in the presence of Fe(II)-NO bound *PaH-NOX*. Only homologs of the EAL and “HD-GYP” domain containing response regulators are present in *Vibrio cholerae*, while all three response regulator proteins are present in *Pseudoalteromonas atlantica*, but only the “HD-GYP” domain containing response regulator protein, however, has been characterized.

Figure 4.

NO regulates HqsK autophosphorylation activity and subsequently influences quorum sensing activity in *Vibrio harveyi* (A) and *Vibrio parahaemolyticus* (B). (A) In the absence and presence of Fe(II) VhH-NOX, VhHqsK autophosphorylation, and therefore kinase activity, remains unchanged enabling phosphate flux to LuxU/LuxO to occur. In the presence of Fe(III), Fe(II)-NO and Fe(II)-CO bound forms of VhH-NOX, VhHqsK autophosphorylation activity decreases. VhHqsK phosphatase activity increases causing phosphate flux to occur in the reverse. The greatest degree of VhHqsK autophosphorylation inhibition occurs in the presence of Fe(II)-NO VhH-NOX. (B) VpHqsK functions as a kinase in the absence of VpH-NOX. In the presence of VpH-NOX, VpHqsK kinase activity decreases while its phosphatase activity increases. The highest degree of inhibition of VpHqsK activity was observed in the presence of Fe(II)-NO VpH-NOX.

Figure 5.

NO regulates *VhHaHK* and *SilH-HaHK* autophosphorylation activity and may ultimately influence their symbiotic relationships with their respective eukaryotic hosts. (A) In *V. fischeri*, Fe(II)-NO bound H-NOX may inhibit *VhHaHK* autophosphorylation activity which not only leads to a change in phosphate flow in the predicted signaling network but also influences its symbiosis with *Euprymna scolopes*, the Hawaiian bobtail squid. (B) In *Silicibacter sp. TrichCH4B*, *SilH-HaHK* autophosphorylation activity and phosphate flow within the pathway remain unchanged in the absence and presence of Fe(II) and Fe(II)-CO bound *SilH-NOX*. NO bound *SilH-NOX* has been shown to decrease *SilH-HaHK* autophosphorylation activity while increasing *SilH-HaHK* phosphatase activity. This causes a decrease in phosphate flow within the *SilH-HaHK* signaling pathway leading to increased biofilm formation, and influences *Silicibacter sp. TrichCH4B* symbiosis with *Trichodesmium erythraeum*, an algal symbiont.