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The Molecular Basis of a Nitric Oxide Responsive Quorum Sensing Circuit in *Vibrio harveyi*

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Abstract of the Dissertation

The molecular basis of a nitric oxide responsive quorum sensing circuit in *Vibrio harveyi*

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The discovery of bacteria's ability to coordinate population-wide undertakings has paved the way to understanding many biological processes once thought to be restricted to multi-cellular organisms. Bacteria use small molecules to communicate with one another and gauge the surroundings to formulate a unified response. This process, known as quorum sensing (QS), regulates diverse functions such as bioluminescence, biofilm formation, and virulence. *Vibrio harveyi* have three parallel QS circuits that regulate bioluminescence. Each system involves the synthesis of specific small molecule called autoinducer that binds to its cognate receptor histidine kinase and regulates phosphorylation. The three pathways converge and exchange phosphate with a common phospho-relay protein called LuxU. LuxU transfers phosphate to LuxO, a response regulator that controls expression of LuxR and,

ultimately, the quorum sensing response. In this thesis project, we have identified nitric oxide (NO) as a signal molecule that participates in regulation of bioluminescence through the LuxU/LuxO/LuxR pathway. We show that *V. harveyi* display a NO concentration-dependent increase in light production that is regulated by expression of a NO-sensitive *hnoX* gene. We demonstrate that VIBHAR_01911, annotated as Heme-Nitric Oxide/Oxygen binding protein (H-NOX) binds NO at approximately picomolar level. NO-bound H-NOX interacts and regulates the phosphorylation of a histidine kinase named HqsK (H-NOX-associated quorum sensing kinase) that, like the other QS kinases, transfers phosphate to LuxU. Thus, NO concentration is factored into the quorum sensing output of *V. harveyi* via H-NOX-HqsK. This study reports the discovery of a fourth quorum sensing pathway in *V. harveyi* and suggests that bacteria use QS to integrate not only the density of bacteria, but also other diverse information, potentially even the presence of eukaryotes, into decisions about gene expression. Furthermore, this study provides characterization of a new pathway that could be targeted for the development of novel antibiotics.

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AB	Autoinducer bioassay
ABC	ATP-binding cassette
AHL	N-acyl homoserine lactone
AI	Autoinducer
AI-2	Autoinducer-2
AIP	Autoinducing peptide
Ala	Alanine
ALA	Aminolevulinic acid
Amp	Ampicillin
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAI-1	Cholerae autoindcer-1
c-di GMP	cyclic-diguanosine monophosphate
c-GMP	cyclic-guanylate monophosphate
CV	Crystal violet
CLSM	Confocal laser scanning microscope
DAP	2,3-diaminopropionic acid
DGC	Diguanylate cyclase
DHMF	2,4-dihydroxy-2-methyldihydrofuran-3-one
DMSO	Dimethyl sulfoxide
DPD	4,5-dihydroxy-2,3-pentanedione

DPTA	Dipropylenetriamine
DTT	Dithiothreitol
GST	Glutathione S-transferase
HAI-1	Homoserine autoinducer-1
His	Histidine
H-NOX	Heme-nitric oxide/oxygen binding protein
HqsK	H-NOX-associated quorum sensing kinase
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
Km	Kanamycin
LB	Luria Bertani
LM	Luria marine
MALDI	Matrix-assisted laser desorption/ionization
MCP	Methyl-accepting chemotaxis protein
MM	Marine media
MS	Mass Spectrometry
MTA	Methylthioadenosine
MTR	Methylthioribose
NO	Nitric oxide
NOA	Nitric oxide analyzer
OMFP	O-methylfluorescienphosphate
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PMSF	Phenylmethylsulfonyl fluoride
Qrrs	Quorum regulatory RNAs
QS	Quorum sensing

SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SRH	S-ribosylhomocysteine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sGC	Soluble Guanylate cyclase
TCS	Two-component signal
THMF	2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
WT	Wild type

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CHAPTER 1

Introduction: Cell-cell communication

Abstract

The discovery of bacteria's ability to coordinate population-wide undertakings has paved the way to understanding many biological processes once thought to be restricted to multi-cellular organisms. It has become apparent that bacteria mainly crowd together in a highly complex yet structured community, where they communicate to assess the surroundings and formulate a unified response. Cell communication is accomplished by sending out chemical signals in the form of small molecules called autoinducers (AI) (1). Accumulation of these AIs, as a result of increased cell population, enables bacteria to gather information about the metabolic potential of the environment and the constituent species present, where they react accordingly by changing their behavior. The diverse and ever-changing environment require various yet specific chemical signals to filter out noise from molecules with similar structures that exist in the environment and avoid cross-talk between signals. This phenomenon, known as quorum sensing (QS), has uncovered a wealth of information on bacteria's social nature and cooperative behavior.

1.1 Quorum sensing in bacteria

The term quorum sensing (QS) is used to describe the synchronous regulation of gene expression in response to changes in cell density and species complexity (2). To assess the local environment, bacteria employ diverse chemical signals to sense, integrate and modulate behavior. Through QS, bacteria are able to coordinate cellular functions such as bioluminescence, biofilm formation, virulence production, type III secretion, which are essential for cell survival (3-5).

The concept of QS was first identified in the bioluminescent marine bacterium *Vibrio fischeri*. Investigation of light production (4, 6) led to the conclusion that bioluminescence can only be achieved once the cell culture has reached high cell density (7). Specifically, *V. fischeri* produce and respond to a minimum threshold concentration of secreted signaling molecules called autoinducers (AI) that accumulate in the environment as a function of increasing cell-population density (8, 9). When the threshold limit of AI is reached, a signal transduction cascade is initiated that leads to the upregulation of the luciferase genes and results to light production (10-12). It is then suggested that bacteria use AIs to communicate with one another to coordinate behavior and act in unison.

This exciting discovery has changed our view of the bacterial lifestyle, where once perceived as asocial and self-sufficient, bacteria are now considered to be capable of establishing a highly social network. In the natural environment, bacteria live in mixed population communities and are constantly exposed to various stimuli. It is therefore not surprising for bacteria to engage in several strategies to ensure survival. Bacteria employ intricate networks such as QS in response to the fluctuating environment. Thus research towards the understanding of QS would improve our knowledge of the microbial world.

Considerable progress has been made in the field of QS and basic fundamental steps involve: 1. synthesis of small molecules known as AIs, 2. release of AIs either passively or actively using a transporter to secrete outside the cell, and 3. response to AIs above the threshold limit that leads to recognition by cognate receptors on target cells which then triggers signal transduction cascade that results to population-wide changes in gene expression (13).

The field of QS has revealed a wealth of information on bacterial social life and this has opened a new avenue for research. Understanding the basic principles and elucidating the molecular mechanisms of bacterial communication could impact how we fight disease. This could be the first step toward human intervention where we can scramble the wires of communication to thwart activities of harmful bacteria or facilitate activities of the helpful ones.

1.2. Bacterial communication networks

Integration of information from the environment to alteration of cellular function involves intricate cellular networks. The diversity of chemical signals recognized by bacteria seems to indicate that they employ several systems to help them respond accordingly. To survey the environment, bacteria release and receive signal molecules to determine the population density as well as the type of species present. Regulation of gene expression is accomplished by the recognition of the cognate AI by specific receptors. Several QS circuits have already been identified which can be classified either as intraspecies, used by same family of bacteria or interspecies, used to identify the presence of other species. QS systems are best studied in representative species, and are therefore used in the discussion to better understand the details of these complex and intricate communication systems.

1.2.1 Intraspecies communication system

Bacteria synthesize specific signals that can only be detected by members of the same species. This confers the intraspecies communication.

1.2.1.1 Acylated homoserine lactone-based QS in Gram-negative bacteria

The QS circuit in *V. fischeri* laid the foundation for all subsequent studies of QS in Gram-negative bacteria (14). Genetic and biochemical studies showed two regulatory components that control QS circuit in *V. fischeri* (10-12). The synthase, LuxI, is responsible for the production of AI, while a transcriptional regulator called LuxR is responsible for binding AI and activating transcription of the luciferase operon at high cell density (10, 11).

The LuxI/LuxR (AI synthase/transcriptional regulator) signal-response system appears to be the standard mechanism used by Gram-negative bacteria to control cell density-dependent functions (15). Many Gram-negative bacteria contain LuxI-type AI synthases that produce homoserine lactone rings carrying acyl chains of C_4 to C_8 (16) in length at the α-position. These side chains are occasionally modified to contain a different functional group such as unsaturated double bonds. In general, natural acylated homoserine lactones (AHL) identified to date, share conserved structural characteristics as shown in Figure 1.1.

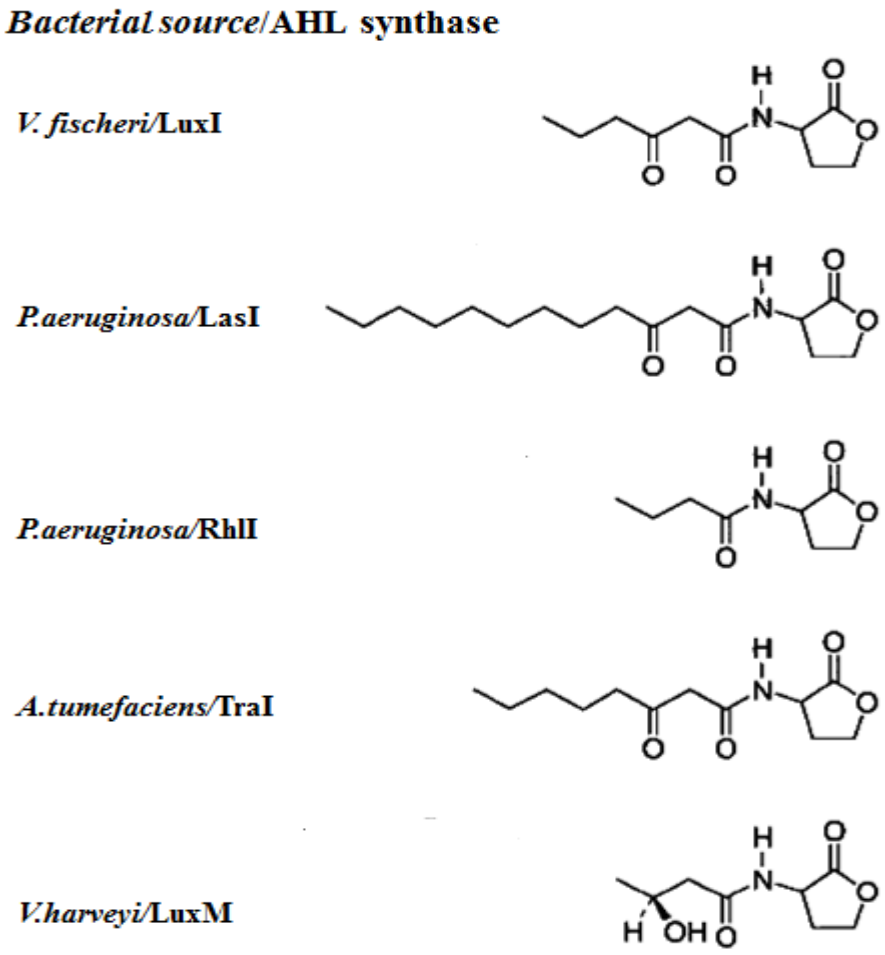


Figure 1-1. Representative structures of Acyl-homoserine lactone AIs in Gram-negative bacteria, identified by the AI synthase in which organism they were isolated (adapted from (17).

The synthesis of AHL is derived by the acylation of a specific acyl-acyl carrier protein from the fatty acid biosynthetic machinery to the homocysteine moiety of S-adenosylmethionine (SAM). This step is catalyzed by LuxI-type enzymes (18) as shown in Figure 1.2. The ligated intermediate lactonizes to form AHL and methylthioadenosine (MTA) (19-21), but the latter is potentially toxic, thus a nucleosidase, Pfs degrades MTA into

adenine and methylthioribose (MTR), both of which are non-toxic. However, the function of MTR is still to be determined. Some AI synthases found in Gram-negative bacteria share no homology with LuxI but it is proposed that these synthases will follow the same mode of action. These include LuxM in *V. harveyi*, RhlI and LasI in *Psuedomonas aeruginosa*, AinS in *V. fischeri*, TraI in *Agrobacterium tumefaciens* and HdtS in *Pseudomonas fluorescence* (1, 22, 23).

Following production, AHLs are sufficiently amphipathic, i.e, they can diffuse freely crossing the cell membrane passively, where they accumulate in the external environment (24). As the cell density increases, so does the concentration of AHLs. Regulation of gene expression is triggered when sufficient AHLs accumulate in the environment for productive binding with the cognate receptors. LuxR-type proteins are the receptor of AHLs in Gram-negative bacteria. Although LuxR is produced at a basal level, LuxR is unstable and is easily degraded. However, the presence of AHL stabilizes LuxR and this complex acts as transcriptional regulator to many genes associated with cellular processes. Specifically, when the concentration is high enough to enable AHL to bind to LuxR-type proteins, dimerization of LuxR occurs and forms the active conformation leading to activation of target DNA. Mutational analyses of *V. fischeri* LuxR show that the amino-terminal regions bind AHL while, the C-terminal domains are responsible for oligomerization and binding to the DNA promoter (25-27).

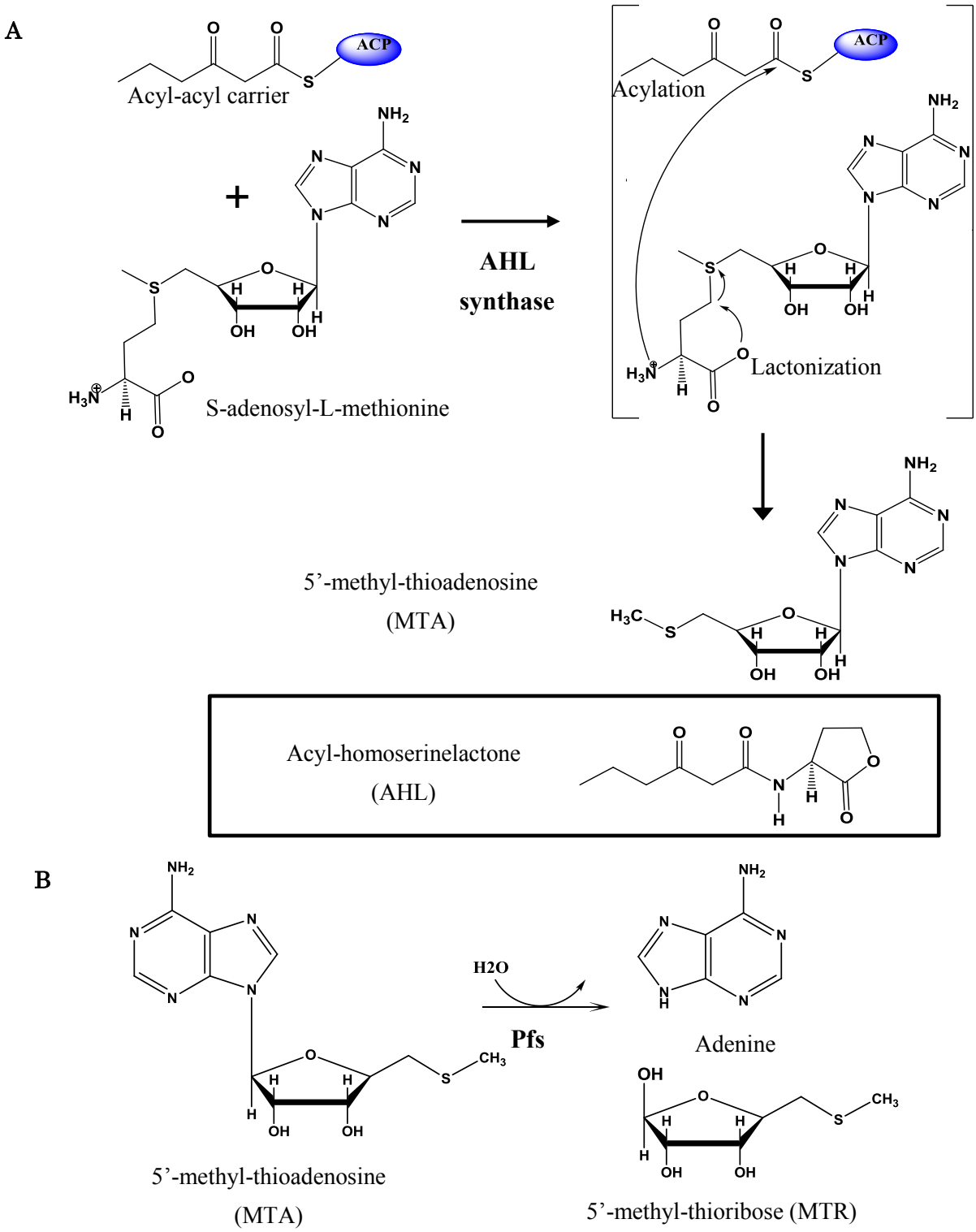


Figure 1-2. Biosynthesis of AHL. (A) The schematic diagram illustrates the general features of the AHL synthesis reaction (The AHL in these diagram is the AHL synthesized by *V. fischeri* LuxI). (B). Degradation of MTA by Pfs.

In the case of *V. fischeri*, LuxR-AHL also activates the expression of LuxI and since it belongs to the same operon as the *luxCDABE* cluster, it generates a positive feedback loop, hence the term autoinduction. Homologs of LuxR have been found in several Gram-negative organisms which also function as the cytoplasmic AI receptors as well as DNA-binding transcriptional regulators (10) as depicted in Figure 1.3..

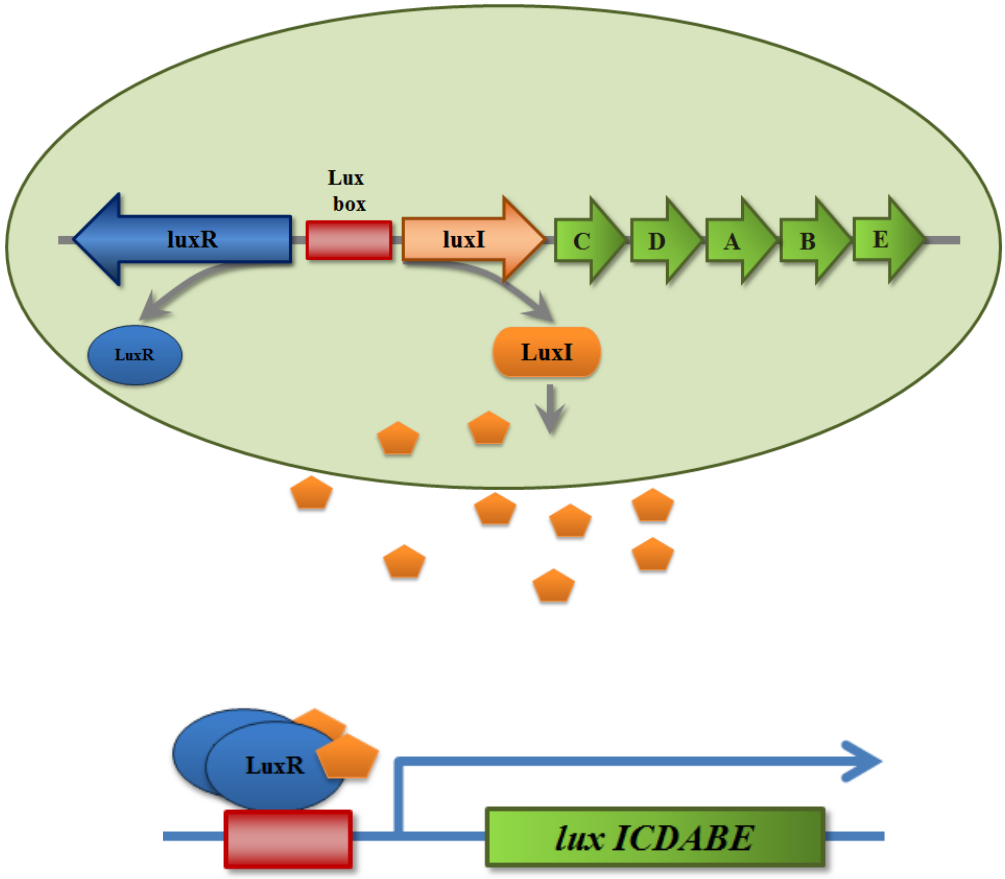


Figure 1-3. A schematic of the LuxI/LuxR Quorum sensing system in Gram-negative bacteria. The orange pentagons represent AHL autoinducers (Adapted from (28).

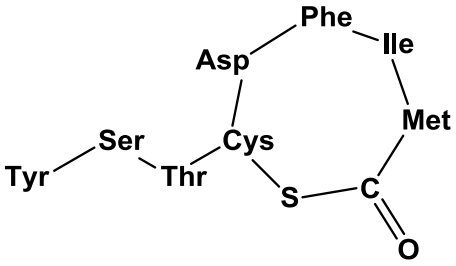
Signaling specificity exists in LuxI/LuxR systems, in spite of the structural similarity of the AHL molecules (29). Typically, AHLs are detected only by the species that produce them, thus it is suggested that AHLs act as intraspecies signals. Differences in the length of the fatty-acyl side chains, backbone saturation and side-chain substitutions contribute to the signal specificity. Structural studies have shown that LuxI-type proteins contain an acyl-binding pocket specific to a particular side chain moiety (18, 30) while LuxR contains a specific acyl-binding pocket that allows each molecule to bind another LuxR to form a dimer, and be activated by its cognate AHL (28, 31, 32). These findings suggest that even in mixed-environments, each species can distinguish a specific AHL.

The LuxI/LuxR system is the paradigm for all AHL-based QS system. It should be noted though that QS signaling process that occurs in some Gram-negative bacteria is often more complex than what is depicted in *V. fischeri* (13). In some cases, QS network involve two or more AHL signals that are detected by specific receptors which act in series or parallel (33) to control global cell-density dependent gene expression (34).

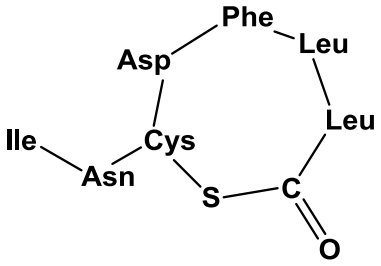
1.2.1.2 Oligopeptide-based QS in Gram-positive bacteria

Quorum sensing in Gram-positive bacteria employs a different set of AIs as a result of different membrane properties possessed by Gram-positive bacteria. They use modified oligopeptides as signal molecules and two-component type membrane-bound kinases as detectors. In Gram-positive bacteria, precursor peptides are chemically modified and cyclized before secretion into the environment. Because these peptides are impermeable to biological membranes, they are secreted via an ATP-binding cassette (ABC) transporter complex (13) and their receptors are at the cell membrane. Concomitant with signal release,

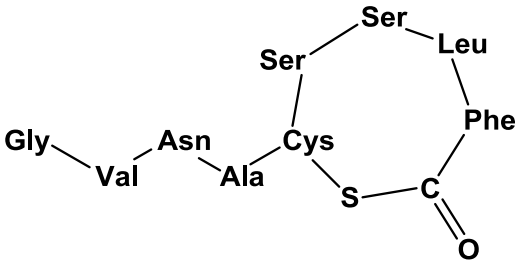
it is known that some peptide AIs are cleaved from larger precursor peptides, which are then modified to contain lactone and thiolactone rings, lanthiones, and isoprenyl groups (35, 36). Representative modified oligopeptides are shown in Figure 1.4.



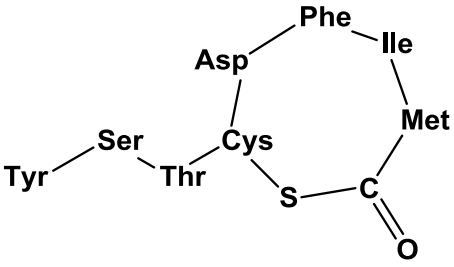
AIP-I *(S.aureus)*



AIP-II *(S.aureus)*



AIP-III *(S.aureus)*



AIP-IV *(S.aureus)*

ADPITRQ$\overset{*}{W}$GD	ComX (*B. subtilis*)
ERGMT	CSF (*B. subtilis*)
EMRLSKFFRDFILQRKK	CSP (*S. pneumoniae*)

Figure 1-4. Representative oligopeptide AIs in gram-positive bacteria designated by the name and bacterial source. The asterisk above ComX represents isoprenyl modification of the tryptophan (Adapted from (13)).

Oligopeptide AIs are detected by cognate receptor histidine kinases. One major difference between the Gram-negative AHL receptor and the oligo-based receptor is the location of the cognate receptors; whereas LuxR-type receptors are cytoplasmic, oligo-based receptors are membrane bound. These membrane-bound receptors, as members of the two-component signal transduction system of kinase receptors, transduce information through a cascade of phosphorylation events (37, 38) (Figure 1.5). Specifically, as the concentration of oligopeptide increases with increasing cell density, the membrane-bound histidine kinase receptors bind the oligopeptide AI, which in turn stimulates autophosphorylation of a conserved histidine residue in the cytoplasmic domain of the receptor. The phosphate is then transferred to a conserved aspartate in the response regulator whose primary function as a transcriptional regulator is to bind to the promoter of the target DNA and influence gene expression. In many cases the histidine kinase receptor and the response regulator are found in the same operon where expression is auto-induced by QS (39, 40) .

This mechanism of signal transduction is conserved in many Gram-positive bacteria where specificity of the receptors for the cognate AI is also observed. Unlike the AHLs in Gram- negative bacteria, the peptides are genetically encoded and not a product of variation of a single molecule, thus each bacterial species are capable of producing a very specific peptide that suits the individualized needs of the species. Moreover, the transmembrane AI binding domain shows little homology with other transmembrane proteins even though the kinase domain is homologous to other sensor histidine kinases. These two factors likely determine the specificity in Gram-positive bacteria (41, 42). Because of this specificity, the oligopeptide autoinducers are also understood to confer intraspecies communication.

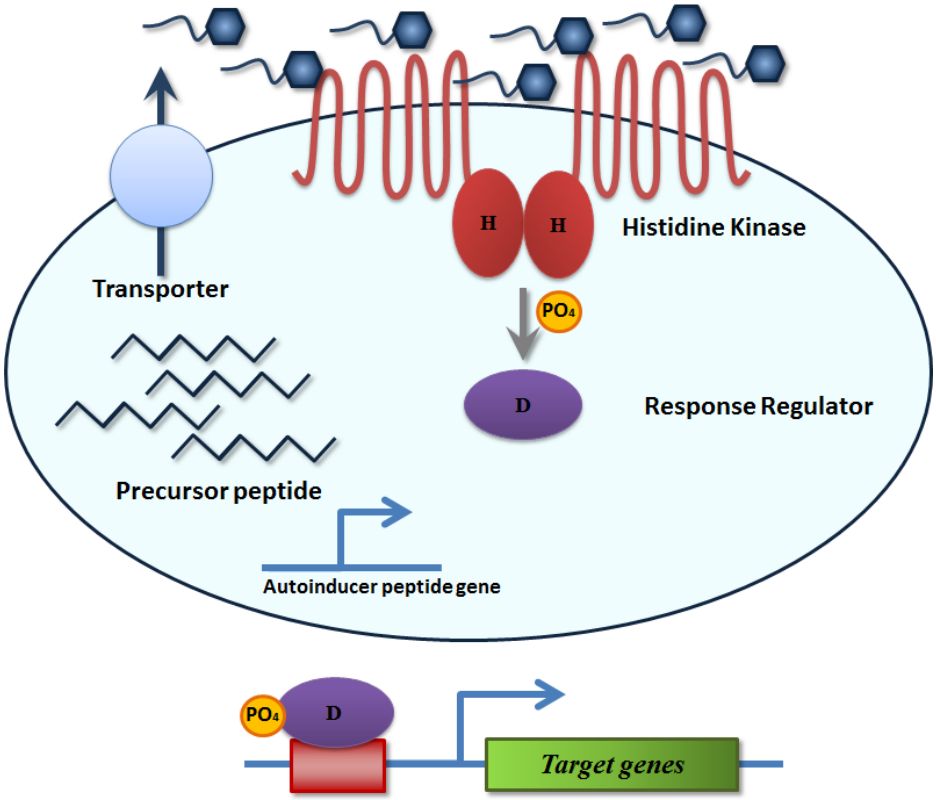


Figure 1-5. A schematic of a canonical QS circuit in Gram-positive bacteria. The hexagons represent the processed/modified peptide autoinducers (Adapted from (13)).

One of the most studied Gram-positive bacteria, *Staphyloccocus aureu*s, carries in its genome sequences encoding an autoinducing system composed of an autoinducing peptide (AIP) and a two-component histidine kinase-response regulator pair, AgrC and AgrA respectively (43). The peptide is encoded by the gene *agrD* (39) while AgrB protein exports and modifies AIP by adding a thiolactone ring as depicted in Figure 1.5 (44). Binding of the AIP to AgrC leads to phosphorylation of AgrA. Phospho-AgrA induces the expression of

many regulatory units such as the regulatory RNA termed RNAIII. This represses expression of cell-adhesion factors while inducing expression of secreted factors and the expression of *agrBDCA* operon(42). Analogous to LuxI/LuxR system, the expression of the *agrBDCA* operon confers a positive loop that enables the entire population to switch from the low-cell density to high cell density state through autoinduction. Interestingly, four different AIPs are found in *S. aureus* that bind to AgrC receptor but inhibit activation of all other competitive binding receptor kinases (45). This suggests that bacterial cell communication possibly plays a role in establishing a specific niche for each strain (42).

Other processes responsive to cell density in Gram-positive bacteria include the competence for DNA uptake in *Bacillus subtilis* and *Streptococcus pneumonia*, conjugation in *Enterococcus faecalis* and microcin production in *Lactobacillus sake*. All of which confirm that QS gives the evolutionary advantage to the bacteria using it.

1.2.2. Interspecies communication system

Bacteria live in a multi-species community where the environment is composed of many others that crowd to form a highly complex structure. So, how do bacteria know who is family and who is not? The answer lies in the discovery that bacteria not only talk to other members of the same species, but also send and receive messages to and from different species. This has attracted attention which led to the discovery of another signaling molecule, otherwise known as auotinducer-2 (AI-2). AI-2 was first identified in *Vibrio harveyi* and so named because it was the second AI to be discovered which contains in its structure a furanosyl borate diester (46). The synthesis of this AI is directed by the LuxS synthase and has been implicated in interspecies signaling (47). Mutational analysis on *luxS*

gene from *E. coli, V. cholerae* and *Salmonella typhimurium* showed elimination of AI-2 production (48) conferring its function as an AI-2 synthase.

Biosynthesis of AI-2 is derived from S-adenosylmethionine (SAM), whose utilization as a methyl donor yields S-adenosylhomocysteine (SAH) (28). SAH is metabolized by SAH hydrolase into adenine and S-ribosylhomocysteine (SRH) (49). LuxS cleaves SRH to generate homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (50). DPD, a highly reactive product, is expected to cyclize spontaneously to form two epimeric furanoses, (2*R*,4*S*)- and (2*S*,4*S*)-2,4-dihydroxy-2-methyldihydrofuran-3-one *(R*- and *S*-DHMF, respectively; Hydration of *R*- and *S*-DHMF would give rise to (2*R*,4*S*)- and (2*S*,4*S*)- 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*- and *S*-THMF, respectively. The biosynthetic pathway is shown in Figure 1.6. The Crystal structure of the Synthase-AI-2 complex in *V. harveyi* LuxP-AI-2 (46) and *S. typhimurium* LsrB-AI-2 (51) showed that the signal molecule is S-THMF borate and R-THMF, respectively. Formation of this molecule from DPD can be explained by a simple reaction. Since borate reacts readily with adjacent hydroxyl groups on the furanosyl rings, it is chemically reasonable that S-THMF borate form spontaneously by addition of borate, which is abundant (c.a 0.4 mM) in marine environments, to form S-THMF borate.

Different bacterial strains, whether Gram-positive or Gram-negative, recognize different DPD derivatives, where bacteria recognize their own AI-2 and also AI-2 produced by other bacterial species (52). This is possible because of the interconversion of molecules within the AI-2 pool. Thus, AI-2 is proposed to be a universal signal for interspecies signaling in bacteria.

The receptor involved in recognizing AI-2 and the QS circuits that control gene expression are best understood in the marine bacterium, *V. harveyi*. Discussion of the

detailed mechanism is given in the next section.

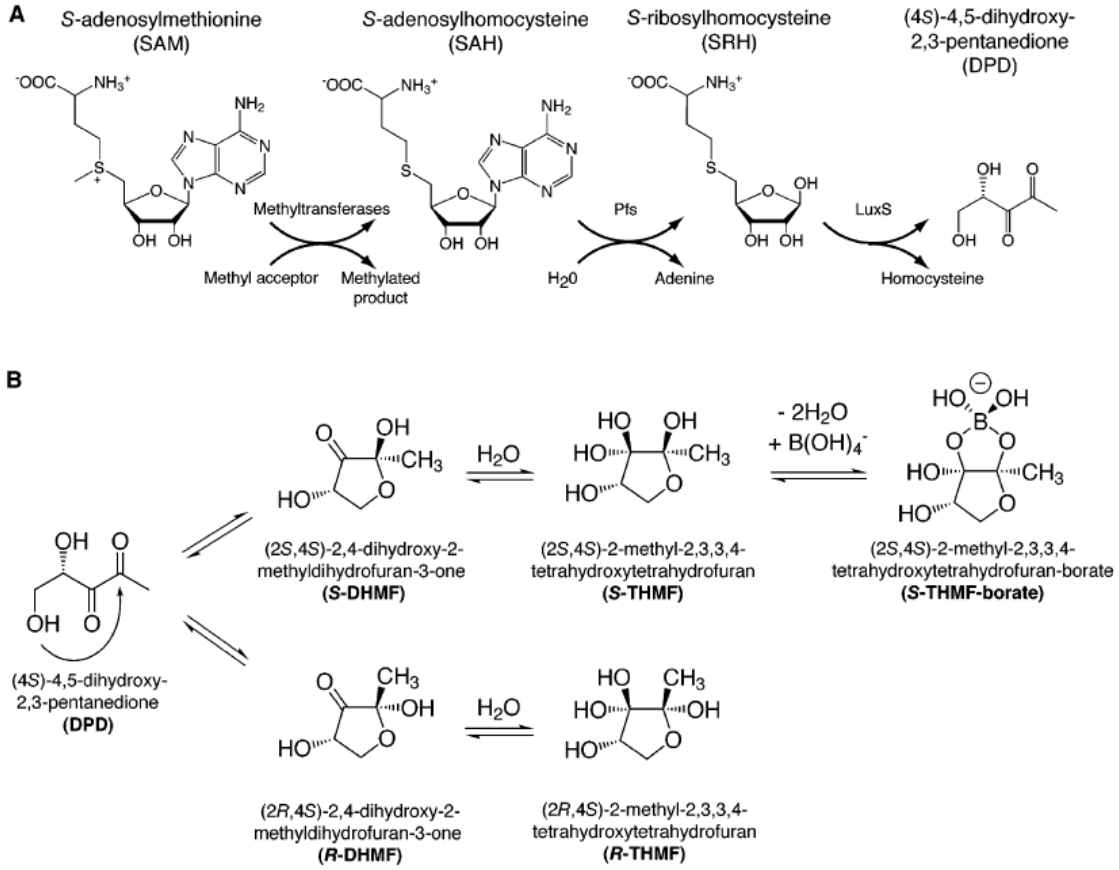


Figure 1-6. Biosynthetic pathway of AI-2. (A) Metabolic pathway leading to DPD (B) Proposed pathway that leads to AI-2 in *V. harveyi* (upper branch) and *S. typhimurium* (lower branch) (Adapted from (51)).

1.3 Quorum sensing circuit in *Vibrio harveyi*

The ability of bacteria to communicate with one another using multiple quorum-sensing signals was first observed in the QS system of the Gram-negative, bioluminescent marine bacterium *V. harveyi.* *V. harveyi,* while closely related to *V. fischeri*,

do not live in symbiotic associations with higher organisms. Rather, *V. harveyi* are found free living in seawater, in shallow sediments and on surfaces and in the gut tracts of various marine animals. However, they are also considered as pathogens of aquatic animals such as shrimp, causing luminescent vibriosis and affecting significant losses in the aquaculture industry worldwide. *V. harveyi* use QS to control bioluminescence albeit differently from the LuxI/LuxR system of *V. fischeri.* The multiple QS circuits in *V. harveyi* are a hybrid of the Gram-positive and Gram-negative QS systems. Analogous to other Gram-negative bacteria, *V. harveyi* produce and detect AHL autoinducers (Figure 1.6) however, the AI sensors are histidine kinases similar to Gram-positive QS sensor kinases, where gene regulation also involves a signal transduction system.

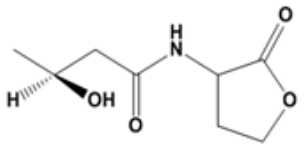
Prior to the work described in this study, *V. harveyi* were known to have three AIs and three cognate receptors functioning in parallel to channel information into a shared regulatory pathway (53). The first channel involves an AHL autoinducer termed HAI-I, 3OHC4-homoserine lactone (9), synthesized by LuxM. However, LuxM does not share any homology with LuxI-type enzymes but it is proposed to catalyze an identical biochemical reaction to generate the AHL from SAM and specific acyl-ACP (5, 19). HAI-1 binds to a membrane-bound sensor histidine kinase (LuxN) similar to sensors in Gram-positive QS signaling circuits (5, 54). Genetic and biochemical studies have shown that LuxN is a two-component hybrid sensor kinase (55, 56) which can also be found in few other very closely related *Vibrio* species, and has been implicated in intraspecies communication (9, 53).

The second *V. harveyi* signal is a furanosyl borate diester known as AI-2 (46, 57), production of which requires the metalloenzyme, LuxS enzyme (48, 51, 58). LuxS contains a divalent ion in the active site, where the type of cation present in the active site varies during the course of the reaction (59). It is suggested that the cation plays a role in stabilizing the

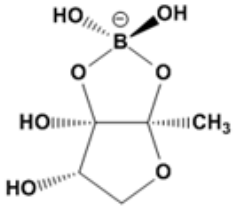
DPD intermediate by binding to them in a bidentate manner (59). DPD is the key intermediate in the generation of AI-2 signal molecules. LuxS is present in many bacterial species, further evidence in support for AI-2 as a signal for interspecies communication (47) or the universal language of bacteria. AI-2, 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate (S-THMF borate), is detected by LuxP, a soluble periplasmic protein that is homologous to the ribose binding proteins of *Escherichia coli* and *S. typhimurium*. LuxP and the histidine sensor kinase, LuxQ (57, 60) form the complex that is used as sensor for AI-2. Crystal structure and functional studies showed that binding of AI-2 to LuxPQ quaternary complex causes a major conformational change within LuxP, which in turn stabilizes the quaternary arrangement of two LuxPQ monomers and form an asymmetric association. The authors proposed that the formation of the asymmetric quaternary structure is responsible for the deactivation of the kinase activity on LuxQ, leaving the phosphatase activity on and draining the phosphate from LuxO (60).

The third *V. harveyi* signal, termed CAI-1 was first identified in *V. cholerae*, thus named cholerae autoinducer-1. The identity of CAI-1 in *V. harveyi* was determined to be (*Z*)-3-aminoundec-2-en-4-one (Ea-C8-CAI-1) carrying an 8-carbon tail. It is produced by the CqsA enzyme (2), and again, this signal interacts with a membrane-bound sensor histidine kinase, CqsS (53). The CqsA/CqsS system is conserved in many *Vibrio* species (53, 61, 62), suggesting it may be used for communication among *Vibrios*. LuxN, LuxQ and CqsS are bifunctional enzymes that contain kinase and phosphatase activities. These two-component enzymes all transduce the information by phosphorylation and dephosphorylation to and from a shared two-component phospho-relay protein, called LuxU (54, 63), which subsequently conveys the signal to the response regulator LuxO (64) and indirectly represses light production. Interestingly, no LuxI/LuxR homologs have been identified so far in the *V.*

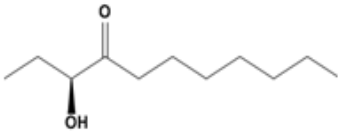
harveyi QS system.



HAI-1



AI-2



CAI-1

Figure 1-7. Chemical structures of the AIs used by *V. harveyi.*

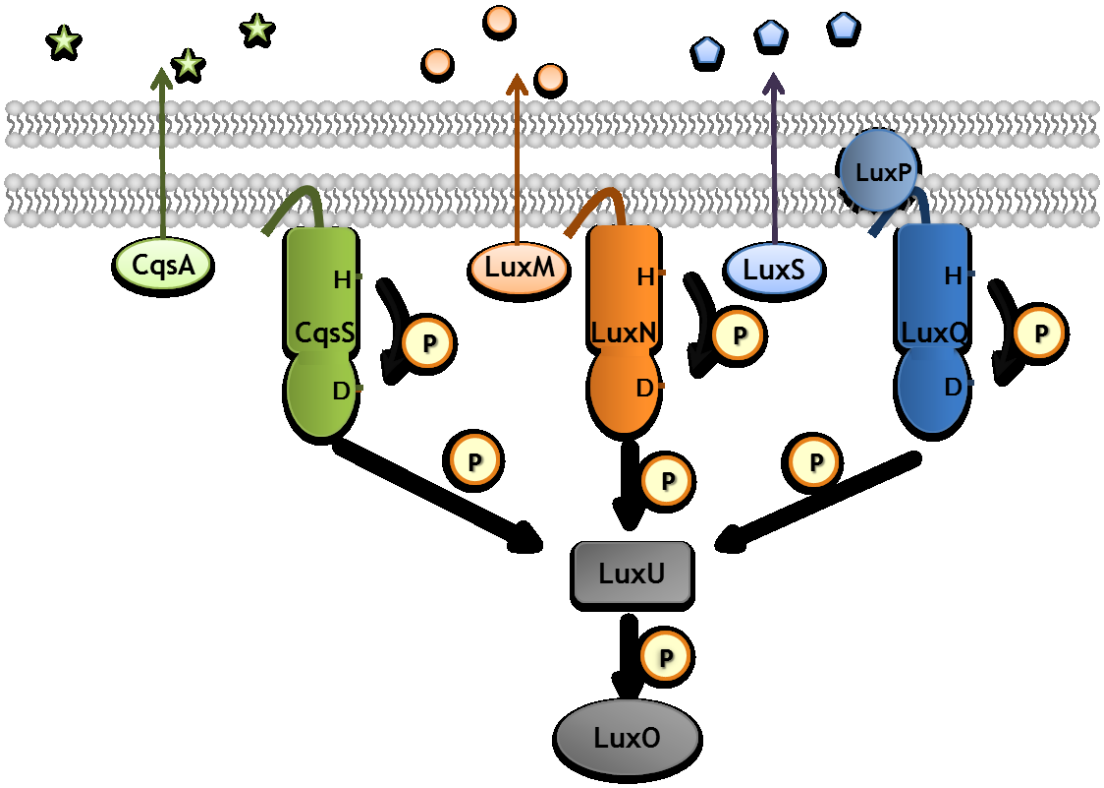
Autoinducer binding triggers a signal transduction cascade that funnels phosphate to and from the same response regulator, LuxO through LuxU. The flow of phosphate depends on the concentration of AIs as a result of cell density, and in consequence the net function of the receptors. At low cell density (Figure 1.8A**)**, when there is negligible concentration of AIs in solution, the three receptors act as histidine kinases which autophosphorylate the histidyl residue on the catalytic domain and then transfer it to the aspartate in the receiver domain. LuxU serves as the common conduit for all three pathways via its histidine residue (H58) where phosphate is finally transferred to the D47 of LuxO. Together with a transcription factor (65), σ^{54}, phosphorylated LuxO activates the production of five regulatory small RNAs (sRNAs) termed Qrr 1-5 (65). These Qrrs bind to Hfq (66), a

member of the Sm family of eukaryotic chaperones involved in RNA splicing (67). Together, they destabilize *luxR* mRNA(66), in effect decreases LuxR, the master transcriptional regulator of the luciferase operon *luxCDABE* (68). This repression of the lux operon leads to a dark phenotype (since no light is produced). The structural unit of the luciferase operon is composed of *luxAB* genes which code for the subunits of luciferase while *luxC, luxD, luxE* code for the reductase, transferase polypeptide, and synthetase, respectively. Together, *luxCDE* form the fatty acid reductase complex which produces and recycles the long chain fatty aldehyde substrate needed for light production (69).

As more cells are produced, *i.e.* at high cell density, (Figure 1.8 B), more AIs are available in the system where the level reaches the threshold required for detection. The interaction of the AIs with the receptors switches the role from net kinases to net phosphatases. This switch in mode reverses the flow of phosphate leading to the dephosphorylation of LuxO, rendering it inactive. As a consequence, Qrrs are not synthesized which ultimately results in the activation of the *luxR* mRNA to produce LuxR (70). This pathway controls many genes in addition to those encoding luciferase, such as biofilm formation, type III secretion, virulence production (3, 53, 71).

The QS systems described rely on the precise recognition of signal molecules by specific cognate receptors to help bacteria identify the component of the surrounding environment. It appears that tight specificity is required to prevent bacteria from responding to noise brought about by the diversity of signals presented. Distinct responses to the intraspecies, interspecies and interkingdom signals allow bacteria to modulate their behavior depending on the niche in which they reside. Having multiple “languages” help them serve different purposes and confer an ecological advantage at any given challenges that they may encounter.

A


CqsA
H
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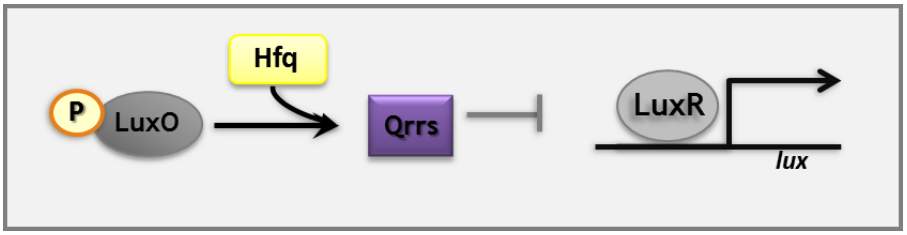
Hfq
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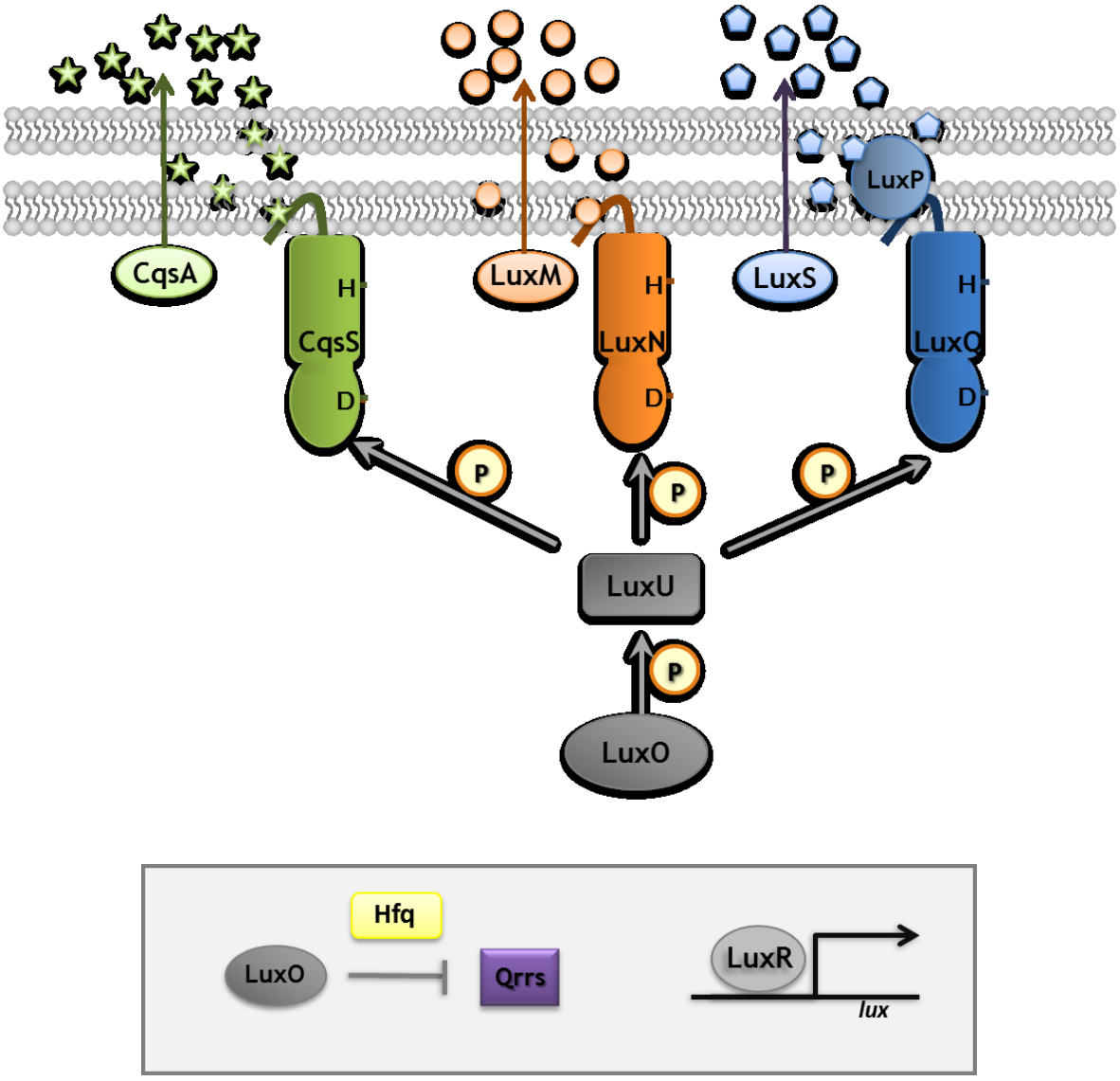


Figure 1-8. Schematic of parallel quorum sensing circuits in *V. harveyi*. (A) depicts the flow of phosphate under low cell density state (B) depicts the flow of phosphate at high cell density state.

1.4. Nitric oxide and the bacterial NO sensor, H-NOX

The various role of NO in every phase of biology and medicine has earned this molecule an award given by Science magazine (72). It may be a simple diatomic molecule, but its function is nevertheless critical to most physiological processes. This gas is known to act as a signaling molecule in eukaryotes; regulating blood flow and thrombosis (73), as well as a role in platelet aggregation and immune response (74). There also is a growing interest in the signaling role of NO in bacteria, for it regulates multiple systems that enable bacteria to avoid cell death (75).

The mechanism by which NO is sensed by eukaryotes is attributed to its sensor, the soluble guanylate cyclase (sGC). sGC is a heterodimer which contains a C-terminal domain that regulates guanylate cyclase and a heme-containing domain in the N-terminus responsible for NO binding (76). Although no sGC has been identified in prokaryotes, primary sequence analyses have shown that the heme-containing domain of sGC is also encoded in some bacterial genomes. Sequence homology has identified these gene sequences to be putative NO/O_2 binding protein, and based on spectroscopic and kinetic characterization of these sGC-like proteins (77), the family is named H-NOX for Heme-Nitric oxide/Oxygen binding protein (78). Most bacterial H-NOX proteins are standalone proteins, but they are usually encoded in the same gene cluster as those responsible for signal transduction such as histidine kinase, diguanylate cyclase or fused to methyl-accepting chemotaxis protein (MCP). Thus, it is proposed that H-NOX/NO is involved signaling pathways that regulate gene expressions that alter cellular function.

The biological role of bacterial H-NOX appears to indicate an important additional modulation of physiological processes. NO and H-NOX have been associated with biofilm

formation in several microorganisms, such as *Shewanella woodyi* (79), *Legionella pneumophila* (80), and *Shewanella oneidensis* (81). In the bacterial genomes of *S. woodyi* and *L. pneumophila*, the *hnoX* gene is in the same operon as a diguanylate cyclase (DGC). DGC, through its di-guanylate cyclase activity and/or phosphodiesterase (PDE) activity, regulates the production of cyclic-diguanosine monophosphate (c-di GMP), a well-documented second messenger associated with biofilm formation. This type of regulation is reminiscent of the functional role of sGC, where the presence of NO activates the cyclase activity which catalyzes the production of cyclic-guanylate monophosphate (cGMP), an important eukaryotic second messenger (82). Phenotypic and biochemical studies showed that NO influences biofilm formation through H-NOX regulation of DGC. In *S. woodyi*, NO-bound H-NOX negatively affects biofilm formation by directly regulating c-di-GMP turnover (79). However, in *S. oneidensis*, *hnoX* gene is in the same operon as a two-component histidine kinase that interacts with a response regulator with PDE activity (81). Phosphotransfer profiling showed that H-NOX associated histidine kinase transfers phosphate to a response regulator containing an EAL effector domain which controls c-di-GMP levels. Moreover, biochemical studies show that NO inhibits the kinase activity (83) thereby affecting the phosphotransfer to its cognate response regulator. This also seems to be the case for another marine bacterium, *Pseudoalteromonas atlantica* (84). Other than biofilm formation, NO/HNOX also plays a role in the symbiosis of *V. fischeri* and *E. scolopes*, where NO bound H-NOX mediates colonization by *V. fischeri*.

In *V. harveyi*, a *hnoX* gene is found in the same operon as a histidine kinase, annotated as LuxQ. LuxQ, together with LuxP, is the bifunctional sensor for AI-2 that participates in the signal transduction cascade of *V. harveyi's* QS circuit. Thus, we hypothesize that H-NOX acts as the sensory domain of the histidine kinase, which we name

HqsK for H-NOX-associated quorum sensing Kinase.

Here we describe the fourth *V. harveyi* QS circuit. We show that NO enters QS circuits analogously to the other AIs, where it binds to its cognate receptor pair: H-NOX/HqsK and participates in the QS by funneling phosphate to and from LuxO through LuxU. It is likely that *V. harveyi* monitor NO as an environmental cue, where it could possibly indicate the presence of a eukaryote. Therefore, we propose that NO is an interkingdom signaling molecule used for eukaryote-to-bacteria communication.

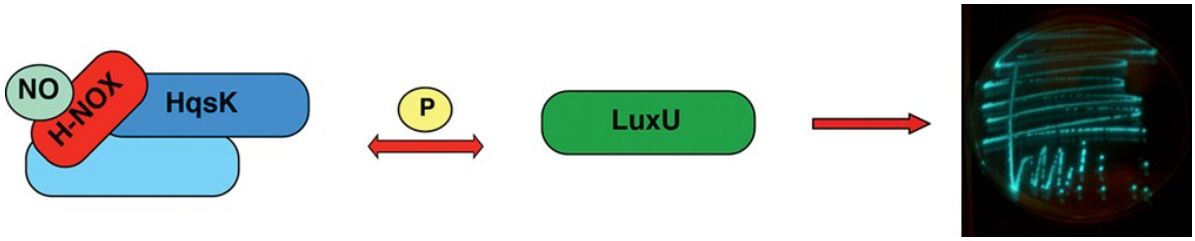


Figure 1-9. Proposed model for NO-regulation of bioluminescence through the NO-H-NOX/HqsK QS circuit in *V. harveyi*

1.5. Overview of research projects included in this dissertation

This manuscript is divided into 4 parts, Chapter 1 contains the literature survey and background material, Chapters 2 and 3 contain the experimental details and explanation of the discovery of a NO-responsive quorum sensing circuit in *V. harveyi,* and in Chapter 4 we explore additional phenotypic phenomenon controlled by NO. Specifically, Chapter 2 explains the effect of NO on light production. Bioluminescence is the canonical read-out for QS, since expression of genes involved in light production is directly controlled by AIs and its receptors as a function of cell-population. Here, we monitored light production in *V. harveyi* strain as a function of NO over time. DPTA NONOate was used as NO donor,

which gives a steady supply of NO in the low nanomolar level. *V. harveyi* display a NO concentration-dependent increase in light production that is regulated by expression of a NO-sensitive *hnoX* gene. Mutational analyses show that a *ΔhnoX* strain is insensitive to additional NO, while plasmid complementation of *hnoX* in the Δ*hnoX* strain restores the WT response to NO. Our findings demonstrate that an NO-responsive sensor, proposed to be H-NOX regulates a histidine kinase that factors phosphorylation into the QS of *V. harveyi*.

In Chapter 3, we explore the possibility that NO/H-NOX and a histidine kinase found in the same operon may function as a two-component system that funnels phosphate into the QS circuit of *V. harveyi*. Spectroscopic and kinetic analyses show that H-NOX is an NO sensor capable of detecting NO at low picomolar range while the histidine kinase, which we named HqsK for H-NOX-associated quorum sensing kinase, shows autophosphorylation activities and factors phosphate into the system through LuxU, the common regulatory system in the QS circuit of *V. harveyi*. Biochemical analyses further show that H-NOX interacts with HqsK, where the NO-bound H-NOX shows regulation of HqsK kinase activity. Taken together, our biochemical, genetic and phenotypic data represent a new pathway which involves an NO signal sensed by H-NOX to modulate the kinase activity and phosphate flow into the QS system through HqsK and phosphotransfer to LuxU to alter light production.

In Chapter 4, we look into another phenotype that is demonstrated to be regulated by NO/H-NOX. Biofilm formation has been shown to be regulated by QS in other organisms. Interestingly, we found that NO/H-NOX can also influence biofilm formation through regulation of c-di-GMP. Here, we try to explore the possibility of an NO/H-NOX-HqsK biofilm regulation through the QS circuit of *V. harveyi*. Colorimetric and microscopic analyses were performed to assess biofilm thickness in the presence of NO. Preliminary studies show an NO dependence of biofilm formation in *V. harveyi*.

CHAPTER 2

Nitric oxide is a quorum sensing signal in *Vibrio harveyi*

Abstract

Bacteria use small molecules to assess the density and identity of other organisms in their surroundings and to make decisions about how to respond to a complex environment. This process, known as quorum sensing (QS), regulates diverse functions such as bioluminescence, biofilm formation, and virulence. In *Vibrio harveyi*, three parallel QS circuits that synergistically regulate bioluminescence have been previously identified. Each system involves the synthesis of a specific small molecule called autoinducer that binds to its cognate receptor histidine kinase and regulates phosphorylation. The three pathways converge and exchange phosphate with a common phospho-relay protein called LuxU. LuxU transfers phosphate to LuxO, a response regulator that controls expression of LuxR and, ultimately, the quorum sensing response. In this study, we have identified nitric oxide (NO) as a signal molecule that participates in regulation of bioluminescence through the LuxU/LuxO/LuxR pathway. Our bioluminescence assays showed that *V. harveyi* exposed to low nanomolar concentration of NO resulted in a concentration-dependent increase in light production. Furthermore, NO-dependent light production was not observed in *hnoX* deletion mutant however, the mutant strain complemented with *hnoX* gene restored the wild type behavior. Our findings suggest that NO regulates bioluminescence through the expression of a NO-sensitive *hnoX* gene. These data provide the basis for a novel NO-regulated pathway in the QS circuit of *V. harveyi.*

2.1 Introduction

It is now widely accepted that bacteria are social in nature, capable of expressing themselves with the use of chemical signals known as autoinducers (AI) to signify their presence and gauge their surroundings (1, 85). Quorum sensing (QS), the popular term for bacterial cell-cell communication, provides a mechanism in which bacteria coordinate group activities as a function of AI concentration to explain communal cellular behaviors such as bioluminescence, biofilm formation, type III secretion and virulence factor production (3, 11, 86, 87). QS is traditionally defined as the synthesis of, detection of and response to AIs to regulate gene expression (complete review (13)). AIs vary in structures and properties that can be genus or species specific, or can be a universal signal recognized by both gram negative and gram positive bacteria. For example, in *V. harveyi*, the three characterized AIs play the roles which specifically identify the type of cell communication; HAI-1, identified as 3-hydroxybutanoyl homoserine lactone (9), is synthesized by LuxM and found in very few other very closely related *Vibrio* species, thus it is proposed to be an intraspecies signal (5, 53). This species-specific signal is recognized by LuxN. The second autoinucer discovered, AI-2, is a furanosyl borate diester, identified (S-THMF-borate) as (2*S*,4*S*)-2methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate (46) is synthesized by LuxS, and exists in many bacterial genomes thus considered the universal, interspecies signal (28, 57). AI-2 is detected by the periplasmic LuxP in complex with LuxQ (53, 57, 60, 88). CAI-1, identified as (*Z*)-3-aminoundec-2-en-4-one (Ea-C8-CaAI-1) (2) is synthesized by CqsA and responsible for same genus interaction (53, 62) is detected by CqsS. The diversity of these signals and the specificity for the corresponding cognate receptors represent and reflect the specific needs of the community living in a particular niche.

Species-specific QS has intuitive and well-understood advantages for bacteria (89); indeed, the terms quorum sensing and autoinduction were coined based on intraspecies QS. That QS plays a role in communication and competition between different bacterial species (33, 85, 90) as well as between eukaryotes and bacteria (33, 90, 91) has also been well-documented. More recently, it has been demonstrated that eukaryotic hosts respond to AIs produced by bacteria. Furthermore, other pathways, such as c-di-GMP signaling pathways, also respond to external signals and affect community behaviors such as swarming (92) and biofilm formation. It appears that QS reciprocally influences these other pathways, and together they provide a complex, integrated network for assimilating numerous external stimuli into the complex regulation of bacterial lifestyle choices (motility vs. sessility, acute vs. chronic infection, etc.) (93). Such studies have raised questions about fundamental aspects of QS, including whether quorum sensing is the appropriate term for this type of cell-cell communication (90).

One such interesting molecule, nitric oxide (NO), a well-known signaling molecule in mammals (94) and a potent antibiotic at high concentrations (95), is a bacterial signaling molecule at nanomolar concentrations. It has been shown to participate in QS-regulated bacterial activities such as biofilm formation in *Shewanella woodyi* (79), *Shewanella oneidensis* (81), *Legionella pneumophila* (80), and in biofilm dispersal and denitrification of *Pseudomonas aeruginosa* (96, 97). It has also been demonstrated that bacteria can detect and respond to NO derived from eukaryotic hosts. *V. fischeri,* symbionts of the Hawaiian Bobtail squid *Euprymna scolopes*, respond to host-derived NO as a cue for colonization of the light organ of *E. scolopes* (98, 99). Based on these observations, we hypothesized that bacteria may have evolved the ability to detect eukaryotic NO during QS, possibly as an inter-kingdom signal.

To this possibility, we investigated the effect of NO on QS in the bioluminescent marine bacterium, *V. harveyi*. *V. harveyi* are Gram-negative, facultative anaerobic bacteria with non-sporulating rods and polar flagella. The polar flagella of the bacteria help them to move in a run and tumble motion during chemotaxis (moving towards chemical attractants and away from chemical repellants). They exist in several milieus where they can exist as free swimming in seawater, attached to abiotic surfaces as a member of a biofilm community in marine animals, or in gut tracts in pathogenic associations with marine hosts (4). In fact, they are the causative agent of luminescent vibriosis in shrimps that cause significant loss in the aquaculture industry worldwide (100) although, *V. harveyi* have no reported pathogenicity in humans. One distinctive characteristic of *V. harveyi* is the ability to produce light which makes them a good model system to study QS because although QS regulates the transcription of at least 50 promoters (101), light production is a quantitative and direct readout of QS. Bioluminescence in *V. harveyi* is brought about by the expression of the luciferase operon *luxCDABE*. Luciferase, encoded by genes *luxA* and *luxB*, catalyzes the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long chain fatty acid aldehyde as shown below:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + hv\ (490nm)$$

The synthesis of the fatty acid aldehyde is carried out by the multienzyme fatty acid reductase complex composed of a reductase encoded by *luxC*, a transferase encoded by *luxD* and a synthase encoded by *luxE* (69). Expression of the *lux* genes is highly regulated by QS.

V. harveyi have three described quorum sensing pathways that act together in parallel to regulate light production. These three pathways are LuxM/LuxN, LuxS/LuxPQ, and CqsA/CqsS, each named for the AI synthase and cognate receptor pair (53). At low cell density (Figure 2.1 represented by black arrows), when the concentration of the AIs (HAI-1, AI-2, CAI-1) is low, the unbound cytosolic kinase/phosphatase (LuxN, LuxPQ, CqsA, respectively) subunit of each receptor has a net kinase and phosphotransfer activities (60, 88), funneling phosphate to a common phospho-relay protein, LuxU (63) (Figure 2.1). Phosphorylated LuxU passes phosphate to LuxO (102), a response regulator of the NtrC family (65) of transcription factors that when phosphorylated indirectly represses the master regulator of quorum sensing, LuxR (103). Phosphorylated LuxO, together with σ^{54}, activates the production small RNAs called Quorum Regulatory RNAs (*qrrs 1-5*) (104) and binds to *luxR* mRNA. Because transcription of LuxR is repressed, at low cell density (low AI), *V. harveyi* are not bioluminescent. However, at high cell density (Figure 2.1 represented by gray arrows), AI concentration is elevated and each binds to its respective receptor, resulting to a change in receptor function, from net kinase activity to net phosphatase activity (60). This change in activity results in the dephosphorylation of LuxO, reversing the flow of phosphate, in turn allowing translation of LuxR, expression of the *lux* operon, and production of light (53).

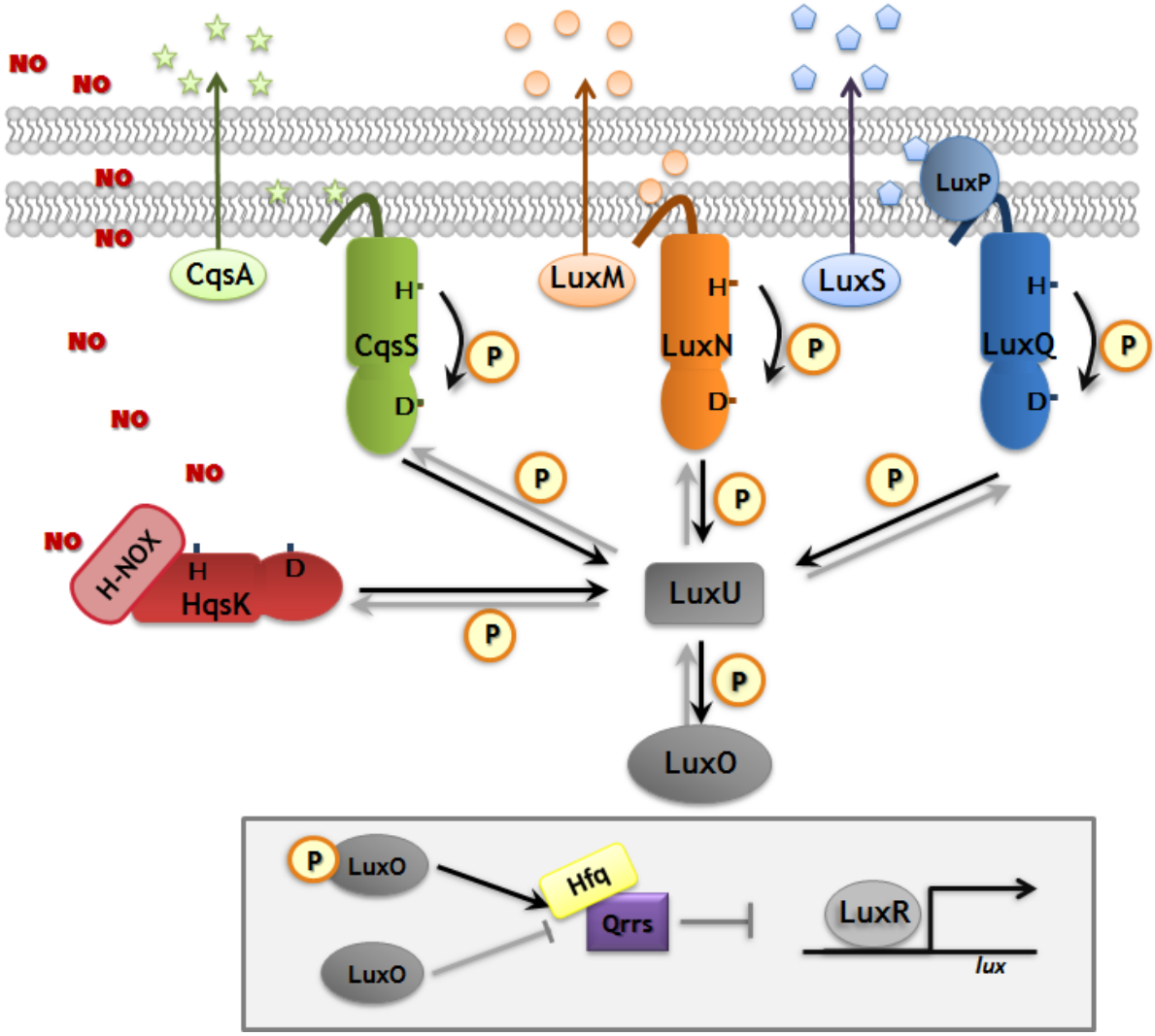


Figure 2.1. A schematic of NO-H-NOX/HqsK QS circuits in *V. harveyi.* The arrows illustrate the flow of phosphate at low cell density state (black arrows) where in the absence of AIs, the receptors act as kinases; at high cell density state (gray arrows) where AIs accumulate and bind to the receptors and reverse the flow of phosphate

2.1.1 Hypothesis

The widely studied eukaryotic NO sensor, sGC, has unveiled the signaling cascade mechanism of great importance to the cardiovascular and nervous system (105). NO/sGC regulates the second messenger cyclic guanosine monophosphate (cGMP) to control vascular smooth muscle relaxation, synaptic transmission, and platelet aggregation (106). Interestingly, bioinformatics and biochemical studies have identified that the NO-sensing domain of sGC is also encoded in the bacterial genomes as a stand-alone protein known as Heme-Nitric Oxide/Oxygen binding protein or H-NOX (107, 108). Several studies on bacterial H-NOX proteins have shown potentials as NO sensors (80, 83) and invoke regulation of downstream cellular processes (79, 81, 96, 98). Upon completion of the *V. harveyi* genome project, a *hnoX* gene (VIBHAR_01911) was identified and downstream is a gene (VIBHAR_01913) annotated as *luxQ,* a putative histidine kinase involved in two-component signaling. Primary sequence analysis studies showed that VIBHAR_01913 contains the important phosphorylation sites similar to that of LuxQ, the characterized AI-2 receptor in *V. harveyi* (88). Thus we hypothesize that H-NOX (VIBHAR_01911) acts as an NO sensor and participates in the QS pathway through the regulation of the histidine kinase. In this study, we demonstrate that NO contributes to light production in *V. harveyi* through H-NOX and the histidine kinase, which we named, HqsK for H-NOX-associated quorum sensing Kinase.

2.2 Materials and Methods

Bacterial strains and growth conditions. Strains used in this study are listed Table 2.1. *V. harveyi* strains WT, Δ*luxO*, and Δ*luxNS* were purchased from the American Type Culture Collection (ATCC), maintained in Marine Media (MM; 28 g/L; BD Difco) and grown at 30 °C with agitation at 250 rpm. For bioluminescence assays, cells were grown in Autoinducer Bioassay media [AB; 0.2% vitamin-free casamino acids, 0.3 M NaCl, 0.05 M $MgSO_4$, 10 mM potassium phosphate pH 7.0, 1 mM L-arginine, 1 %(v/v) glycerol] (5, 103). *E. coli* strains XL1 and BL21(DE3)pLysS were used for plasmid amplification and protein purification, respectively. *E. coli* strains were typically grown in Luria Broth (LB; 20 g/L; EMD chemicals) at 37 °C with agitation at 250 rpm. *E. coli* strain WM3064 was used as a donor for conjugation and was grown in LB complemented with 2,3-diaminopropionic acid (DAP; 0.36 mM; Sigma Aldrich) at 37 °C with agitation at 250 rpm.

Construction of in-frame gene disruption mutant strains. PCR was used to amplify regions of genomic DNA flanking the target gene (VIBHAR_01911) from *V. harveyi* genomic DNA using Phusion® polymerase (New England Biolabs). Upstream and downstream primers (koH-NOX-fwd and koH-NOX-rev, Table 2.1) contained ApaI and SpeI restriction sites, respectively. The amplified PCR product was cloned into pSMV3 (109) (gift from Jeffrey Gralnick, University of Minnesota). The plasmid was modified to yield *pΔhnoX* by introducing two BamHI restriction sites (siteA, near the *hnoX* start codon; siteB, near the *hnoX* stop codon) through site-directed mutagenesis (koH-NOX-site-fwd, koH-NOX-siteArev, koH-NOX-siteB-fwd, koH-NOX-siteBrev, Table 2.1), digestion with BamHI and ligation with T4 DNA ligase. In *pΔhnoX*, *hnoX* is replaced by DNA coding for a

37-amino-acid polypeptide consisting of the first 7 and last 28 amino acids of H-NOX and two additional amino acids encoded by the BamHI site. *pΔhnoX* was transformed into *E.coli* WM3064(109) and grown on LM/DAP plates [Luria Marine; 10 g/L tryptone, 5 g/L yeast extract (Difco) and 20 g/L NaCl, 15 g/L Agar (RPI)] with kanamycin added to a concentration of 100 μg/ml. WM3064 transformed with the deletion vectors were mated with *V. harveyi* in a 1:5 ratio on LM/DAP agar overnight at ambient temperature. *V. harveyi pΔhnoX* transconjugants containing the deletion vectors were selected on LM plates supplemented with 100 μg/ml kanamycin and verified by colony PCR. The selected colonies were then plated on LM plates containing 5% sucrose at ambient temperature to select for double recombination events. Colonies were then replica plated onto LM plates with added kanamycin (100 μg/ml) and incubated overnight at ambient temperature; kanamycin sensitive colonies were screened by colony PCR for gene deletion (79).

Construction of gene disruption mutant complementation plasmid. PCR was used to amplify VIBHAR_01911 from *V. harveyi* genomic DNA using Phusion® polymerase (New England Biolabs). Upstream and downstream primers contained EcoRI and BamHI restriction sites, respectively (*hnoX*comp-fwd and *hnoX*comp-rev, Table 2.1). The amplified PCR products were cloned into the broad host range plasmid pBBR1MCS-2 (110)(gift from Jeffrey Gralnick, University of Minnesota) and sequenced (Stony Brook sequencing core). The resulting plasmid, p*hnoX*, was introduced into the gene disrupted strain via conjugation as previously described (79).

Determination of NO concentration from the decay of DPTA NONOate. NO concentration in AB medium was determined at 30 °C. Stock solution of dipropylenetriamine

(DPTA) NONOate ($t_{1/2}$ = 3 and 5 at 37 °C and 22 °C, respectively, Cayman Chemicals) was prepared inside the anaerobic chamber, dissolved in 10 mM NaOH and stored in -20 °C for not more than 1 day. NONOates are small molecules that release NO at neutral or acidic pH, but are stable as solids or in alkaline solution. NO concentration was determined by diluting the stock solution into fresh AB medium to a final concentration of 10 μM, 50 μM, or 100 μM. The solutions were incubated at 30 °C and using a gas-tight Hamilton syringe, samples were drawn and analyzed at the indicated time points. NO concentration was determined by Sievers nitric oxide analyzer NOA 280i (GE Analytical Instruments) and quantified by comparing to NO standards. Error bars represent standard deviation from triplicate samples. A relatively constant NO concentration was reached after ~3 hrs and maintained for the next ~9 hrs under these experimental conditions.

Cell growth dependence on NO. Cell growth in the presence of NO was determined by diluting overnight cultures of *V. harveyi* strains 100-fold in fresh AB medium supplemented with varying concentrations of DPTA NONOate. DPTA NONOate was predecayed in AB for 3 hrs at 30 °C for all cell-based assays in order to have a constant NO concentration during the bioluminescence experiments. Viability of cells in the presence of DPTA NONOate was monitored over time with aeration at 30 °C. OD_{600nm} was determined on an Ultrospec 10 Cell Density Meter (Biochrom). All measurements were done in triplicate.

Bioluminescence assays. Bioluminescence assays were carried out as previously described (5, 103) with a few modifications. Briefly, overnight cultures of *V. harveyi* were diluted 2000-fold into fresh AB medium supplemented with either 50 or 100 μM DPTA NONOate. Prior to inoculation, varying DPTA NONOate concentrations were predecayed in AB

medium for 3 hrs at 30 °C to reach a concentration of NO (~60-40 nM or ~200-100 nM, respectively). Cell cultures were grown in sterile 50-ml Falcon Tubes (BD Falcon) at 30 °C and aerated by shaking at 250 rpm. Bioluminescence was monitored over a period of 10-12 hrs by aliquoting 100 µl into black 96-well Microfluor® 1 plates (Thermo Scientific). Light production was measured using a VictorX5 microplate reader (Perkin Elmer) using the luminescence mode. Light intensity was measured in triplicate. Cell density was expressed as colony forming units (CFU) determined by diluting the same cultures into fresh AB medium and plating on Luria Marine medium [LM; 10 g/L tryptone, 5 g/L yeast extract (Difco) and 20 g/L NaCl, 15 g/L Agar (RPI)]. Plates were incubated at 30 °C and colonies were counted the following day. Light intensity is reported as relative light units, defined as light intensity ml^{-1} x 1000 CFU^{-1} ml^{-1}. Cell free supernatant, used as positive control, was prepared as previously described (5). For single time-point experiment, cell cultures grown overnight at 30 °C were diluted 5000-fold into fresh AB medium containing 50 µM DPTA NONOate that had been predecayed prior to inoculation (~50 nM NO). Cells were grown until cell density reached ~$5x10^6$ cells/ml. Light intensity and CFU were determined as described above. At least three independent biological trials were done and shown are representative experiments. Error bars represent one standard deviation from triplicates of a representative experiment.

Table 2-1. Strains, plasmids and primers used in this work.

Strains and plasmids	**Relevant characteristics**	**Ref.**
Bacterial strains		
V. harveyi		
WT	BB120, *V. harveyi* WT, ATCC BAA-1116	ATCC
Δ*luxO*	BB721, *luxO*:Tn5 ATCC 700106	ATCC(102)
Δ*luxNS*	MM30, luxN::Cm, luxS::Tn5Kan, ATCC BAA-1120	ATCC(51)

Δ*hnoX*	ATCC BAA-1116 ΔVIBHAR_01911	This work
Δ*hnoX*/p*hnoX*	ATCC BAA-1116 Δ*hnoX* p*hnoX*, Km^r	This work
E. coli		
WM3064	Mating strain	(109)
Plasmids		
In-frame deletion		
pSMV3	Deletion vector, Km^r, *sacB*	(109)
pBBR1MCS-2	Broad range cloning vector, Km^r	(110)
pΔ*hnoX*	pSMV3 with 1 kbp upstream and downstream of *hnoX*	This work
p*hnoX*	pBBR1MCS-2 with *hnoX*	This work
Primers		
Gene deletion		
koH-NOX-fwd	GCGCCGAGGGCCCCATCACTTTAAAGTGATGTGTTTTTGC	
koH-NOX-rev	CTATCGCGGCCGCACTAGTGATCTCCATCATGCCTGC	
koH-NOX-siteA-fwd	GGGATAATATTTACTGGATCCATGGAGTTAGTCGAGAAG	
koH-NOX-siteA-rev	CTTCTCGACTAACTCCATGGATCCAGTAAATATTATCCC	
koH-NOX-siteB-fwd	CACGTGTGTTTAGGATCCATTCACGGTTGTGCGAGTCAC	
koH-NOX-siteB-rev	GTGACTCGCACAACCGTGAATGGATCCTAAACACACGTG	
Complementation		
*hnoX*comp-fwd	AGCGGAATTCATGAAAGGGATA	
*hnoX*comp-rev	TATTGGATCCTTATTGCGCCTC	

Abbreviation used: Km^r: Kanamycin resistance, Amp^r: Ampicillin resistance.

2.3 Results

V. harveyi respond to NO

If NO has any influence on the fate of bacteria, it should be present at a level that would not endanger the cell's life but rather elicit functions important for its survival. In this study, we would like to focus on the biological role of NO as a signaling molecule thus it is imperative to determine the concentration in which it is not detrimental to the cells. To investigate the biological role of NO in QS, light production by *V. harveyi*, a model organism commonly used to study QS, was monitored using DPTA NONOate as NO donor. Each DPTA NONOate releases 2 molecules of NO (Figure 2.2) under acidic/neutral conditions via first order kinetics and has a half-life of 2 hrs at 37°C and 5 hrs at 22 °C (111), making this an ideal source of NO. But because the system is not a closed system and the concentration of NO may vary under different conditions (temperature, pH), NO was measured over time using a Nitric oxide analyzer (NOA).

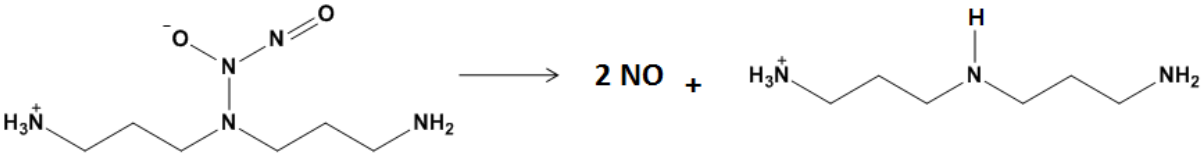


Figure 2-2. Dissociation of DPTA NONOate liberates two molecules of NO and one molecule of dipropylenetriamine.

NO concentrations from DPTA NONOate used in the bioluminescence assay were in the nanomolar level as quantified by NOA. Under the conditions used to monitor bioluminescence (AB media, 30 °C, shaking at 250 rpm) NO concentration was determined

to be: 50 μM DPTA = ~ 40-60 nM NO; 100 μM DPTA = 100-200 nM NO and remained constant 3 hrs into the decay of DPTA NONOate. DPTA NONOate provided a steady state supply of NO during the course of the assay (Figure 2.3).

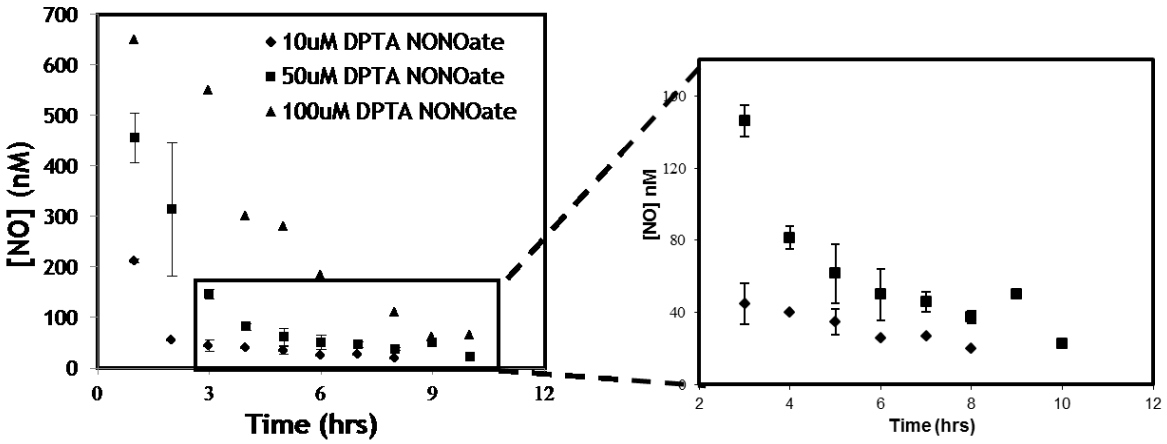


Figure 2-3. Determination of NO from DPTA NONOate at 30 °C in AB medium using Nitric oxide Analyzer

To assess the viability of *V. harveyi* WT and the mutant strains of *ΔhnoX* and the *phnoX/ΔhnoX* in the presence of NO, different DPTA NONOate concentrations were added to AB media. The treated media were predecayed for 3 hrs at 30 °C before adding an overnight culture, since NO concentration from the above experiment remained constant only after 3 hrs of incubation. Cell density was monitored over time and no significant delay in cell growth was observed over time up until a concentration of 100 μM DPTA NONOate at 30 °C (figure 2.4). This implies that at this concentration range, NO acts as a signaling molecule and does not impair the bacteria's normal growth cycle. However, a much slower growth rate was observed in the presence of 500 μM DPTA NONOate (Figure 2.4), therefore bioluminescence assays were carried out using 100 μM DPTA NONOate as the highest concentration.

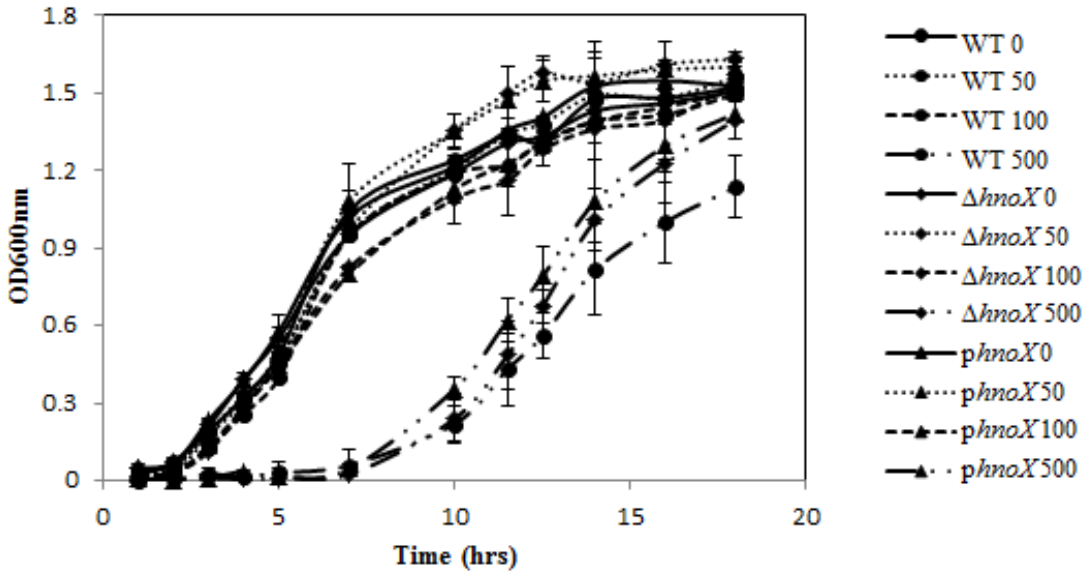


Figure 2-4. Growth curve of *V. harveyi* WT, *ΔhnoX* and the *phnoX/ΔhnoX* at 30 °C with DPTA NONOate (broken lines) and without DPTA NONOate (solid lines).

NO at low concentration acts as a signaling molecule in V. harveyi

To investigate a possible QS role for NO, *V. harveyi* bioluminescence was monitored in the presence of NO. Figure 2.5 (solid line) illustrates a typical *V. harveyi* cell density-dependent bioluminescence curve; upon dilution of a dense overnight culture, cells are bright, but light production decreases with awareness of dilution until the culture reaches ~$5x10^6$ cells/mL, at which point sufficient AI has accumulated in the media, and light production commences. Interestingly, the presence of NO resulted in advanced light production relative to the absence of NO. This increase in bioluminescence at lower cell density is dose-dependent in NO. As expected, addition of AIs resulted in an increase in light production regardless of cell density, this mimics the high cell density state. This is because AI concentration dictates the net activity of the receptors which in turn determines light

production. At low cell density, the receptors have net kinase activities which funnel phosphate to LuxU then toLuxO and eventually repress light production. On the other hand, at high cell density, the receptors have net phosphatase activities which revert back the flow of phosphate thus allowing induction of light production. Thus, the observed earlier onset of light production in the presence of NO implies that LuxO is being dephosphorylated at a lower cell density. This can be interpreted as a decrease in the net flux of phosphate from LuxU to LuxO, which is consistent with decreased kinase activity and/or increased phosphatase activity of a protein capable of exchanging phosphate with LuxU (i.e., a QS receptor). Thus, we reasoned that *V. harveyi* must code for a NO-specific QS receptor.

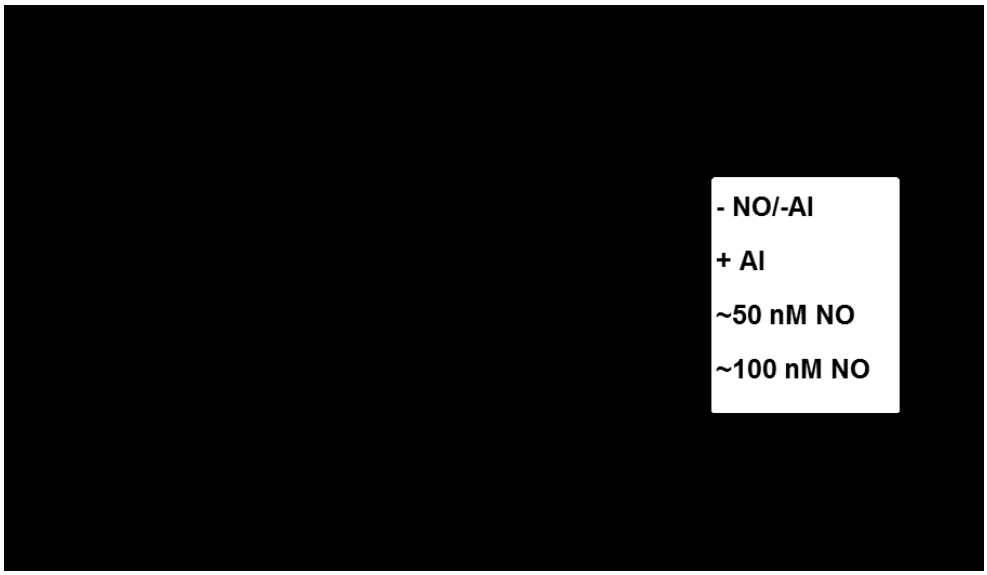


Figure 2-5. Effect of nitric oxide (NO) on light production in *V. harveyi*. Wild type ATCC strain (BAA-1116) of *V. harveyi* was diluted 1:2000 into fresh AB medium containing different concentrations of DPTA NONOate predecayed for 3 hrs at 30 °C. Light production and cell density were subsequently measured over time. Cell density was measured at each time point by plating onto LM agar and colonies were counted after overnight growth. Earlier onset of light production is observed in cultures containing NO (50 μM: triangle, 100 μM: circle) than without added NO (diamond). Cell free culture supernatant (10%) from WT strain was used as positive control (AI, square). Relative light units are defined as counts min^{-1} $ml^{-1}x10^3$ per colony forming units (cfu) ml^{-1}

An NO-sensitive hnoX gene regulates bioluminescence

The above results suggested that an NO-sensitive gene was responsible for the NO-dependent bioluminescence regulation, and based on bioinformatics studies we predict that H-NOX may serve as an NO-sensor to a histidine kinase, HqsK, and together participate in the quorum sensing pathway of *V. harveyi*. To test this prediction, we constructed a *hnoX* in-frame gene deletion mutant through homologous recombination and a complementation strain where a plasmid containing a *hnoX* gene is transformed into the mutant strain, *ΔhnoX*. We also tested the *ΔluxO,* bright mutant, and *ΔluxNS*, dim mutant, as controls. The *ΔluxO* strain is constitutively bright because LuxO represses, though indirectly, *lux* expression while *ΔluxNS* strain is dim because LuxPQ acts as a kinase in the absence of AI-2 (synthesized by LuxS) and therefore funnels phosphate into the QS system. The CqsA/CqsS system may also contribute to the system, but this is observed only at very low cell densities (53) and thus negligible at the time points we monitored NO-regulation of light production.

Deletion of the *hnoX* gene resulted in a delay in light production compared to *V. harveyi* wild-type (WT), while control strains (Δ*luxO* is a constitutively bright strain and Δ*luxNS* is a dim strain) displayed the expected trends (Figure 2.6a). This data is in agreement with our hypothesis, that deletion of the *hnoX* gene results in HqsK funneling phosphate into the system thereby delaying bioluminescence.

In the above results, the effect of NO is more apparent before *V. harveyi* become very bright thus, light production in the presence of ~50 nM NO was monitored at a cell density of ~$5x10^6$ cells/ml (just as bioluminescence is initiated). The addition of NO did not result in a difference in Δ*hnoX* light production, whereas NO caused a significant increase in light production in the WT strain (Fig. 2.6B). Expression of H-NOX from a plasmid in the Δ*hnoX* mutant restored the wild-type behavior. The dim mutant, Δ*luxNS*, also displayed an

increase in light production in the presence of NO, which is expected if NO is acting through a receptor kinase operating parallel to LuxN/LuxQ and upstream of LuxO in the *V. harveyi* QS circuit (Figure 2.1). NO did not have any effect on Δ*luxO*, which indicates that NO/H-NOX enters QS upstream of LuxO. The inability of Δ*hnoX* to induce an earlier onset of bioluminescene in response to NO, as seen in the WT strain, suggests that HqsK relieves the NO-induced flow of phosphate away from LuxU, consistent with HqsK maintaining kinase activity in the absence of NO/H-NOX. A reasonable mechanism to explain these data is that NO is sensed by H-NOX, and in response, regulates the activity of HqsK. Further, HqsK enters the QS pathway by transferring phosphate to LuxU, analogous to LuxQ, LuxN, and CqsS (Figure 2.1).

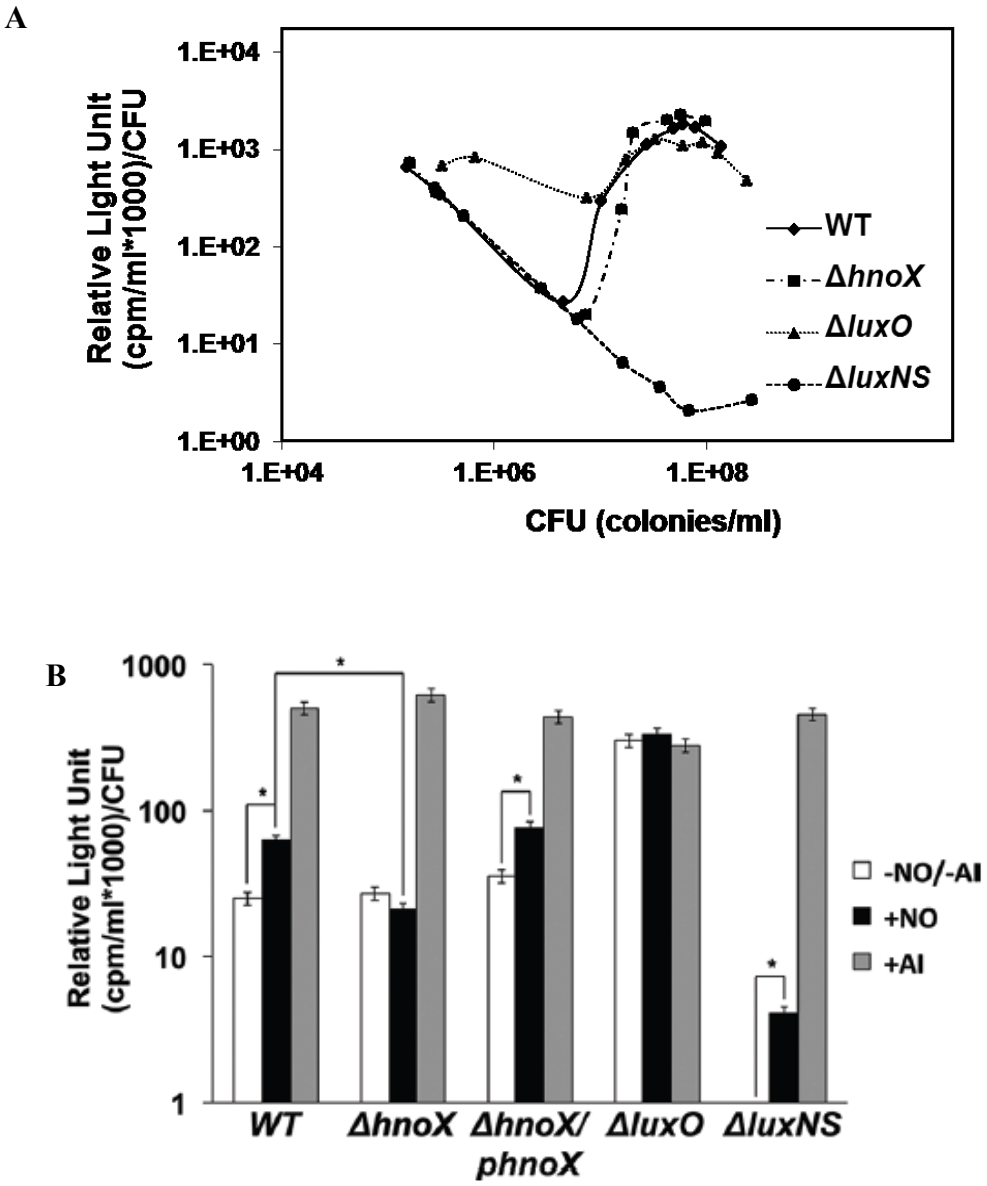


Figure 2-6. Effect of nitric oxide (NO) on light production of WT and mutant strains. Overnight cultures were diluted 1:5000 into fresh AB medium and grown at 30 °C. (A) Light production and cell density were monitored over time. Cell density was measured at each time point by plating onto LM agar and colonies were counted after overnight growth. (B) Effect of NO was determined by adding 50 μM DPTA NONOate into fresh AB medium as described in the experimental procedure. Light production in the *ΔhnoX* mutant culture was not affected by the addition of NO whereas an increase in light production was observed in *V. harveyi* WT. Light production was measured at a cell density of about 5-10 x10^6, the state in which the onset of bioluminescence has the largest difference.

2.4 Discussion

Despite the plethora of environmental cues received by bacteria, recognizing and responding to specific chemical signals remain the principal means to regulate gene expression which leads to changes in cellular function. One such molecule known to affect cellular behavior is the gaseous molecule NO. Although dubbed as an antimicrobial agent (95, 112), the interest in NO as a signaling molecule in bacteria has elicited great interest among scientists. Thus the pathway in which it participates in is an ideal target for drug design and discovery. Here, we propose a novel pathway in which NO acts as a signaling molecule through a two-component signal transduction system involving H-NOX/HqsK that intersects the *V. harveyi* QS circuit through LuxU. In this report, genetic and phenotypic analyses were employed to support this new proposed pathway that may have implications on *V. harveyi'*s response to environmental stress.

Results of our bioluminescence experiments suggest that NO acts in parallel with the other AIs that have been previously identified to control a common set of target genes in the QS pathway. Having an earlier onset of light production in the presence of non-lethal NO concentrations implies that LuxO, a QS repressor, is being deactivated. This effect on LuxO and consequently, on light production, has been shown to be the case when high concentrations of AIs are present in solution (64), where the AIs render the receptor kinases inactive and switch to a net phosphatase. This reverses the flow of phosphate thereby leaving LuxO dephosphorylated and inactive, permitting LuxR to transcribe the *lux* operon. Thus what we observe as an earlier onset can be interpreted as a function of a specific kinase being switched off and/or a phosphatase being switched on in the presence of NO, which we hypothesized to be HqsK. We have determined that the effective NO concentration where

there is considerable change without compromising cell growth to be between 50-100 μM DPTA NONOate, and this corresponds to 50-100 nM NO. However, 100 μM DPTA NONOate seemed to be the upper limit, for specific NO-concentration dependent bioluminescence assays, 50 μM DPTA NONOate was used. While it may seem that the effect of NO on light production is very subtle under the conditions done in the lab, it is highly likely that bacteria are exposed to different ecological challenges *in vivo* that would warrant different responses requiring one, or more or the simultaneous presence of different AIs. Although there is no known or reported NO-regulated bioluminescence in other microorganisms; the NO concentration capable of inducing a change in cellular function is comparable to other cellular phenomenon such as biofilm formation/dispersal, where a change is observed in the presence of low nanomolar NO (79, 81, 96).

Bioluminescence is the canonical readout for QS because it is a direct measure of the association of the signal with the repertoire of proteins in the signal transduction cascade involved in QS. We used bioluminescence to determine the biological role of NO but it is also possible that the real cellular function in which NO may have the most impact would be on other biological processes that are controlled indirectly by QS. The Lux regulon targets genes other than *lux*CDABE (71, 101) and it is not surprising that QS controls a lot of group behaviors as the transition from a solitary life to community existence would entail changes in the global gene expression. For example, biofilm formation in *V. cholerae* is modulated by HapR, the homolog of LuxR, through repression of *vpsT*, *vpsR* and 14 other genes that code for the proteins that regulate c-di-GMP (113) while VpsR and VpsT induces the *vps* genes (114).

This work provides, to our knowledge, the first evidence of an NO-H-NOX-HqsK regulated signaling in the QS circuit. The biological role of bacterial H-NOX has been

elucidated to regulate biofilm formation through c-di-GMP expression in *S. woodyi* (79), *S. oneidensis* (81), *L. pneumophila* (80). However, bioinformatics studies show that H-NOX is not limited to *Vibrios* nor to specific classes of bacteria, neither is it in the same operon as only one type of enzyme. Instead, it appears in the genomes of many bacteria and eukaryotes. This may seem to indicate that H-NOX was evolutionarily acquired to sense NO in the environment or the presence of a eukaryote, and that it helps the organisms respond as a community to such changes in order to survive the niche that they reside.

We may have identified a new pathway, but what does it really mean for *V. harveyi* and most bacteria to have multiple circuits? Multiple circuits with coincidence detectors enable bacteria to respond with high fidelity even as fluctuations of signals occur (53). In this case, it appears that NO has taken a role which is more than to evaluate who is around, but also monitors the environment it is in. Our data seem to suggest that *V. harveyi* detect NO and that it is used to signal the presence of other organisms in the environment (analogous to other AIs). The most likely sources of this signal are incomplete reduction of NO produced during anaerobic respiration using nitrate or nitrite in an oxygen limited environment (115) and NO produced enzymatically from NO synthase (NOS) (116). We do not rule out the possibility that NO may be used to signal an anaerobic environment (97). However, we note that NO has the greatest effect on QS at low cell density; anaerobic respiration is more likely at high cell density where QS circuits are overwhelmed by a high concentration of other AIs. Also, *V. harveyi* have no predicted NOS gene, and at present there are only a few Gram-positive bacteria that are known to possess a NOS gene, (116). Thus we propose that the NO source may be eukaryotic and that using QS circuits to detect the presence of eukaryotes during the early stages of symbiosis or pathogenesis (before iNOS is producing large concentrations of NO, which would obviate the need for a picomolar NO

sensor) is a likely physiological function for NO as an inter-kingdom signaling molecule. This hypothesis is consistent with the known role of NO in establishing symbiosis between *V. fisheri* and *E. scolopes* (98, 117). This is also consistent with the *V. harveyi* lifestyle, where it is found in the gut, in pathogenic association with marine hosts (4).

2.5 Conclusion

NO has been implicated with several signaling pathways at low concentration. Here, we have demonstrated that NO acts as a signaling molecule that regulates bioluminescence in *V. harveyi* through H-NOX/HqsK pathway. *V. harveyi* increase their light production in the presence of NO, analogous to the function of the previously described autoinducers. We show that deleting the *hnoX* gene resulted to loss of sensitivity to NO while complementing this with a plasmid-containing *hnoX* gene restores the WT behavior. These results indicate that H-NOX acts as the sensory domain which recognizes NO, and invokes regulation of the HqsK activity to alter light production in *V. harveyi.* This is the first study to demonstrate an NO-responsive QS circuit in *V. harveyi.*

CHAPTER 3

Characterization of a NO-regulated QS circuit in *Vibrio harveyi*

Abstract

The diversity of environmental stimuli received by bacteria requires an adaptive response to modulate cellular processes as a function of signal molecules. Bacteria use two-component signal transduction systems (TCS) to relay information via a series of phosphorylation events. In *V. harveyi,* QS circuits employ multiple two-component proteins that participate in the phosphorylation/dephosphorylation cascade for signal transduction. The receptors, LuxN, LuxQ, CqsS function as two-component proteins that channel phosphate to and from a common phosphorelay protein called LuxU which is then transferred to and from the master response regulator, LuxO. This phosphorylation/dephosphorylation cascade is mediated by the kinase/phosphatase activity of the sensors as a function of the respective chemical signals, also known as autoinducers. In our previous study, we have shown that NO acts a signal that regulates light production through an NO-sensitive gene annotated as *hnoX*. In this study, we demonstrate that H-NOX is the sensory domain of HqsK that functions as the receptor for NO in the H-NOX/HqsK sensor system. Biochemical studies on the enzymatic activities of HqsK reveal that it is a hybrid two-component protein capable of exchanging phosphate with LuxU. Mutational studies show two phosphorylation sites, H174 on the transmitter domain and D459 on the receiver domain. Here, we probe the molecular mechanism of the signal transduction that leads to regulation of bioluminescence and the discovery of a fourth QS circuit in *V. harveyi.*

3.1 Introduction

Bacteria are continually exposed to a wide range of physical and chemical signals that need to be recognized and processed in order to adapt to changes in the local environment. Central to detecting and responding to the right chemical signals are receptors of the histidine kinase family and response regulators; together, they comprise the basic transduction system in bacteria, known as the two-component signal (TCS) transduction system (37, 38, 118). Quorum sensing (QS), a process of bacterial cell-cell communication, employs a network of TCS to coordinate community-wide changes in gene expression, commonly regulating outputs such as biofilm formation, virulence gene production, and bioluminescence (1, 3) (Review in (13)).

The success of a concerted response to group behavior rests on the ability to detect signal molecules, known as AIs (AIs) by their specific receptors. Signal-receptor specificity prevents cross-talk between related signals and eliminates noise from molecules of similar structure in the environment (13). In *V. harveyi*, the central QS signaling machinery involves multiple receptors that recognize specific AIs where they act in parallel to regulate gene expression. Specifically, LuxN recognizes HAI-1(57), LuxPQ is the receptor for AI-2 (46) and CqsS is the receptor for CAI-1(2, 53). The information contained in the three AIs is transduced through a phosphorylation cascade which ultimately activates LuxR. However, QS system in *V. harveyi* is composed of multiple TCS where the hybrid sensor kinases, in the absence of AIs, act as kinases which autophosphorylate itself on the His residue in the transmitter domain and transfer phosphate on Asp in the receiver domain (54). Phosphate is then funneled to a common phosphorelay protein, LuxU on His58 (63, 119). LuxU then transfers phosphate to the response regulator, LuxO at position D47 and triggers the

deactivation of LuxR (64, 102), the master regulator of the *lux* operon (53) through the destabilization of *luxR* mRNA by Qrrs produced by phosphorylated LuxO (65, 104). However, binding of AIs to the sensor kinases, as a result of increased cell population, causes a major conformational change rendering the kinase inactive and permitting the receptors to assume a net phosphatase function (60). This reverses the flow of phosphate from LuxO to LuxU, which deactivates the production of Qrrs, thereby leaving LuxR active, and induces the expression of the *lux* operon as well as regulation of at least 50 other genes involved in QS (33, 53, 71, 101, 120).

Genetic and structural studies done on LuxPQ and LuxN have shed light on the possible mechanism of the multiple-component signal transduction pathway in *V. harveyi*. These two proteins are shown to function as hybrid two-component receptors, with kinase and phosphatase activities (60, 121, 122). Crystallographic and functional studies on LuxP-AI-2 complex (46) and LuxPQ showed that LuxP is bound to LuxQ independent of AI-2. Binding of AI-2 resulted to a major conformational change on the quaternary structure in which 2 LuxPQ monomers associated asymmetrically (60). It was proposed that this asymmetric structure causes the repression of the kinase activities of both LuxQ (60). This is corroborated by the biochemical studies on LuxN, where enzymatic and phosphotransfer activities showed that LuxN contains autokinase activity and that it can transfer phosphate to the phosphorelay protein LuxU (56). ,

We have shown in our previous work that NO contributed to light production in *V. harveyi*, and we proposed that an NO-sensitive gene and a histidine kinase act as the sensor/receptor system that enters the QS pathway similar to the previously identified systems. Here we use biochemical characterization to probe the molecular mechanism of the phoshpho-relay system involving NO-H-NOX/HqsK pathway.

Upon completion of the *V. harveyi* genome project in 2007, we discovered a gene annotated by NCBI as *luxQ*, (VIBHAR_01913) that is predicted to encode a soluble histidine kinase/phosphatase with homology to the cytosolic kinase/phosphatase domain of the well characterized QS receptor LuxQ (Appendix 1) (55). Unlike the other QS receptors, VIBHAR_01913 is predicted to be soluble. This is supportive of our hypothesis and our observation that *V. harveyi* respond to NO, because NO is readily membrane-permeable and thus does not require an extracellular sensor. Interestingly, however, we determined that the gene coding for VIBHAR_01913 is in the same operon as a gene (VIBHAR_01911) coding for a putative soluble H-NOX (nitric oxide/oxygen binding domain) protein, a member of the NO-sensors homologous to soluble guanylate cyclase (sGC) (108) . Like LuxQ, VIBHAR_01913 does not have a sensory domain in its primary sequence (AI-2 binds to LuxP, which associates with LuxQ in the periplasm to regulate LuxQ activity) (88), and we propose that H-NOX acts as the sensory domain that recognizes NO.

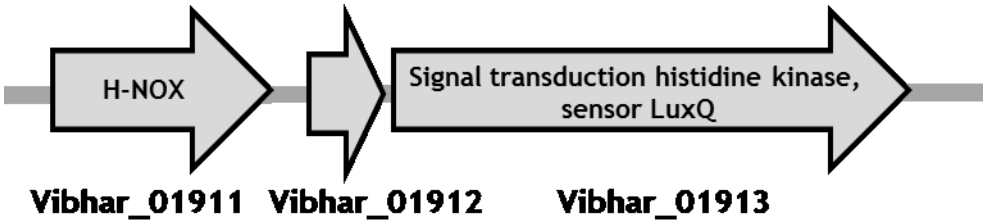


Figure 3-1. Domain organization of H-NOX (VIBHAR_01911) and HqsK (VIBHAR_01913). H-NOX (VIBHAR_01911) and HqsK (VIBHAR_01913) are encoded in the same operon. VIBHAR_01911 is a 561 bp gene that codes for a H-NOX protein (186 amino acids); VIBHAR_01912 is a 108 bp gene that codes for a hypothetical protein annotated as signal transduction histidine kinase, sensor LuxQ (35 amino acids); VIBHAR_01913 is a 1581 bp gene that codes for a protein annotated as signal transduction histidine kinase, sensor LuxQ (526 amino acids)

H-NOX is a member of a family of nitric oxide (NO) sensing hemoproteins discovered by genetic homology (15-40% identity) with the well-characterized mammalian NO sensor soluble guanylate cyclase (sGC) (123). Although H-NOX is a stand-alone protein, it is commonly found in the same operon as a histidine kinase (*Shewanella oneidensis, Pseudoalteromonas atlantica*), a diguanylate cyclase (*Shewanella woodyi, Legionella pneumohila*) or fused to a methyl-accepting chemotaxis protein (MCP) (*Thermanaerobacter tencongenesis*) (Figure 3.2). With the discovery of H-NOX, fundamental questions about the role of NO/H-NOX signaling in bacteria are being considered (79-81, 83, 98). Interestingly, although as a class bacterial H-NOX proteins are not well-characterized, regulation of group activities, such as those classically regulated by QS, has been a strong theme in the studies to date. In *V. fischeri*, H-NOX appears to be used to sense the light organ environment during colonization of *E. scolopes* (98). In *S. woodyi* (79), *S. oneidensis* (81) and *L. pneumophila* (80), NO/H-NOX has been shown to be involved in regulation of biofilm formation.

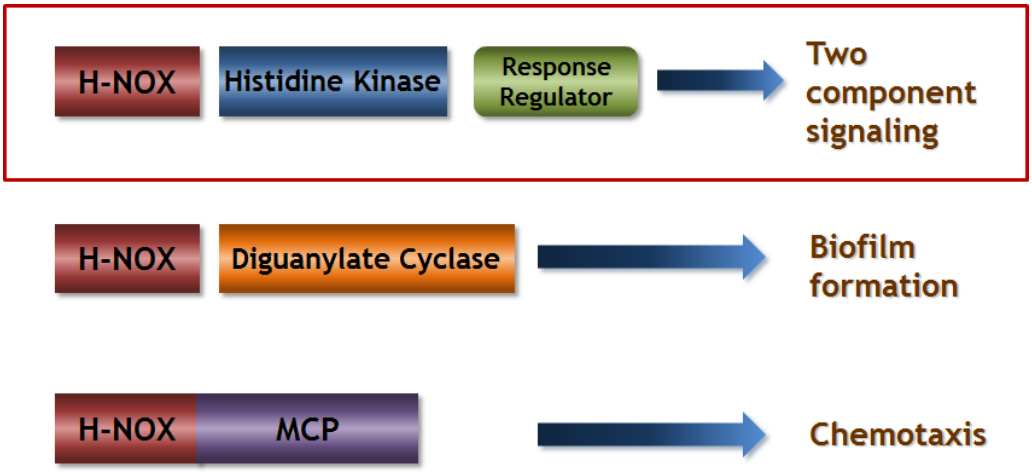


Figure 3-2. Domain organization of bacterial H-NOX and its effector proteins.

3.1.1 Hypothesis

Based on these bioinformatics discoveries, we predict that VIBHAR_01913, which we named HqsK for H-NOX-associated quorum sensing kinase, will behave like the sensor kinase, LuxQ. We expect VIBHAR_01913 to act as a kinase in the absence of NO and as a phosphatase in the presence of NO. Being in the same operon, we hypothesize that H-NOX serves as the sensory domain of HqsK, which serves as the NO sensor system. The NO-H-NOX/HqsK system participates in the QS pathway of *V. harveyi* by exchanging phosphate with the common phosphotransfer protein, LuxU. Specifically, we predict that NO/H-NOX regulation of HqsK kinase activity would explain the effect of NO on *V. harveyi* light production. Here we demonstrate that HqsK is a two-component sensor kinase, whose kinase function is inhibited by NO-bound H-NOX, and channels phosphate into the QS pathway parallel to the previously identified circuits,

3.2 Materials and Methods

Bacterial strains and growth conditions. Strains used in this study are listed in Table 3.2 *V. harveyi* wild type strain (BB120) was purchased from the American Type Culture Collection (ATCC), maintained in Marine Media (MM; 28 g/L; BD Difco) and grown at 30 °C with agitation at 250 rpm. *E. coli* strains XL1 and BL21(DE3)pLysS were used for plasmid amplification and protein purification, respectively. *E. coli* strains were typically grown in Luria Broth (LB; 20 g/L; EMD chemicals) at 37 °C with agitation at 250 rpm.

Protein cloning, expression, and purification. To express recombinant proteins, the open reading frames of VIBHAR_01911 (*hnoX*), VIBHAR_01913 (*hqsK*) and VIBHAR_02958 (*luxU*) were amplified from *V. harveyi* genomic DNA using Pfu Turbo polymerase (specific primers listed in Table 3.1). The amplified PCR products were cloned into appropriately digested pET20b (Novagen) for H-NOX to produce a C-terminal His_6-tag and into pET-23a-HisTEV (Novagen) for HqsK and LuxU to produce an N-terminal His_6-tag. HqsK and LuxU mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's guidelines. The resulting vectors (Table 3.1) were sequenced (Stony Brook DNA sequencing facility) and HqsK mutations were further confirmed by MS/MS. Expression of H-NOX was carried out as previously described for other H-NOX proteins (98). Briefly, cells were grown in phosphate-buffered yeast extract and induced with the addition of 50 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) supplemented with 100 μM aminolevulinic acid (ALA, Sigma-Aldrich). Cells were grown at 25°C and harvested after 12 hours. For HqsK and LuxU production, cells were grown in 2X YT (16 g/L Tryptone, 10 g/L yeast extract, 5 g/L NaCl) supplemented with 100 μg/ml ampicillin. Overexpression of HqsK and LuxU was induced by the addition of 100 μM IPTG at 18°C for 8 hours. Purification of His_6-tagged proteins was carried out using metal affinity chromatography (Ni^{2+}-NTA, GE healthcare). Protein concentrations were determined by bicinchoninic acid (BCA) assay (Pierce) using BSA as standard.

Table 3-1. Strains, plasmids and primers used in this work.

Strains and plasmids	Relevant characteristics	Ref.
Bacterial strains		
V. harveyi		
WT	BB120, *V. harveyi* WT, ATCC BAA-1116	ATCC

E. coli		
XL1-Blue	Cloning strain	Lab stock
BL21(DE3)pLysS	Expression strain	Invitrogen
Plasmids		
Cloning/Expression		
pHisH-NOX	pET-20b(+) with *hnoX*, C-terminal 6x His-tag, Amp^r	This work
pHisHqsK	pET-23a(+) with *hqsK,* N-terminal 6x His-tag, Amp^r	This work
pHisLuxU	pET-23a(+) with *luxU*, N-terminal 6x His-tag, Amp^r	This work
pGSTHqsK	pGEX-4T2 with *hqsK*, N-terminal GST-tag, Amp^r	This work

Primers	
Cloning	
pETH-NOX-fwd	GCGGCGGCCATATGAAAGGGATAATATTTACTGAGTTC
pETH-NOX-rev	GCGGCGGCCTCGAGTTGCGCCTCCAGCCAAAAG
pETHqsK-fwd	GCGAATTCGTGGCGTTAAAGAAACTCG
pETHqsK-rev	CAATGAAGCTTTTCGCCGAGCCATTTACAC
pETLuxU-fwd	ACGTACCCATGGGCATGAATACGG
pETLuxU-rev	ATTCCGCTCGAGGTTTGTCCAAGAA

Abbreviation used: Km^r: Kanamycin resistance, Amp^r: Ampicillin resistance.

Mass spectrometric characterization of HqsK, HqsKH174A, and HqsKD459A. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained using a Bruker Autoflex II MALDI/TOF/TOF. Proteins were first separated on a 12.5%T SDS-PAGE and stained with Coomassie brilliant blue. The band that corresponds to the protein was excised and destained overnight. In-gel trypsin digestion was performed by treating the sample with 0.1 μg/μL of trypsin at 1:50 (protein:protease) ratio and digestion was allowed to proceed overnight at 37 °C. The reaction was quenched with 1/10th volume of 100% formic acid and concentrated to 1/10th of the reaction volume.

Electronic spectroscopy. All electronic spectra were recorded on a Cary 100 spectrophotometer equipped with a constant temperature bath. Preparation of the various ligation complexes of H-NOX was carried out as previously published (124).

NO dissociation rate. To determine NO dissociation kinetics, rates were measured as previously described (98). Briefly, Fe^{2+}-NO complexes of protein (final heme concentration of 5 μM) diluted in anaerobic 50 mM HEPES, 50 mM NaCl buffer (pH 7.5) were rapidly mixed with an anaerobic solution of 30 mM (final concentration) dithionite ($Na_2S_2O_4$) saturated with carbon monoxide (CO) in the same buffer . It has been previously established that CO binding is not rate-limiting in these experiments (124); this was confirmed in experiments using only 30 mM $Na_2S_2O_4$ without CO as a trap. Data were acquired by scanning periodically on a Cary 100 spectrophotometer equipped with a constant-temperature bath set to 20 °C. The dissociation of NO from the heme was monitored as the formation of the Fe^{2+}-CO complex at 423 nm. Difference spectra were calculated by subtracting the first scan from each subsequent scan. The NO dissociation rate was determined from the increase in absorbance at 423 nm versus time and fit with a single or two parallel exponentials of the form $f(x) = A(1-e^{-kx})$. Each experiment was performed a minimum of six times, and the resulting rates were averaged. The dissociation rates measured are independent of CO and dithionite concentration (3, 30, and 300 mM dithionite were tested).

Pull-down assay. A pull-down assay of H-NOX and HqsK was carried out using glutathione S-transferase (GST) fusion of HqsK and His_6-tagged H-NOX. GST-HqsK was subcloned from HqsK pET23a-HisTEV using BamHI and NotI restriction enzymes, respectively and ligated into a properly digested pGEX4t-2 (GE Life Sciences) vector. GST constructs were

purified from BL21(DE3)pLysS cells (induced with 500 μM IPTG overnight at 18 ºC) using glutathione sepharose beads. Captured GST-tagged proteins were washed with 50 mM Tris/HCl (pH 8), 500 mM NaCl, 1 mM PMSF, 2 mM DTT, and 0.5% Triton X-100. *E. coli* cell lysates (200 μl) containing overexpressed His_6-H-NOX was added to the beads to a final volume of 1 mL in the same buffer and incubated for 2 hrs at 4 ºC with gentle rocking. Beads were then washed 3 times with the same buffer and then boiled in 50 μL of SDS sample buffer. 10 μL of this reaction was resolved in a 12.5%T polyacrylamide gel Tris glycine and subsequently processed for Western Blot analysis. Polyclonal anti-His_6 antibody (Abcam) was used to detect the presence of His_6-H-NOX (79).

Autophosphorylation assays. For autophosphorylation assays, 15 μM of HqsK wild-type or mutant proteins were incubated with 500 μM $MgCl_2$, 1 mM ATP, and 10 μCi [γ-^{32}P] ATP (3000 μCi of 10 μCi ml^{-1} purchased from Perkin Elmer) at room temperature in phosphorylation buffer (50 mM Tris/HCl, 500 mM KCl, 10 %(v/v) glycerol, 2 mM DTT). Reactions were terminated at the indicated time points with SDS loading dye and boiled for 3 min followed by separation on a 12.5%T Tris-glycine SDS-PAGE and detected by autoradiography. Dried gels were exposed to a phosphoscreen overnight and scanned using Phosphoimager (Amersham Biosciences). Kinase inhibition assays by H-NOX were carried out by incubating 11 μM of $HqsK_{D45A}$ with varying concentration of NO-bound H-NOX. Reactions were initiated by the addition of 500 μM $MgCl_2$, 1 mM ATP, and 10 μCi [γ-^{32}P]ATP (56). Assays were terminated after 30 min and phosphorylation signals were detected by autoradiography as described above. Band intensities were quantified using ImageJ and levels of proteins in solution were determined by Coomassie brilliant blue stain.

Phosphatase assays. Autophosphatase activity of HqsK, $HqsK_{H174A}$, $HqsK_{D459A}$ was determined using O-methylfluorescienphosphate (OMFP, 0.1 mM final concentration) as a substrate and 15 μM protein in phosphatase buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM DTT, 2.5 mM EDTA, 0.1 mg/ml BSA). The reaction mixture was incubated at 37 °C and hydrolysis of phosphate was monitored over time by measuring the absorbance at 450 nm.

Phosphotransfer assays. To assay the time course of phosphotransfer from HqsK to LuxU, a reaction mixture containing 10 μM HqsK in phosphorylation buffer was first phosphorylated by the addition of 500 μM $MgCl_2$, 1 mM ATP, and 10 μCi [γ -^{32}P]ATP for 1 min prior to the addition of LuxU (100 μM final concentration). Incubation was continued and samples were quenched with SDS loading dye at the indicated time points. Reaction mixtures containing HqsK only or LuxU only were allowed to incubate for 60 min. Phosphorylated proteins were separated on a 15%T Tris-glycine SDS-PAGE gel and detected by autoradiography. Single time point phosphotransfer assays with HqsK and LuxU point mutants were performed as described above except that the reaction mixture was incubated for 30 min at room temperature (56).

3.3 Results

To explain the observed NO-regulation of light production in *V. harveyi*, we propose that bioluminescence is regulated by H-NOX and HqsK, where NO is sensed by H-NOX, and in response, regulates the activity of HqsK. Further, HqsK enters the QS pathway by

transferring phosphate onto H58 of LuxU, analogously to LuxQ, LuxN, and CqsS. To test these possibilities, we cloned, expressed, and purified the recombinant *V. harveyi* proteins H-NOX, HqsK, and LuxU from *E. coli* and tested their proposed functions in solution.

Ligand binding properties V. harveyi H-NOX is consistent with an NO sensor

Multiple primary sequence alignment of VIBHAR_01911, annotated by NCBI as H-NOX, with other bacterial H-NOX proteins predicted that it will bind NO and CO but not O_2 due to the lack of a tyrosine residue in the distal pocket which stabilizes oxygen binding in *Thermoanaerobacter tencongenesis* (Figure 3.3) (107). Further, it contains the residues important for heme binding: **I** – hydrophobic contact with pyrolle ring A; **H** – proximal ligand for the heme iron; **P** – hydrophobic contact with pyrolle ring D; **YxS/TxR motif** – involved in H-bonding interactions with the propionate groups of the protophorphyrin (full sequence alignment in Appendix B).

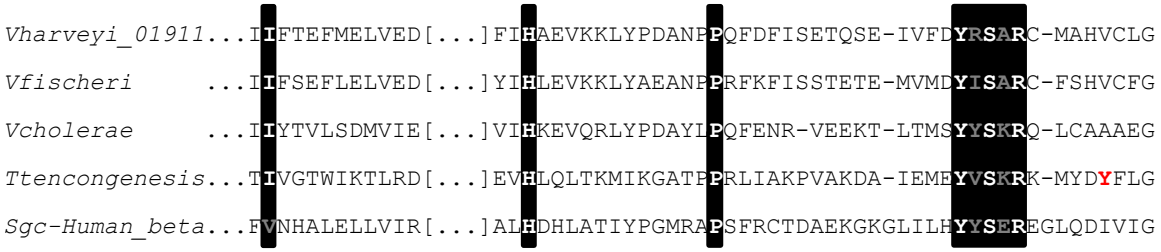


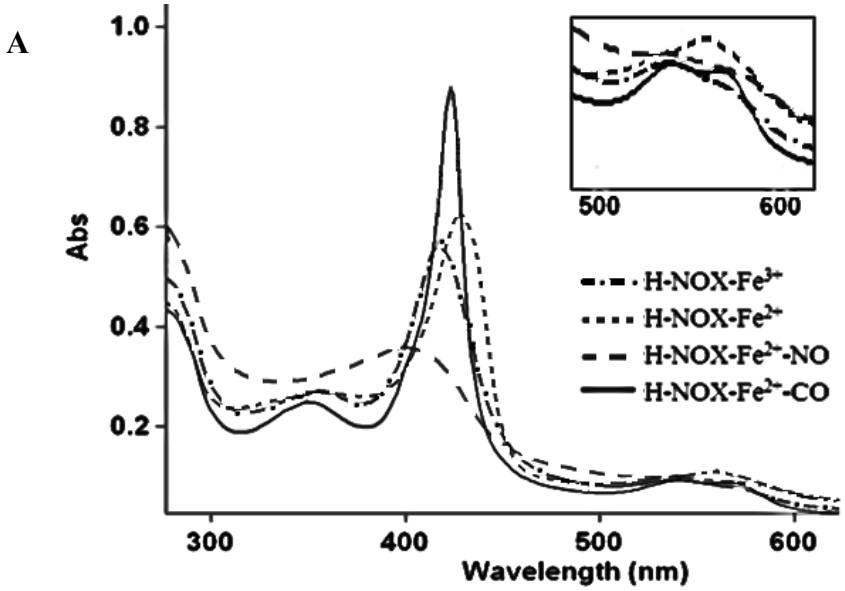
Figure 3-3. Sequence alignment of VIBHAR_01911 with other histidyl-ligated heme proteins using ClustalW. Shown is the selected sequence which contains the residues important for heme binding; residues boxed in black make direct contact with the heme; tyr residue that stabilizes Fe^{2+}-O_2 complex in *Thermoanaerobacter tencongenesis* is highlighted in red.

To confirm that VIBHAR_01911 has the same ligand binding properties, spectroscopic and kinetic analyses of purified H-NOX were determined. H-NOX proteins are easily characterized by electronic spectroscopy because binding of the ligand to the ferrous iron center of the histidine-ligated protoporphyrin IX – ferrous complex gives off characteristic absorption peaks (Soret peaks) in the visible spectrum that can be monitored using spectroscopy. *V. harveyi* H-NOX showed spectroscopic properties similar to sGC and other members of the H-NOX family from aerobic organisms. UV-visible spectra of the different oxidation and ligation states at room temperature are shown in Figure 3.4 and compared to sGC and other bacterial H-NOX proteins in Table 3.2. *V. harveyi* H-NOX binds NO and CO (Soret peaks at 399nm and 423nm, respectively) and did not bind O_2. Furthermore, we measured the dissociation rate constant [k_{off} (NO)] to be 4.6×10^{-4} s^{-1} at 20 °C for the Fe^{2+}-NO complex (Figure 3.2 b). The k_{on} (NO) for H-NOX domains is predicted to be ~10^8, putting the K_D (NO) for H-NOX in the low picomolar range (125). These data indicate that H-NOX has ligand binding properties consistent with a sensitive and selective NO sensor.

Table 3-2. Ligand binding properties of ferrous H-NOX domains from multiple species.

	Soret Band (nm)			k_{off} (NO) ($x10^{-4}$ s^{-1})	Ref.
protein	Fe^{2+}-unligated	Fe^{2+}-CO	Fe^{2+}-NO		
*Vh*H-NOX[a]	429	423	399	4.6 ± 0.9	This work
sGC[b]	431	423	398	3.6 ± 0.9	(126)
*Vf*H-NOX[c]	428	423	398	21 ± 0.6	(98)
*Vc*H-NOX[d]	429	423	398	ND	(77)
*Sw*H-NOX[e]	430	423	399	15.2 ± 3.5	(79)
*Lp*H-NOX[f]	428	420	398	10.3 ± 1.4	(108)
*So*H-NOX[g]	427	424	398	ND[h]	(83)

[a]H-NOX from *V. harveyi*, VIBHAR_01911, [b]H-NOX from soluble guanylate cyclase from bovine lung (H-NOX domain only), [c]H-NOX from *Vibrio fischeri*, [d]H-NOX from *Vibrio cholerae,* [e]H-NOX from *Shewanella woodyi,* [f]H-NOX from *Legionella pneumophila*, [g]H-NOX from *Shewanella oneidensis*, [h]Not determined.



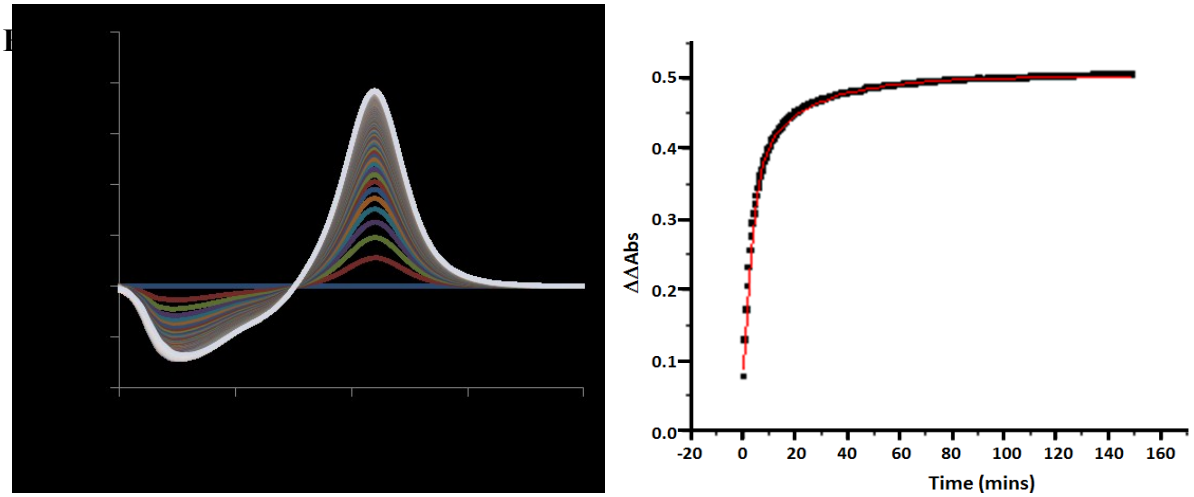


Figure 3-4. Ligand binding properties of *V. harveyi* H-NOX. (A)Electronic absorption spectra of H-NOX (VIBHAR_01911). The different H-NOX complexes showed a soret maximum at: Fe^{2+}-CO: 423 nm, Fe^{2+}-NO: 399 nm, Fe^{2+}-unligated: 429 nm, Fe^{3+}: 417 nm. Characteristic α/β bands are shown as inset. (B) Kinetic analysis of the dissociation rate for the Fe^{2+}-NO complex of H-NOX. The absorbance spectrum of a Fe^{2+}-NO sample of H-NOX at 20 °C was measured at various time points by electronic absorption spectroscopy using saturating CO and 30 mM $Na_2S_2O_4$ as a trap for the released NO. The absorbance difference spectrum (the spectrum at time = 0 min was subtracted from the spectrum at each subsequent time point) of the Fe^{2+}-CO complex growing in over time was generated. A plot of the change in absorbance at 423 nm minus 393 nm (the maximum and the minimum in the difference spectrum) verses time along with the exponential fit of that data is illustrated. The data were fit to the equation $y = y_0 + a_1*(1 - e^{(-b_1*x)}) + a_2*(1-e^{(-b_2*x)})$ using Origin 7.0 software. The slower of the two rates measured is reported as k_{off}. The measured k_{off} for the H-NOX Fe^{2+}-NO complex is $(4.6 \pm 0.9) * 10^{-4}$. Error analysis was determined from at least three independent trials. The measured rates were independent of CO and dithionite at all concentrations tested (3 – 300 mM).

HqsK is a hybrid histidine kinase

We hypothesize that HqsK will behave similarly to the LuxQ protein of *V. harveyi*. Primary sequence alignment with LuxQ reveals two predicted phosphporylation sites, H174 in the transmitter domain and D459 in the receiver domain in HqsK (Figure 3.6). To test

our hypothesis we performed *in vitro* autophosphorylation assays of the WT and mutant proteins. We generated alanine mutations using site directed mutagenesis and confirmed the mutation through MS/MS analysis (Figure 3.7)

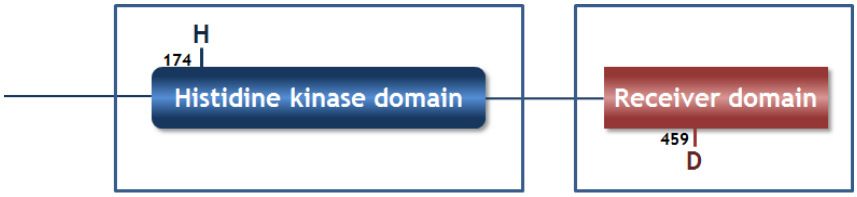
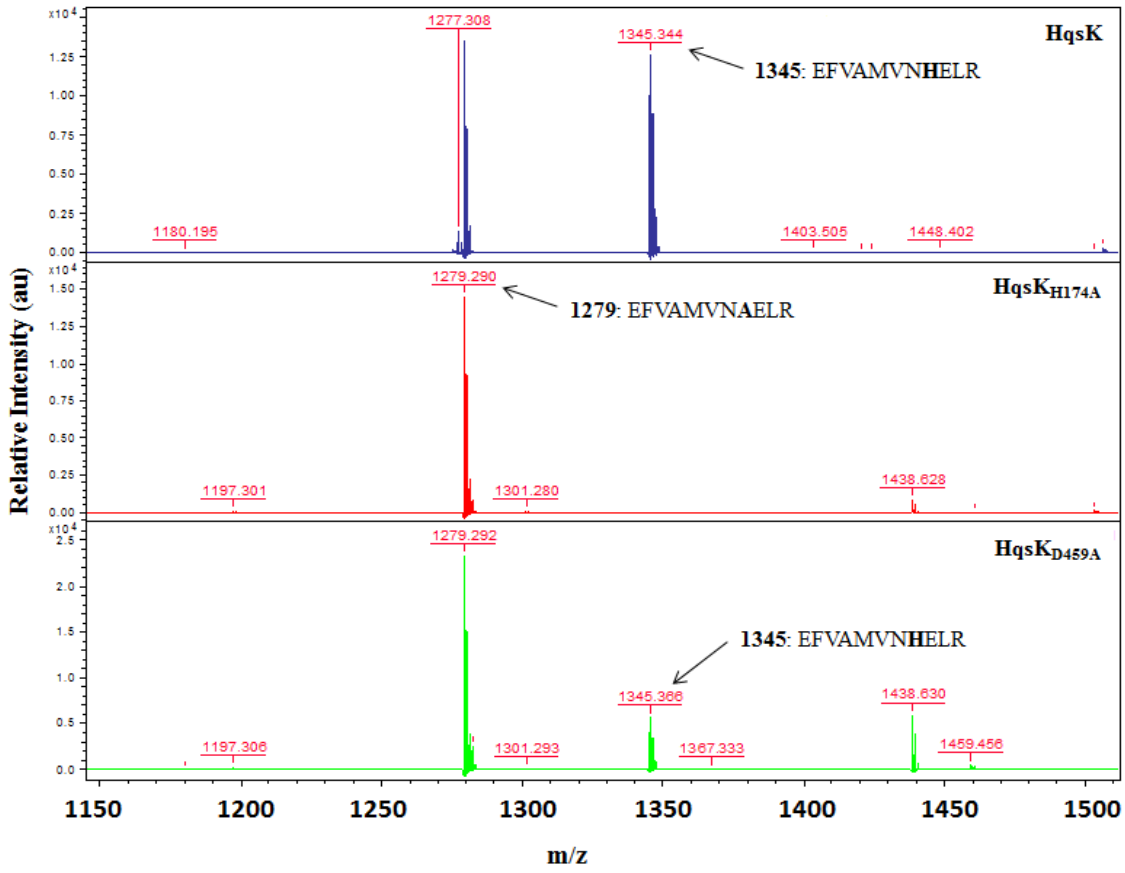


Figure 3-5. Domain organization of HqsK with the predicted phosphorylation sites at H174 in the transmitter domain and D459A in the receiver domain.

A



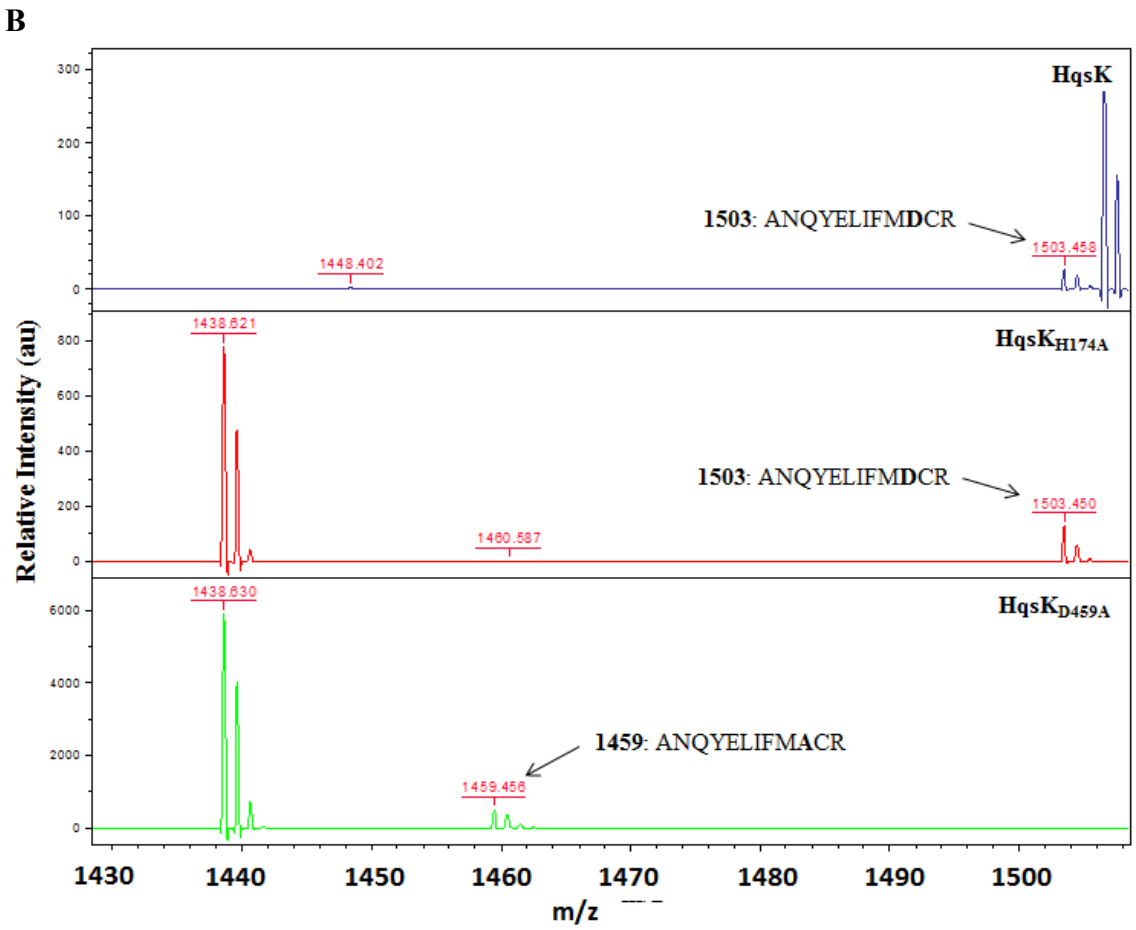


Figure 3-6. Mass spectrometric characterization of HqsK, HqsK$_{H174A}$, and HqsK$_{D459A}$. **a** m/z range 1150 – 1500. **b.** m/z range 1430 – 1540.

MS/MS spectra are shown for HqsK, HqsK$_{H174A}$, and HqsK$_{D459A}$ in Figure 3.7. The peptide that contains the H174 to A174 is in the m/z range 1150 – 1500. HqsK and HqsK$_{D459A}$ each have a peak at m/z = 1345, which corresponds to the peptide EFVAMVN**H**ELR, while the loss of this peak in HqsK$_{H174A}$ suggests the loss of His residue, however the peptide which contains the Ala mutation, EFVAMVN**A**ELR, is not a unique identification of this peptide since another peptide has the same m/z = 1279. Mutation of D459 to A459 can be identified in the m/z range 1430 – 1540. HqsK and HqsK$_{H174A}$ (but not

$HqsK_{D459A}$) each have a peak at m/z = 1503, which corresponds to the peptide, ANQYELIFM**D**CR, containing D459. On the other hand, the sample has a peak at m/z = 1459, corresponding to the peptide, ANQYELIFM**A**CR, containing the D459A mutation.

To determine the kinase activity of HqsK (VIBHAR_01913) and its corresponding Ala mutants, *in vitro* phosphorylation was carried out by incubating the purified proteins with radiolabelled [γ-^{32}P] ATP. Autophosphorylation assays exhibit time-dependent, stable autokinase activity (Figure 3.7). This is in contrast to LuxN, the AI-1 receptor, which has short-lived *in vitro* autokinase activity (56). Mutation of the conserved His residue (H174) to Ala completely abolished the autokinase activity, whereas mutation of D459 to Ala achieved higher levels of phosphorylation than WT. The lack of kinase activity in the $HqsK_{H174A}$ mutant could not be attributed to differences in protein concentration nor protein stability (Figure 3.8 shows that $HqsK_{H174A}$ is active as a phosphatase), instead it is interpreted as the loss of the kinase phosphorylation site. We assume that the increased kinase activity of $HqsK_{D459A}$ was due to the stability of the phosphoramidate bond (His-P) relative to the mixed acid ester (Asp-P) under basic conditions (56, 127, 128), however we do not rule out the possibility that this is due to the loss of the autophosphatase activity in $HqsK_{D459A}$ (129).

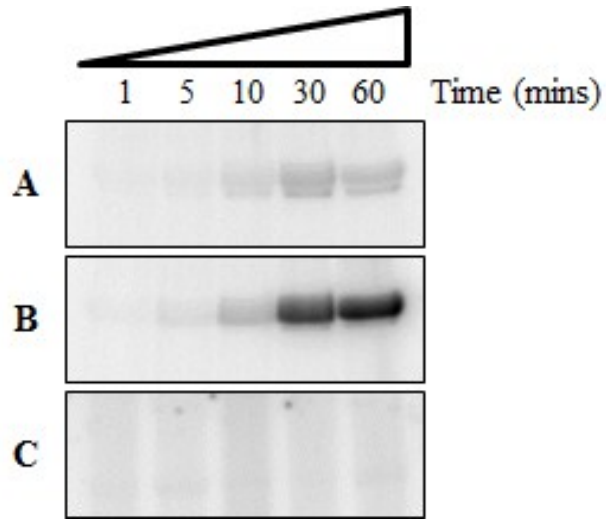


Figure 3-7. *In vitro* autokinase activity of HqsK. VIBHAR_01913 is a hybrid histidine kinase with phosphorylation sites at H174 in the kinase domain and D459 in the receiver domain. Autophosphorylation of (A) $HqsK_{WT}$, (B) $HqsK_{D459A}$, (C) $HqsK_{H174A}$ was carried out with radiolabelled [γ-^{32}P]ATP as substrate and kinase activity was monitored over time. The reaction was terminated at each indicated time point with gel loading dye, and radiolabelled proteins were separated by SDS-PAGE followed by autoradiography.

We did not specifically investigate the phosphatase activity of HqsK using its natural substrate, but we did examine the phosphatase activity of each mutant towards the generic substrate 3-O-methylfluorescein (Figure 3.9). WT and $HqsK_{H174A}$ demonstrated OMFP phosphatase activity, while $HqsK_{D459A}$ did not. These results imply that like the other QS receptors, HqsK has autophosphatase activity. Taken together, we conclude that H174 is phosphorylated in the kinase domain and then the phosphate is transferred to D459 in the receiver domain. These results are in agreement with the three other known QS receptors in *V. harveyi* (53, 56), all of which are hybrid dual sensor kinases.

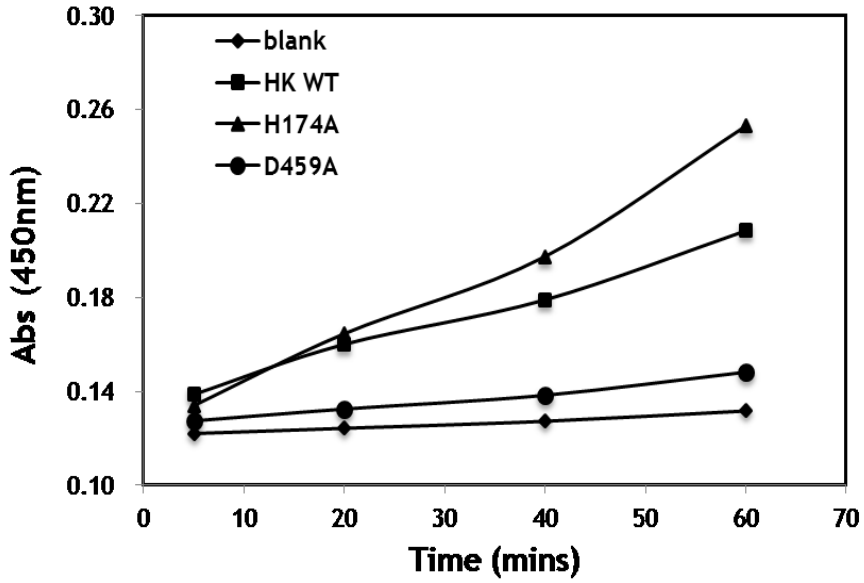


Figure 3-8. Autophosphatase activity of HqsK, $HqsK_{H174A}$, $HqsK_{D459A}$ using O-methylfluorescienphosphate (OMFP) as a substrate. The formation of the product was monitored by measuring the absorbance at 450 nm over time. HqsK and $HqsK_{H174A}$, but not $HqsK_{D459A}$, display phosphatase activity towards OMFP.

H-NOX and HqsK interact with one another

Being in the same operon we predict that H-NOX interacts with HqsK to form the NO sensor. To investigate the interaction, co-immunoprecipitation of GST-tagged HqsK and His_6-tagged H-NOX was performed. Using GST-tagged HqsK bound to glutathione as bait, His_6-tagged HNOX was captured from *E. coli* lysate containing overexpressed His_6-tagged H-NOX. Western blot analysis using anti-His_6 antibody to detect H-NOX showed a band which corresponded to H-NOX in the solution containing GST-tagged HqsK but not in the solution containing GST-glutathione beads only (Figure 3.10). This clearly suggests a strong interaction between HqsK and H-NOX. We did not investigate protein interaction using

different oxidation or ligand binding states of H-NOX, but it has been shown in *S. oneidensis* that H-NOX and *So*HK interaction is independent of the H-NOX ligation state (83).

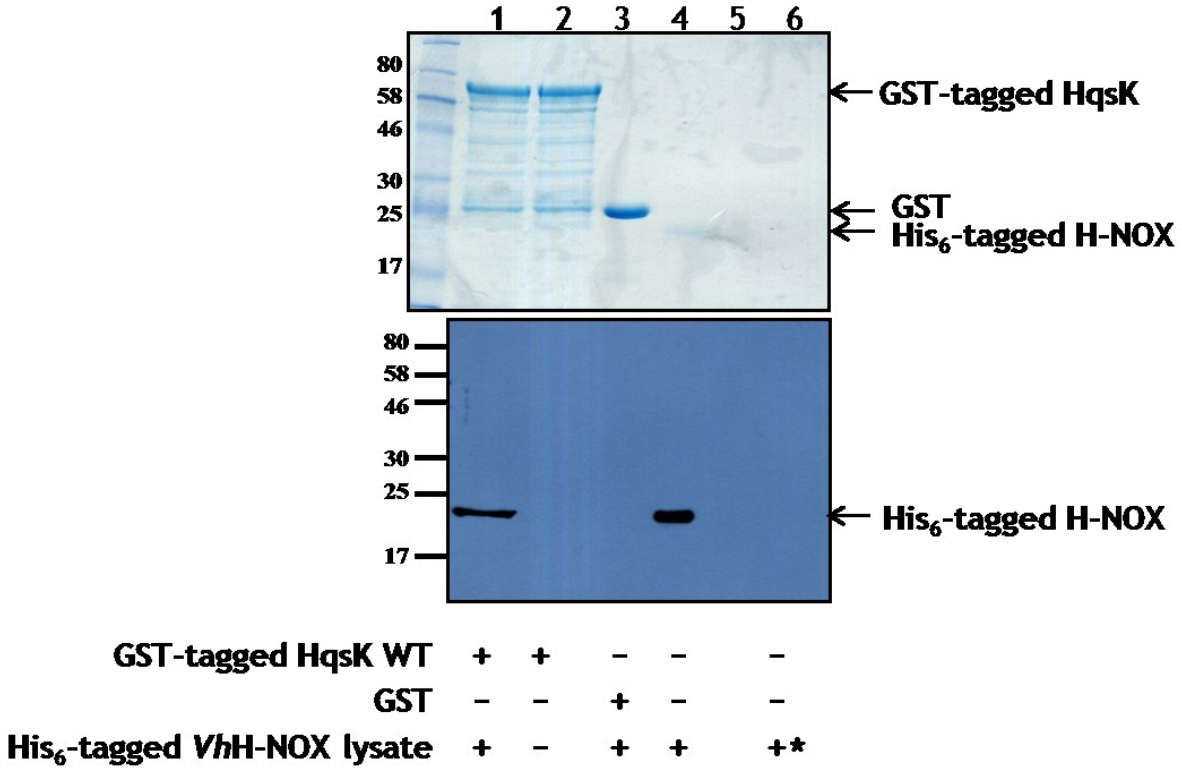


Figure 3-9. H-NOX interacts with HqsK. Interaction of H-NOX and HqsK was detected using pull-down assay. GST-HqsK bound to glutathione beads, but not GST-glutathione beads only, precipitates His_6-H-NOX from *E. coli* cell lysates as analyzed by SDS-PAGE. Top: protein loading detected by Coomassie brilliant blue staining. Bottom: detection of His_6-H-NOX by Anti-His_6 Western blot. Lane 1, molecular weight marker; lane 2, GST-HqsK-glutathione beads + His_6-H-NOX lysate; lane 3, GST-HqsK-glutathione beads (GST-HqsK is ~81 kDa; GST is ~23 kDa); lane 4, GST-glutathione beads + His_6-H-NOX lysate; lane 5, His_6-H-NOX lysate (His_6-H-NOX is ~22 kDa); lane 6*, glutathione beads + His_6-H-NOX lysate.

NO bound HNOX inhibits kinase activity of HqsK

To examine the effect of H-NOX on HqsK activity, we used the $HqsK_{D459A}$ because it yielded a more stable product for quantification and isolated kinase activity from phosphotransfer and phosphatase activities. Different ligation and oxidation states of H-NOX (80 μM) were prepared and incubated with $HqsK_{D459A}$ (11 μM) for 30 mins before addition of ATP. The kinase activity of $HqsK_{D459A}$ was inhibited by all forms of H-NOX, but NO-bound H-NOX had the most significant effect (Figure 3.11A). The unligated Fe^{2+} and Fe^{3+} H-NOX complexes inhibited kinase activity by ~50%, the CO-bound *Vh*H-NOX caused ~70% inhibition, and NO-bound H-NOX almost completely abolished kinase activity under the experimental conditions used in this experiment. The inhibitory effect of NO-bound H-NOX on HqsK kinase activity was even more apparent when 11 μM D459A was incubated with increasing concentrations of NO-bound H-NOX (Figure 3.11B). These results are consistent with previous work examining the effect of H-NOX on histidine kinase activity in *S. oneidensis* (83) and *Pseudoalteromonas atlantica* (84). Furthermore, inhibition of kinase activity upon AI binding is the pattern observed for all other characterized *V. harveyi* QS receptor kinases (53, 56).

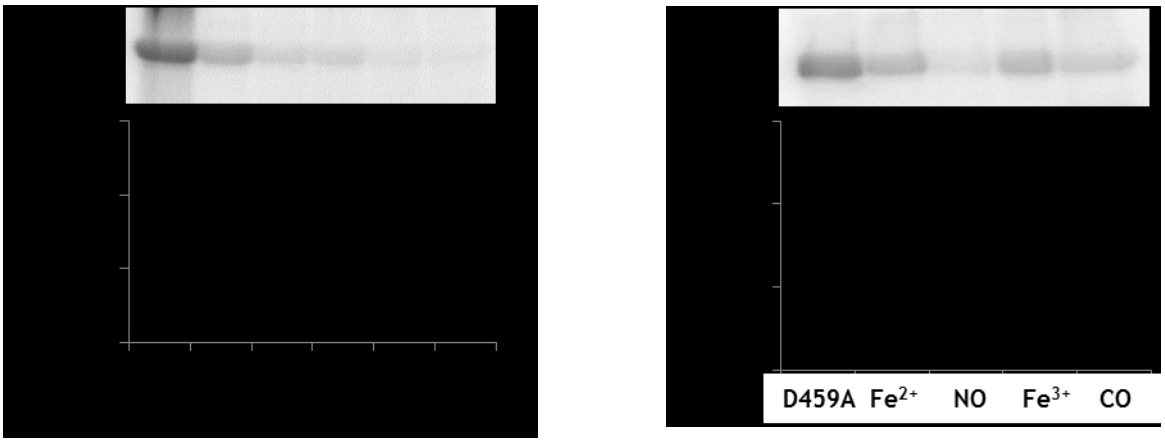
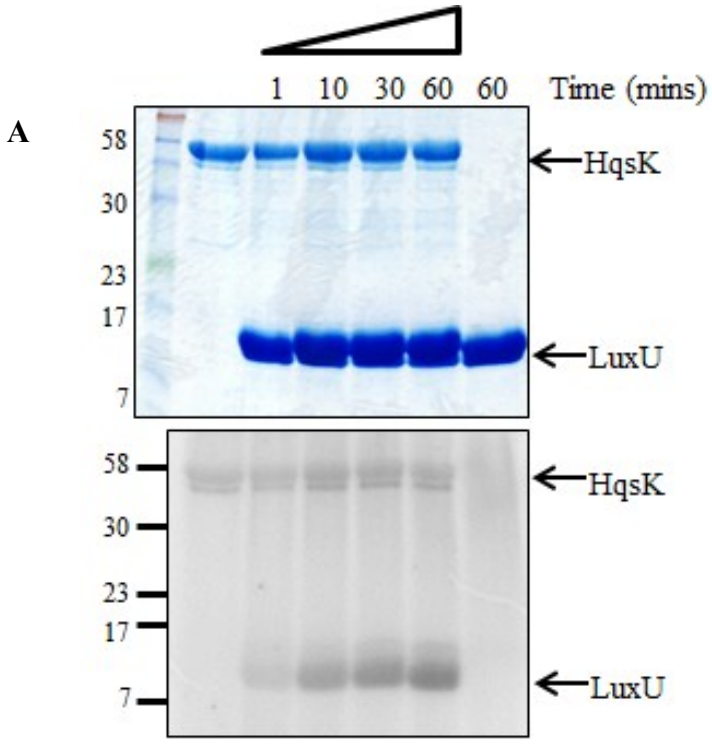


Figure 3-10. Histidine kinase regulation by NO-HNOX. Regulation of histidine kinase activity was monitored by incubating 11 μM $HqsK_{D459A}$ and *Vh*H-NOX using radiolabelled [γ-^{32}P]ATP as substrate. Histidine kinase activity was done as described previously. The radiolabelled proteins were analyzed by SDS-PAGE followed by autoradiography. (A) The NO-bound H-NOX has the highest inhibition towards histidine kinase activity when compared to same concentration (80 μM) of CO-bound H-NOX, H-NOX-Fe^{2+} -unligated and Fe^{3+}. (B) Dose-dependent kinase inhibition by NO-bound *Vh*H-NOX. HqsK was incubated with increasing concentrations of NO-bound *Vh*H-NOX for 30 mins. followed by the addition of radiolabelled [γ-^{32}P]ATP. Kinase inhibition was determined as described previously.

HqsK transfers phosphate to LuxU

Finally, because we hypothesize that HqsK enters *V. harveyi* QS by phosphotransfer to LuxU (parallel to LuxQ, LuxN, and CqsS), we investigated the ability of HqsK to engage in phosphotransfer with LuxU. WT HqsK (10 μM) was incubated with 1 mM ATP (trace [γ-^{32}P]ATP) for 1 min. before addition of LuxU. As illustrated in Figure 3.12, phosphate was transferred from HqsK to LuxU in a time-dependent manner. Interestingly, we observed a more intense signal for phosphorylated LuxU at the end of the time course than for phosphorylated HqsK at the beginning of the time course. This could not be attributed to

differences in protein concentration (Figure 3.12A); rather we concluded that because there was excess ATP in the reaction, HqsK could continually autophosphorylate and transfer that phosphate to LuxU. Therefore the flux of phosphate is towards LuxU where it accumulates due to the relative stability of P-His on LuxU (compared with P-Asp in the receiver domain of HqsK). Mutation of the site of phosphorylation on LuxU (H58) (119) to Arg did not result in phosphotransfer, neither did mutation of H174 or D459 to Ala in HqsK (Figure 3.12B). These results, along with the observation that LuxU incubated with ATP without HqsK does not become phosphorylated (Figure 3.12A) strongly indicate that phosphorelay between HqsK and LuxU takes place as predicted ($HqsK_{H174}$ to $HqsK_{D459}$ to $LuxU_{H58}$). Evidence that HqsK is able to transfer phosphate from H174 to D459 in HqsK to H58 in LuxU confirms the phospho-relay typical of a TCS.



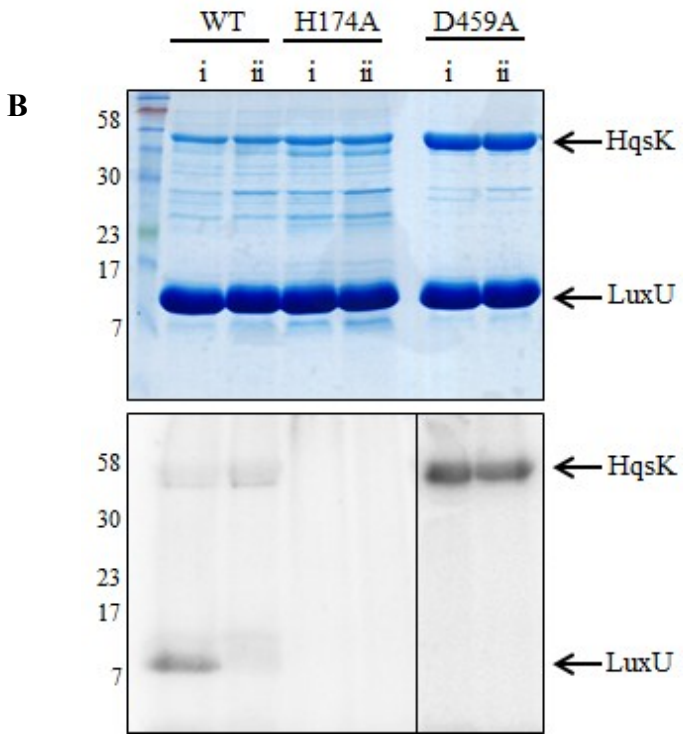


Figure 3-11. HqsK exchanges phosphate with LuxU. (A) Phosphotransfer from HqsK to LuxU monitored over time and analyzed by SDS-PAGE. Top: protein loading detected by Coomassie staining (His_6-HqsK is ~60 kDa; His_6-LuxU is ~13.5 kDa). Bottom: detection of radiolabeled phosphoproteins by autoradiography. Lane 1, molecular weight marker. Lanes 2−7, the following proteins were incubated with [γ-^{32}P]ATP and monitored over time: lane 2, HqsK only; lane 3−6, HqsK + LuxU; lane 7, LuxU only. (B) The phosphotransfer path is $HqsK_{H174}$ to $HqsK_{D459}$ to $LuxU_{H58}$. Phosphate transfer from HqsK, $HqsK_{H174A}$, and $HqsK_{D459A}$ to (i) LuxU and (ii) $LuxU_{H58R}$.

3.4 Discussion

In this study we have provided evidence that H-NOX is an NO-sensor and HqsK is a kinase/phosphatase that participates in *V. harveyi* QS by exchanging phosphate with LuxU in response to the presence of nanomolar NO. Our data indicate that NO behaves exactly like other AIs in *V. harveyi* QS: it binds its cognate receptor (H-NOX/HqsK) and inhibits kinase activity, thus contributing to phosphate flow away from LuxU/LuxO, de-repressing LuxR,

and contributing to light production. We did not attempt to test the phosphotransfer to LuxO, the final acceptor in the phospho-relay, since a previous report stated that getting a soluble LuxO proved to be difficult (56). However, based on our earlier findings, the inability of Δ*hnoX V. harveyi* mutant to induce an earlier onset of bioluminescence in response to NO, in contrast to WT, could only mean that HqsK remained as kinase and contributed to the phosphorylation of LuxO. Furthermore, down-regulation of the kinase activity of the kinase only mutant, $HqsK_{D459A}$, by NO-bound H-NOX in contrast with the other oxidation or ligand-binding states of H-NOX clearly shows that the kinase activity is influenced by NO. Although we did not investigate the phosphatase activity of HqsK using its natural substrate, the hydrolysis of OMFP, a generic phosphatase substrate, implies that it is capable of autodephosphorylation. Taken together, our results strongly suggest that HqsK participates in *V. harveyi* QS circuits by channeling phosphate to and from LuxU then to LuxO, and 2) NO shuts off the kinase activity thereby leaving the phosphatase activity to contribute to the dephosporylation of LuxO similar to the other known *V. harveyi* QS sensor kinases. These results indicate that the phosphorelay: $HqsK_{H174}$ → $HqsK_{D459}$ → $LuxU_{H58}$ takes place as predicted, in accordance with the other *V. harveyi* QS receptors.

3.5 Conclusion

The results presented here reveal an NO-regulated kinase through the NO sensory domain H-NOX. HqsK is a two-component hybrid protein with kinase and phosphatase activities that can transfer phosphate to LuxU. H-NOX is an NO-sensor that inhibits the HqsK activity. These biochemical data are consistent with the bioluminescence and genetic data. *V. harveyi* increase their light production in the presence of NO because HqsK activity

is inhibited contributing to phosphate flow away from LuxU/LuxO and derepression of LuxR. In the absence of NO, HqsK acts as a kinase, channeling phosphate toward LuxU, increasing the population of P-LuxO and contributing to a decrease in light production.

CHAPTER 4

Determining the role of NO in biofilm formation of *Vibrio harveyi*

Abstract

Cell signaling plays an important role in the survival of bacterial colonies. They use small molecules to coordinate gene expression in a cell density dependent manner. This process, known as quorum-sensing, helps bacteria regulate diverse functions such as bioluminescence, biofilm formation and virulence. In *Vibrio harveyi*, a bioluminescent marine bacterium, four parallel quorum-sensing systems have been identified to regulate light production. In this study, we have shown that nitric oxide (NO) not only contributes to light production but also enhances biofilm formation. Biofilm formation involves a complex organization of structure and function, thus there is more than one mechanism employed by bacteria to achieve a highly organized structure. We propose that NO regulation of biofilm formation is through QS via the H-NOX/HqsK pathway. Our data suggest that *V. harveyi* is capable of switching between lifestyles to be able to adapt to changes in its environment.

4.1 Introduction

Bacteria were once viewed as independent and self-sufficient single-celled organisms but research in recent years has revealed that they do live a rich social life. In fact, bacteria communicate with one another using signal molecules, known as autoinducers (AI), to synchronize gene expression in a manner similar to multicellular organisms. Social interactions among bacteria allow them to coordinate behaviors by secreting, detecting and responding to AIs, a phenomenon popularly known as quorum sensing (QS). QS enables bacteria to adapt to changes in the environment by changing their behavior in response to population density. For example bacteria are capable of switching between growth lifestyles such as planktonic or as surface attached communities known as biofilms (130). Biofilms are populations of sessile bacteria that form a complex yet ordered three-dimensional structures known to confer on their members advantages such as access to nutrients, resistance to antimicrobial agents and protection against predators (131-133). Thus it is not surprising for most bacteria to prefer the biofilm mode of growth.

*Vibrio*s are marine bacteria that exist as free-living or in symbiotic or pathogenic relationships with eukaryotic hosts. Most *Vibrios* are notorious pathogens that can cause diarrheal disease that occurs in epidemic form, such as *V. cholerae* the causative agent of cholera. Recent studies have shown that *Vibrios* have developed strategies to grow, survive and proliferate in a given niche. One key factor for survival and transmission is the ability to form a biofilm. Formation of a biofilm (Figure 4.1) begins with the attachment of planktonic bacteria to a surface through a reversible, weak adhesion via van der Waals forces. They then anchor themselves using cell adhesion structures called pili and adhere irreversibly to the substratum. It has been suggested that at the initial stages of biofilm formation, bacteria

are already communicating with one another, since rapid development and phenotypic differentiation occur during this stage and up until they form the mature biofilms (130, 134). At this stage, biofilm structure and mechanical properties are defined by extracellular polymeric substances (EPS), which contain DNA, proteins and polysaccharides, that serve as scaffolding or glue that keep the biofilm together (131, 134). However, the regulatory mechanisms involved in biofilm formation are complex and therefore necessitate better understanding.

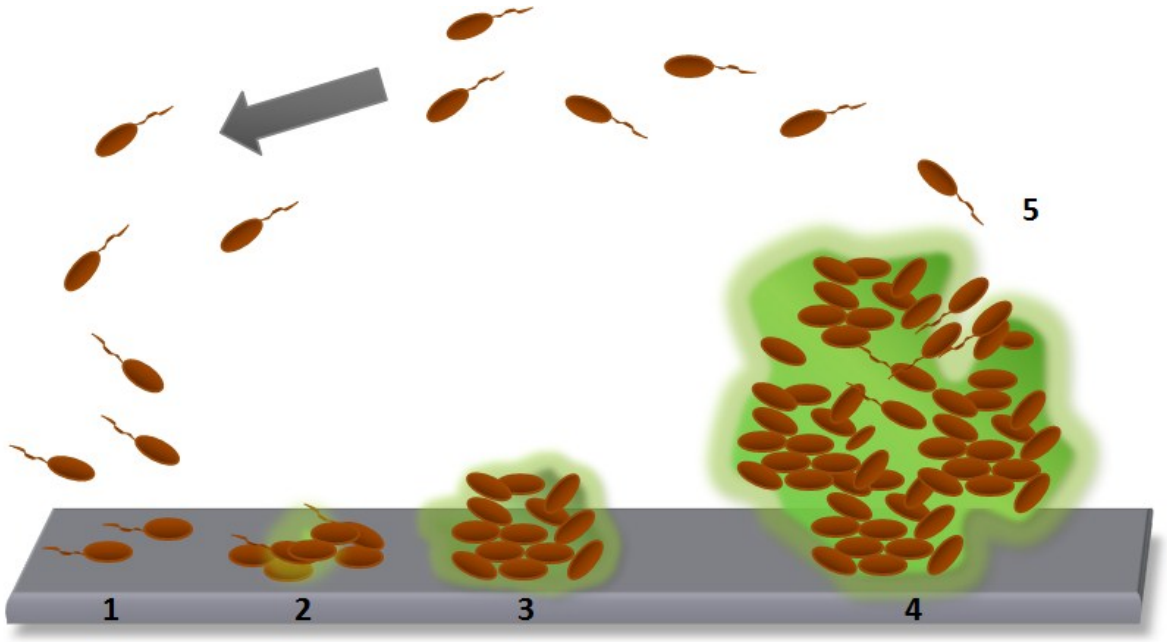


Figure 4-1. Lifecycle of bacteria in biofilm (Adapted from(134)). The biofilm life cycle begins when 1: individual cells populate the surface 2: attachment onto the surface using adhesion structures 3 & 4: biofilm architecture develops and matures 5: single cells are released from the biofilm.

Biofilm formation is closely associated with QS. The sequential steps involved in biofilm formation require regulation of many genes (130) thus, efficient an mechanism of communication is required to ensure that the ordered processes to form the complex are

followed. To achieve this remarkable feat, bacteria send out signals as soon as they enter the confines of the biofilm, since proximity to each other makes it more feasible for bacteria to communicate with one another and engage in the maintenance of the well-being of the community.

In *Vibrios*, several structural genes and regulatory processes have been shown to regulate biofilm formation, such as EPS production by specific polysaccharide genes. *Vibrio* biofilms use QS circuits to regulate EPS production (135). Genetic studies done on *V. parahaemolyticus*, *V. vulnificus* and *V. fischeri* reveal that QS positively regulates biofilm formation (136, 137) through OpaR, SmcR, LitR (138) (homologs of *V. harveyi* LuxR), respectively. However, a different scenario is observed in *V. cholerae*. In fact, an opposite regulation is observed where vibrio polysaccharide (VPS, equivalent of EPS) gene expression is more apparent in the absence of HapR (LuxR homolog in *V. cholerae*), indicating a negative regulation of biofilm formation (139, 140). The ecological importance of this regulation in *V. cholerae* is still being investigated but it has been suggested that being able to detach from the community is important in the transmission, colonization, and persistence of the next generation.

The widely studied model bacteria for QS, *V. harveyi,* are also able to exist as free-swimming, attached to abiotic surfaces, or in association with a host as pathogen of marine animals (4). They are not associated with human pathogenesis, but they cause infection in marine animals such as Tiger shrimp, as a disease called vibriosis, which causes economic loss in the shrimp industry (141). Although it has been shown that *V. harveyi* can switch between lifestyles, very limited information is available about biofilm formation and only limited studies that correlate QS with biofilm formation are available. In one study, a QS-negative *V. harveyi* strain (LuxO D47E, constitutively phosphorylated) showed negligible

biofilm formation as opposed to the QS-positive mutant (constitutively bright mutant), where biofilm formation is observed but much lower than WT strain. The authors suggest that biofilm formation is positively regulated by QS. This is the first demonstration of a QS-regulated biofilm formation in *V. harveyi*. However, it is not clear why a lower biofilm is observed for the QS-positive strain than the WT strain. The limited information available on *V. harveyi* biofilm formation makes it difficult to draw a conclusion on the mechanism of action. Thus there is much to be explored to understand QS and biofilm formation in *V. harveyi*.

V. harveyi employ QS circuits to regulate gene expression such as bioluminescence, virulence production and biofilm formation (3, 86, 87). We have identified a fourth pathway which involves NO as the signal molecule that is sensed by the H-NOX/HqsK sensor pair (142). We have shown that NO acts analogously with the other AIs and positively regulates light production. However, as discussed above, QS can also regulate other cellular processes. It has been shown that LuxR in *V. harveyi* can regulate at least 50 other genes (33). In a genetic study, the presence of LuxR is shown to negatively regulate *flagellar* operons in *V. harveyi* (101). A single polar flagellum provides the bacteria with an effective means of motility. This implies a positive regulation for biofilm formation, since loss of flagella would result to attachment on a surface – the initial stage of biofilm formation. Thus we hypothesize that NO would also contribute to biofilm formation through the QS pathway.

Involvement of NO in biofilm formation has been shown in many bacterial species such as *Shewanella woodyi* (79), *Shewanella oneidensis* (81) and *Legionella pneumophila* (80), through cyclic-di-GMP-mediated biofilm formation. In *Pseudomonas aeruginosa,* however, NO causes QS-mediated biofilm dispersal (96). In this study, we explore the

possibility that NO regulates biofilm formation in *V. harveyi.* Although there is more than one way to build a biofilm, we propose that one of the ways to regulate a NO-mediated biofilm formation is through QS.

4.2 Materials and Methods

Bacterial strains and growth conditions. Strains used in this study are listed Table 2.1. *V. harveyi* strains WT, Δ*luxO*, and Δ*luxNS* were purchased from the American Type Culture Collection (ATCC), maintained in Marine Media (MM; 28 g/L; BD Difco) and grown at 30 °C with agitation at 250 rpm. For bioluminescence assays, cells were grown in Autoinducer Bioassay media [AB; 0.2% vitamin-free casamino acids, 0.3 M NaCl, 0.05 M $MgSO_4$, 10 mM potassium phosphate pH 7.0, 1 mM L-arginine, 1 %(v/v) glycerol] (5, 103). *E. coli* strains XL1 and BL21(DE3)pLysS were used for plasmid amplification and protein purification, respectively. *E. coli* strains were typically grown in Luria Broth (LB; 20 g/L; EMD chemicals) at 37 °C with agitation at 250 rpm. *E. coli* strain WM3064 was used as a donor for conjugation and was grown in LB complemented with 2,3-diaminopropionic acid (DAP; 0.36 mM; Sigma Aldrich) at 37 °C with agitation at 250 rpm.

Construction of in-frame gene disruption mutant strains. PCR was used to amplify regions of genomic DNA flanking the target gene (VIBHAR_01911) from *V. harveyi* genomic DNA using Phusion® polymerase (New England Biolabs). Upstream and downstream primers (koH-NOX-fwd and koH-NOX-rev, Table 2.1) contained ApaI and SpeI restriction sites, respectively. The amplified PCR product was cloned into pSMV3 (109) (gift from Jeffrey Gralnick, University of Minnesota). The plasmid was modified to yield

pΔhnoX by introducing two BamHI restriction sites (siteA, near the *hnoX* start codon; siteB, near the *hnoX* stop codon) through site-directed mutagenesis (koH-NOX-site-fwd, koH-NOX-siteArev, koH-NOX-siteB-fwd, koH-NOX-siteBrev, Table 2.1), digestion with BamHI and ligation with T4 DNA ligase. In *pΔhnoX*, *hnoX* is replaced by DNA coding for a 37-amino-acid polypeptide consisting of the first 7 and last 28 amino acids of H-NOX and two amino acids encoded by BamHI site. *pΔhnoX* was transformed into *E. coli* WM3064(109) and grown on LM/DAP plates [Luria Marine; 10 g/L tryptone, 5 g/L yeast extract (Difco) and 20 g/L NaCl, 15 g/L Agar (RPI)] with kanamycin added to a concentration of 100 μg/ml. WM3064 transformed with the deletion vectors were mated with *V. harveyi* in a 1:5 ratio on LM/DAP agar overnight at ambient temperature. *V. harveyi pΔhnoX* transconjugants containing the deletion vectors were selected on LM plates supplemented with 100 μg/ml kanamycin and verified by colony PCR. The selected colonies were then plated on LM plates containing 5% sucrose at ambient temperature to select for double recombination events. Colonies were then replica plated onto LM plates with added kanamycin (100 μg/ml) and incubated overnight at ambient temperature; kanamycin sensitive colonies were screened by colony PCR for gene deletion (79).

Construction of gene disruption mutant complementation plasmid. PCR was used to amplify VIBHAR_01911 from *V. harveyi* genomic DNA using Phusion® polymerase (New England Biolabs). Upstream and downstream primers contained EcoRI and BamHI restriction sites, respectively (*hnoX*comp-fwd and *hnoX*comp-rev, Table 2.1). The amplified PCR products were cloned into the broad host range plasmid pBBR1MCS-2 (110)(gift from Jeffrey Gralnick, University of Minnesota) and sequenced (Stony Brook sequencing core). The resulting plasmid, p*hnoX*, was introduced into the gene disrupted strain via conjugation

as previously described (79).

Biofilm Imaging by Confocal Microscopy. Microscopy images were recorded on a Zeiss LSM 510 Metanlo Two-Photon Laser Scanning Confocal Microscope System. Overnight culture of *V. harveyi* in marine media was diluted (1:1000) into fresh medium containing different concentrations of DPTA NONOate predecayed for 3 hrs at 30 °C in a sterile 50 mL conical centrifuge tube containing a glass microscope slide. Biofilms were grown under steady-state conditions at 30 °C with slow agitation at 50 rpm for 24 hrs. Following the growth period, the slide was thoroughly rinsed with distilled water and the adhered biofilm cells were stained with the 1% calcoflour white to stain EPS and LIVE/DEAD BacLight kit (Invitrogen) to stain the cells, according to the manufacturer's protocol, for 15 min. The biofilm formed at the air−liquid interface was then imaged and analyzed. The air−liquid interface was ~3 mm wide (in the X dimension, along the longest side of the microscope slide), as determined from crystal violet staining of identically obtained biofilms on microscope slides. The biofilm thickness (X−Z dimension, i.e., the height of the biofilm measured from the surface of the microscope slide to the top of the biofilm) was measured at three different locations in each experiment and averaged to determine the mean biofilm thickness. The locations were chosen randomly, but generally one spot near the middle of the slide and one from each edge of the slide (in the Y dimension) were chosen. Multiple locations were measured because bacterial biofilms are often not of uniform thickness. These measurements may not account for all the variation in biofilm thickness, but they provide an estimate of biofilm thickness for comparison between different NO concentrations. Biofilm mass was quantified using crystal violet staining (see below). Confocal images for each of three independently grown biofilms of each NO concentrations were separately obtained and analyzed. The mean thickness from each trial was determined from measurements at multiple

locations. The mean thickness from three independent trials ± one standard deviation is reported.

Crystal Violet (CV) Staining for Biofilm Quantification. Steady-state biofilm formation, at the air−liquid interface, in a shaking culture was examined in 96-well polyvinyl chloride (PVC) plates as previously described (79), with a few modifications. A 100 µl subculture (1:100 dilution of an overnight culture of *V. harveyi*) in marine media was incubated at 30 °C for 12 h with slow agitation (50 rpm). The planktonic cells and media were then removed, and the remaining biofilm was rigorously washed with water followed by staining with 150 µl of 0.1% CV in water for 15 min. Next the CV solution was removed, and the wells were rinsed three times with distilled water and allowed to thoroughly dry. Then 100 µl of DMSO was added to each well to solubilize the CV adsorbed by the biofilm cells. The DMSO/CV solution was removed from the PVC plate and added to a polystyrene 96-well plate, and the OD570 was measured with a Perkin-Elmer Victor X5 multilabel reader. The data are reported as the CV absorbance at 570 nm divided by the optical density of the planktonic and biofilm cells at 600 nm. Each biofilm condition was run a minimum of 10 times in one experiment, and the entire experiment was performed a minimum of three times. The mean from 3 independent experiments is reported.

4.3 Results

Vibrios live in several different niches and assume different lifestyles; they are found both as planktonic, free-swimming cells and as sessile, attached to surfaces in biofilms. In order to adapt to changes in the environment, all *Vibrios* switch between planktonic and

biofilm lifestyles. However, due to the differences in the specific niches in which they reside, it is not surprising that different species of the *Vibrio* genus have evolved different mechanisms for regulating this lifestyle switch. For example, *V. cholerae* prefer to form biofilms at low cell density and leave these communities at high cell density, whereas *V. parahaemolyticus* form biofilms at high cell density. The evolutionary advantage of these differences lies in the nature of their infection strategies (143); it has been suggested that *V. cholerae* cause acute infection, and need to disperse out of the community in order to colonize new sites (144), while *V. parahaemolyticus* are infective as biofilm.

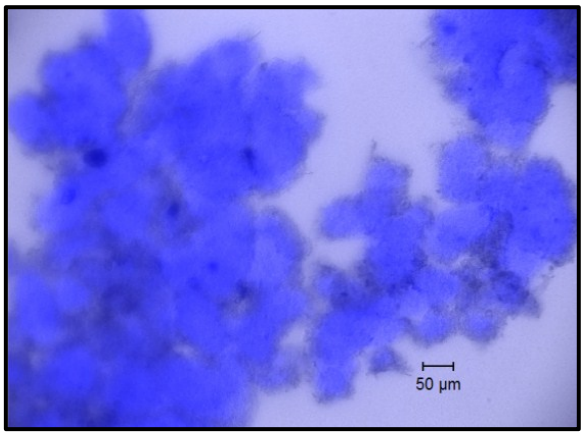
NO enhances biofilm formation in V. harveyi

We have shown in our previous work that NO enhances light production at the initial stage of bioluminescence. However, *lux* operon is not the only set of genes regulated by QS. Thus we hypothesize that NO may regulate biofilm formation. Our hypothesis is based on a genetic study where LuxR, in the presence of AIs, has been shown to down regulate the *flagellar* operon, an important feature in the first stage of biofilm formation (101, 145). Thus, we expect QS to upregulate biofilm formation in the presence of NO.

To test this hypothesis, we investigated the effect of NO on biofilm formation by the *V. harveyi* wild type strain BB120. It has been previously established that NO acts as a signaling molecule at low nanomolar concentrations. Biofilm architecture, biofilm thickness and cell viability were determined using confocal laser scanning microscope (CLSM). The cells were allowed to grow on microscope slides for 12 hours at 30 °C. Biofilms that formed at the air-liquid interface were visualized by staining with SYTO 9 (green; stains live cells and propidium iodide (red; stains dead cells only) for observation under confocal microscope

while EPS production was viewed using calcoflour white under the phase-contrast method. Under the conditions in which the biofilms were obtained, most cells were viable and *V. harveyi* were able to form EPS and thick, robust biofilms under aerobic conditions, however, individual cells are difficult to see due to the other substances present in the EPS matrix (DNA, protein). Biofilm formation is indeed positively regulated by QS as evidenced by the higher biofilm formed by *ΔluxO* strain. This strain contains a *ΔluxO* mutation which renders the production of LuxR independent of AI production, thus this strain is constitutively bright and locked at the high cell density state. Cells exposed to 50 nM NO (determined by NOA) showed a remarkably thick biofilm in comparison to the culture grown without added NO (Figure 4.2 B and C). On the other hand, biofilm formation decreases at higher NO concentrations. Although presently it is not clear why there is a concentration-dependent switch in biofilm regulation in response to NO, it is possible that at high NO concentrations, NO induces dispersal of biofilm through a different pathway. This is shown to be the case in *Psuedomonas auruginosa*, where NO enhances biofilm dispersal through QS (96, 146). In, *N. europaea*, an NO concentration-dependence switch in growth modes was also observed. Biofilm formation is induced at 30ppm while a NO concentration below 5 ppm promotes dispersal (146). However, it is also possible that another regulatory mechanism is being upregulated by NO and possibly H-NOX, such as regulation of a diguanylate cyclase (DGC)/phosphodiesterase (PDE) to affect cyclic-di-GMP production. C-di-GMP is a secondary messenger widely used by bacteria to regulate biofilm formation (79, 147). However, we have shown that an NO-mediated regulation of biofilm formation is apparent at low NO concentrations.

A



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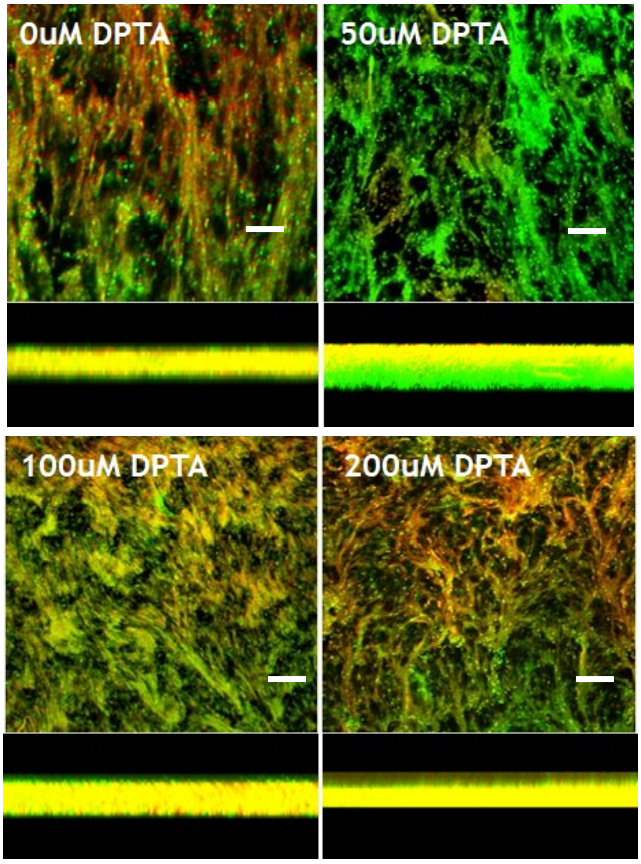

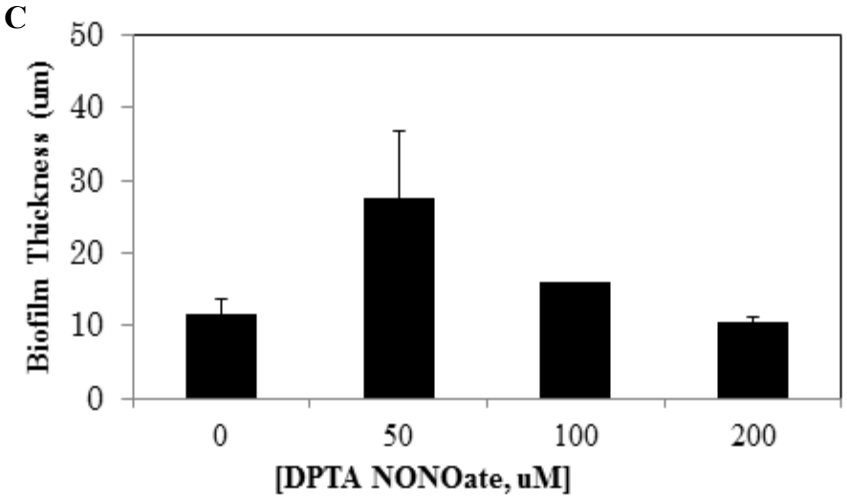

Figure 4-2. NO enhances biofilm formation of WT *V. harveyi.* The effect of NO on biofilm thickness, cell viability was quantified by CLSM. NO regulates biofilm formation in *V. harveyi.* Biofilms of WT exposed to different NO concentrations. (A) Phase-contrast image of biofilm at 10x magnification stained with calcofluor to show EPS production (B) Confocal image of biofilm at 40x magnification. Cells were stained with SYTO 9 (green; stains all cells) and propidium iodide (red; stains dead cells only) Top pictures are the x-y view, bottom pictures show the y-dimension as viewed on the side. Bar is 10μm. (C) Quantitative measure of biofilm thickness. Biofilm thickness was measured using Zeiss LSM Image browser.

This observation is further corroborated when we quantified biofilm formation of *V. harveyi* WT strain grown on 96-well plates using the crystal violet staining method (145) . A similar trend was observed, where at 50nM NO exhibited higher biofilm formation as shown in Figure 4.3. These observations suggest that NO enhances biofilm formation at low NO concentration, consistent with our hypothesis that biofilm is positively regulated by NO, possibly through QS. We have shown in our previous studies that NO is sensed by the H-NOX/HqsK sensor system which participates into QS of *V. harveyi* by exchanging phosphate with LuxU, and indirectly induces light production through LuxR. Thus we

hypothesize that NO regulates biofilm formation through QS through H-NOX/HqsK pathway.

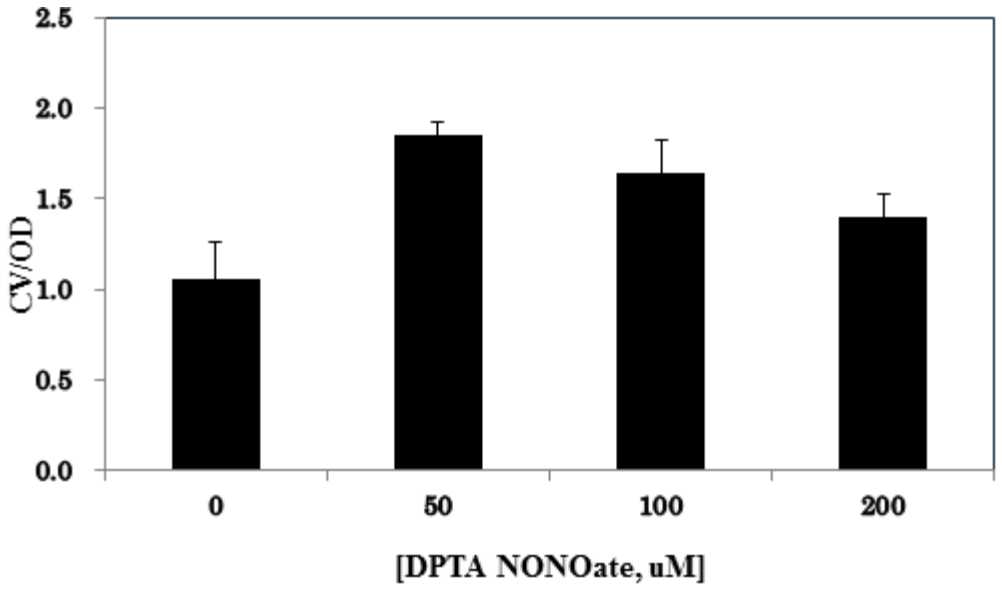

Figure 4-3. Effect of NO on biofilm formation of *V. harveyi* WT strain quantified using crystal violet staining method. Overnight culture of *V. harveyi* WT was diluted 1:100 into fresh MM containing different concentrations of DPTA NONOate predecayed for 3 hours at 30 °C. Cells were grown for 12 hours at 30 °C and cells at the air-liquid interface were stained with 0.1% crystal violet (CV). DMSO was used to solubilize CV absorbed by the cells. CV absorbance was determined at 570nm. Normalized CV is reported as CV divided by OD at 570nm. Error bars represent one standard deviation from the mean of triplicate experiments.

NO regulates biofilm formation through H-NOX

We determined in our previous studies that NO is sensed by the H-NOX/HqsK sensor pair. We have shown that NO-bound H-NOX inhibits HqsK kinase activity, which drains phosphate away from LuxO and as a result, LuxR is translated and induces the lux

operon. Here, we extend the function of LuxR as regulator of biofilm formation through NO and H-NOX/HqsK. We tested this hypothesis by investigating the effect of NO on the WT strain, *ΔhnoX* strain and the complementation strain which contains the *phnoX* plasmid in the *ΔhnoX* strain.

As shown in Figure 4.4, biofilm formation is enhanced in cultures grown in the presence of NO which is consistent with our CLSM assays. As expected, deletion of *hnoX* gene did not elicit the same biofilm enhancement in the presence of NO, and complementation of the *ΔhnoX* mutant strain with *phnoX* restores the wild type behavior. However, a very slight increase in biofilm is observed in the *ΔhnoX* strain in the presence of NO. This as mentioned earlier could possible due to regulation of a DGC/PDE.

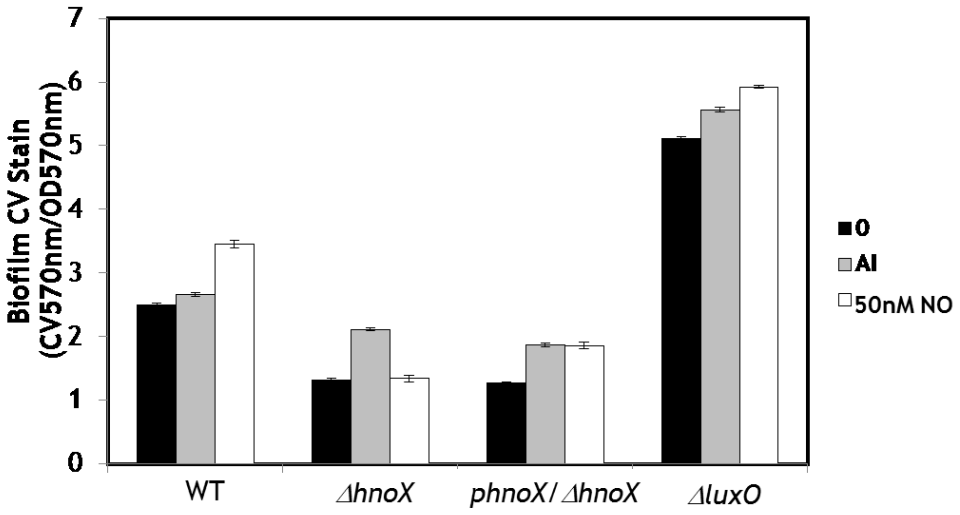

Figure 4-4. Effect of NO on biofilm formation of *V. harveyi* WT, *ΔhnoX,* phnoX/*ΔhnoX* and *ΔluxO* strains quantified by CV staining. Overnight culture of *V. harveyi* WT was diluted 1:100 into fresh MM containing different concentrations of 50 μM DPTA NONOate predecayed for 3 hours at 30 °C and cell free extracts of *V. harveyi* WT (AI). Cells were grown for 12 hours at 30 °C and cells at the air-liquid interface were stained with 0.1% crystal violet (CV). DMSO was used to solubilize CV absorbed by the cells. CV absorbance was determined at 570nm. Normalized CV is reported as CV divided by OD at 570nm. Error bars represent one standard deviation from the mean of triplicate experiments.

The above results seem to indicate a possible NO-mediated regulation of biofilm formation through QS via the H-NOX/HqsK system. This study provides a starting point and a glimpse of the possible correlation between QS and biofilm formation in *V. harveyi*.

4.4 Discussion

In the natural environment, bacteria are often part of a multicultural community. Probably, they spend most of their time in social communes where they are often covered with a polysaccharide matrix that confers protection against environmental stress. The ability of these organisms to coordinate these highly specialized networks called biofilms seems to indicate a QS property. It appears that a global regulatory mechanism is needed to achieve the task at hand, since biofilm formation involves the concomitant expression/repression of tens or even hundreds of unlinked cells in a cell-density dependent manner.

Our data suggest that NO can act as a stimuli to signal *V. harveyi* to go into biofilm. The role of NO as a signaling molecule to regulate biofilm formation is not new. In previous studies, our laboratory and others have reported NO is involved in the regulation cyclic-di-GMP, a second messenger widely used by bacteria to regulate biofilm formation (147, 148). In many bacteria, NO is sensed by an H-NOX that regulates the function of a bifunctional diguanylate cyclase (DGC)/phosphodiesterase (PDE) to control the concentration of c-di-GMP in solution (79, 80) or regulates a histidine kinase involved in two-component signaling, which ultimately, also regulates c-di-GMP (81).

Here, we report a QS-mediated biofilm formation in *V. harveyi* through the H-NOX/HqsK pathway. The results of our experiments contribute to understanding the

complex mechanisms involved in biofilm formation. Biofilms involve a community wide-setting thus QS must impart a crucial step in coordinating several of the genes that need to be expressed to in order to alter cellular function.

CHAPTER 5

Insights and future directions

Vibrio harveyi use cell communication to facilitate interaction by exchanging signal molecules known as autoinducers, in order to assess population density and diversity. However, the present model only accounts for bacterial communication. In nature, bacteria are found in several other milieus, be it with same species, mixed species or in an environment with eukaryotes. In this research study, we have expanded *V. harveyi's* repertoire of signal molecules to include nitric oxide as signal, possibly to indicate the presence of a eukaryote. We have demonstrated that NO regulates bioluminescence and biofilm formation and we have determined the molecular basis of an NO-mediated quorum sensing pathway. The discovery of the fourth QS circuit seems to indicate that the physiological function for NO in *V. harveyi* is as an interkingdom signal. However, we do not rule out the possibility that NO signals an anaerobic environment. This work shows that *V. harveyi* use QS to integrate not only the density of bacteria but also other diverse information about the environment into decisions about gene expression.

The aspect of an interkingdom communication is not an unusual assumption. Eukaryote-to-bacteria cell signaling is an important aspect of the *Vibrio fischeri – Euprymna scolopes* symbiotic relationship. The Hawaiian bobtail-squid, *E. scolopes*, provide nutrients to *V. fischeri* residing in their light organ while *V. fisheri* return the favor by providing light. However, light production happens only when bacterial cells reach high cell density which is not possible if free floating in the ocean. The squid uses the light as counterillumination to

camouflage with the environment and this enables the squid to catch prey and avoid predators. But how do *E. scolopes* acquire *V. fischeri*? It has been shown that NO derived from the squid serves as a cue to signal colonization of juvenile squid by *V. fisheri*. This symbiotic relationship demonstrates that bacteria can respond to signals generated by eukaryotes. In the case of *V. harveyi*, no symbiotic partner has been reported to date. However, a phenomenon called milky sea is an intriguing event where *V. harveyi* associated with a microalgal bloom is proposed to be responsible for the constant light that was observed in a wide area in the northwestern Indian Ocean (Figure 5-1.). The working hypothesis is that algae provide solid substrate for the bacteria to grow. It has also been proposed that the bacteria gain nutrients from living with microalgal blooms. Association with microalgal blooms facilitated the accumulation of AIs which may not be possible if bacteria are free floating in the sea. Moreover, bacteria need to be kept close together in order to sense the AIs. Thus algal-bacterial association aided the bacteria to grow into a high cell density state in order to induce light production. It may not be far-fetched to assume that *V. harveyi* can respond to chemical signals released by algae to signify the presence of an algal bloom. It is also tempting to speculate that signals are being sent for the bacteria to know that a eukaryote is just in the vicinity. However, the benefit that the algae get from this association is still unclear. We can speculate that microalgae use the light from the bacteria to attract prey. The exact nature of this association needs further studies but it will not be surprising if NO is used by microalgae as a signal for *V. harveyi* to inhabit the surface and grow in number.

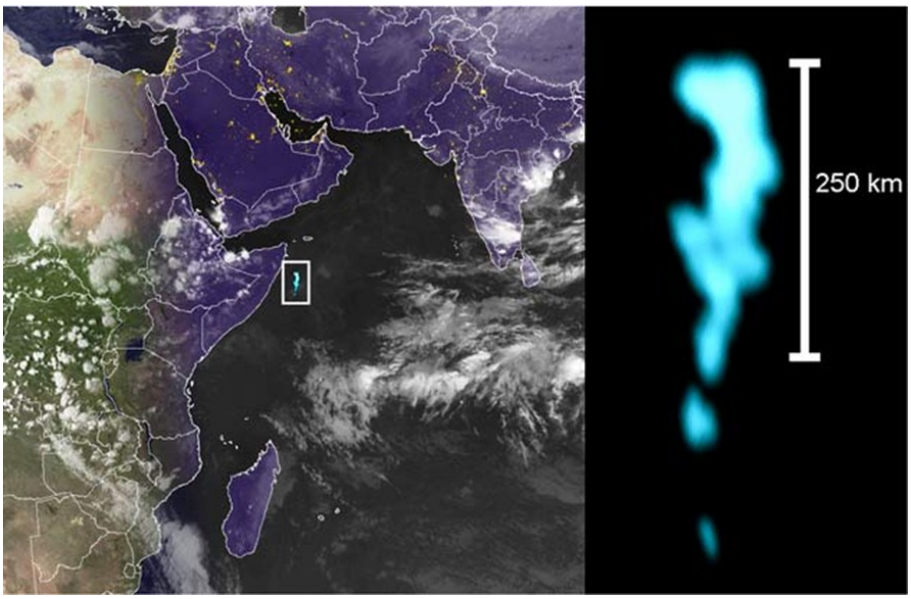

Figure 5-1. A satellite image taken by the U.S. Defense Meteorological Satellite Program (149).

Another possibility for eukaryote-to-*V. harveyi* cell interaction involves pathogenicity. *V. harveyi* are responsible for vibriosis, a shrimp infection that causes the shrimp to glow accompanied by loss of limb function and appendage degradation, is considered to be the leading cause of death in commercially farmed shrimp. This implies that bacteria grow in the gut of the shrimp to a number that enable them to reach a concentration high enough to trigger light production and by extension, proteins and toxins responsible for pathogenesis. QS has been an integral part of a successful host-pathogen interaction but since the existing model identifies only bacterial interaction, it is possible that *V. harveyi* take the host-derived signal, possibly NO, as a cue to start to launch its defense mechanisms. These trigger parallel or sequential schemes that regulate the expression of genes responsible for a variety of proteins in-charge of host invasion. Evading host defenses is a major goal of pathogens thus, timely expression of virulence factors concurrent with cell density is an

important asset to make a concerted attack to overwhelm the host defense system. A better understanding of this phenomenon will help us identify possible pathways to intercept which can be used as a possible drug target.

In general, the ability to coordinate community-wide behavior has obvious advantages to the members of the community. Take for example virulence production; bacteria have to act en masse to overwhelm the host defense system. In the case of bioluminescence, sensing the environment so that together the cells are able ensure that light production, a highly energetic process, is expressed only when necessary and at the same time will be sufficiently high to cause an impact thus making the whole process cost-effective.

Overall, the discovery of bacteria's social nature offers a whole new opportunity to find ways to combat harmful bacteria while keeping the beneficial ones. This opens a number of opportunities for intervention and exploitation of the regulatory networks with a wide variety of applications in medicine, specifically in the field of drug discovery and development. However, the road to finding new drugs is long and entails a better understanding of the complex social network. Thus it is not surprising that more pathways will unravel as interest in the elucidation of cell-cell communication continues to grow.

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APPENDIX 1A. Sequence alignments of VIBHAR_01912 with LuxQ (VIBHAR_05352) and VIBHAR_01913 show that VIBHAR_01912 neither has the conserved phosphorylation sites (in bold red letters) required to act as a histidine kinase nor receives phosphate.

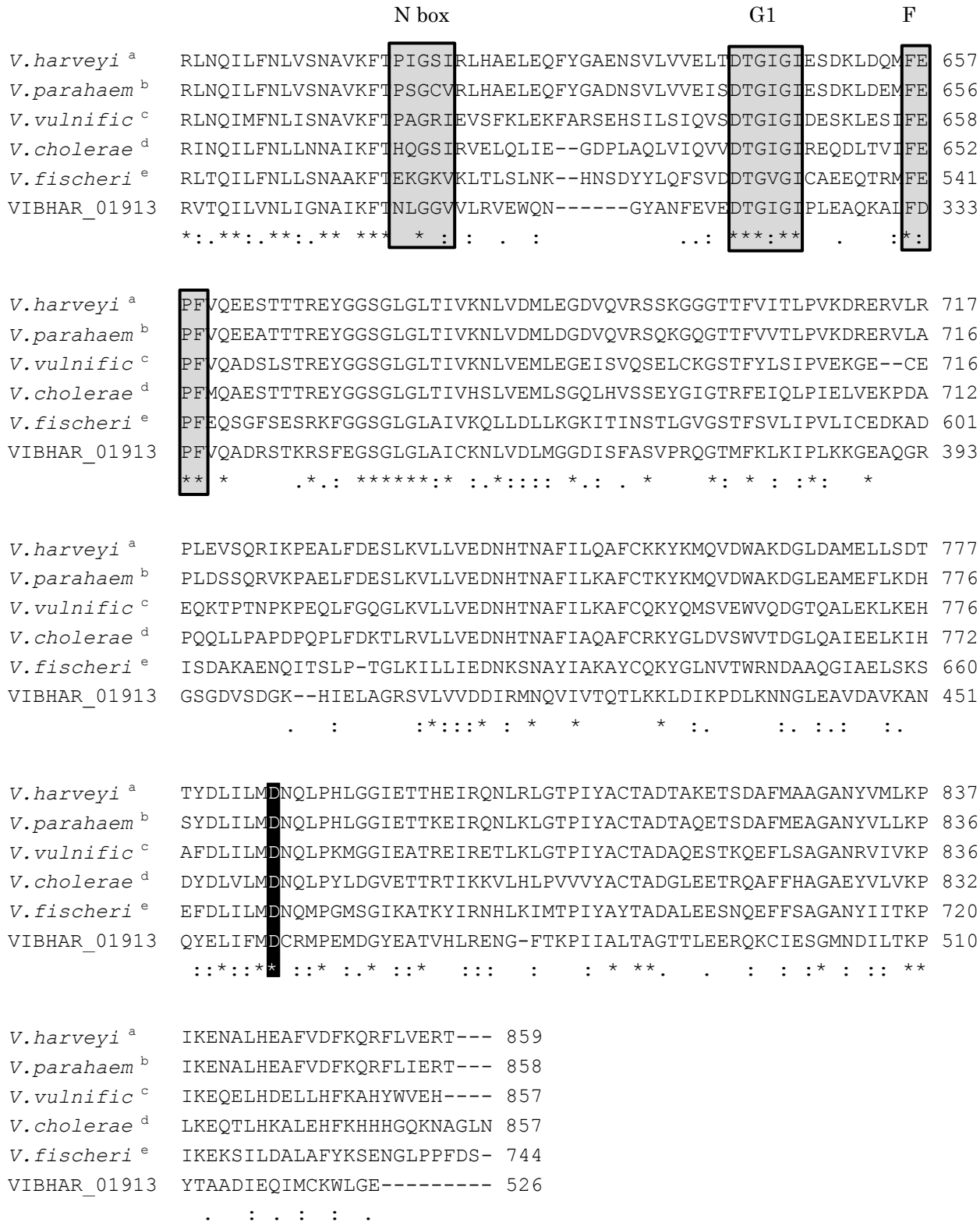
```
VhLuxQ          MTTTRSNIKKRRSLATLITKIIILVLAPIILGIFIQSYYFSKQIIWQEVDRTKQQTSALI 60
VIBHAR_01913    ------------------------------------------------------------
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          HNIFDSHFAAIQIHHDSNSKSEVIRDFYTDRDTDVLNFFFLSIDQSDPSHTPEFRFLTDH 120
VIBHAR_01913    ------------------------------------------------------------
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          KGIIWDDGNAHFYGVNDLILDSLANRVSFSNNWYYINVMTSIGSRHMLVRRVPILDPSTG 180
VIBHAR_01913    ------------MALKKLEMKSQKDLCKFEFEEQIDATLIKFGR--------TFLSSTFG 40
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          EVLGFSFNAVVLDNNFALMEKLKSESNVDNVVLVANSVPLANSLIGDEPYNVADVLQRKS 240
VIBHAR_01913    ESMITSFLEQLTLNSIVLGAYLY--------LVPENVSPLRNHQFGYLQLANNRPIQSKP 92
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          SDKRLDKLLVIETPIVVNAVTTELCLLTVQDNQSVVTLQIQHILAMLASIIGMIMIALMS 300
VIBHAR_01913    HWRKN----CLHLPIIIGSTTFGELIFSLELDQVEQEFIFKQMVLVSDLVHGVISRHLSV 148
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          REWIESKVSAQLESLMSYTRSAREEKGFERFGGSDIEEFDHIGSTLESTFEELEAQKKSF 360
VIBHAR_01913    QREVK------------------------------------------------------- 153
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          RDLFNFALSPIMVWSEESVLIQMNPAARKELVIEDDHEIMHPVFQGFKEKLTPHLKMAAQ 420
VIBHAR_01913    ------------------------------------------------------------
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          GATLTGVNVPIGNKIYRWNLSPIRVDGDISGIIVQGQDITTLIEAEKQSNIARREAEKSA 480
VIBHAR_01913    ----------------------------------------------------LRKRAEASE 162
VIBHAR_01912    ------------------------------------------------------------
```

```
VhLuxQ          QARADFLAKMSHEIRTPINGILGVAQLLKDSVDTQEQKNQIDVLCHSGEHLLAVLNDILD 540
VIBHAR_01913    KATKEFVAMVNHELRTPLNGVLGSADLLDKTLLDDEQQQYLSNLIHSGDLLRVIINDLLD 222
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          FSKIEQGKFNIQKHPFSFTDTMRTLENIYRPICTNKGVELVIENELDPNVEIFTDQVRLN 600
VIBHAR_01913    FSKMNAGMMEIIDKVFAWKDLENALTGVFAAKAAEKRIHFSIDKKLGIPEFLIGDFERVT 282
VIBHAR_01912    ---MTSS-----------SSLEKKLK---REIASRKAAEVLLEQKS-------------- 29
                    :  .           ..  .  *      .:.*   ..::::

VhLuxQ          QILFNLVSNAVKFTPIGSIRLHAELEQFYGAENSVLVVELTDTGIGIESDKLDQMFEPFV 660
VIBHAR_01913    QILVNLIGNAIKFTNLGGVVLRVEWQNGY------ANFEVEDTGIGIPLEAQKALFDPFV 336
VIBHAR_01912    ---FELYSN---------------------------------------------------- 35
                   .:* .*

VhLuxQ          QEESTTTREYGGSGLGLTIVKNLVDMLEGDVQVRSSKGGGTTFVITLPVKDRERVLRPLE 720
VIBHAR_01913    QADRSTKRSFEGSGLGLAICKNLVDLMGGDISFASVPRQGTMFKLKIPLKKGEAQGRGSG 396
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          VSQRIKPEALFDESLKVLLVEDNHTNAFILQAFCKKYKMQVDWAKDGLDAMELLSDTTYD 780
VIBHAR_01913    DVSDGKHIELAGR--SVLVVDDIRMNQVIVTQTLKKLDIKPDLKNNGLEAVDAVKANQYE 454
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ          LILMDNQLPHLGGIETTHEIRQNLRLGTPIYACTADTAKETSDAFMAAGANYVMLKPIKE 840
VIBHAR_01913    LIFMDCRMPEMDGYEATVHLRENG-FTKPIIALTAGTTLEERQKCIESGMNDILTKPYTA 513
VIBHAR_01912    ------------------------------------------------------------

VhLuxQ            NALHEAFVDFKQRFLVERT 859
VIBHAR_01913      ADIEQIMCKWLGE------ 526
VIBHAR_01912      ------------------
```

APPENDIX 1B. Sequence alignment of VIBHAR_01913 with LuxQ from various *Vibrio* species using ClustalW. Phosphorylation sites (highlighted in black) are conserved in the sequences of selected LuxQ proteins, including VIBHAR_01913.

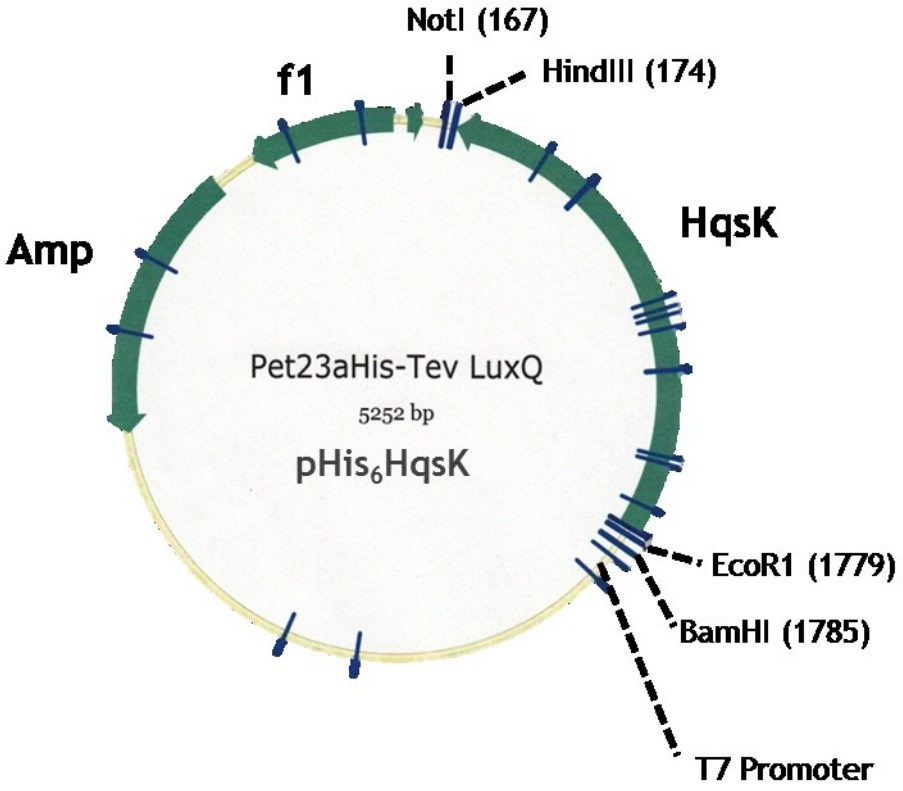
```
V.harveyi [a]    MTTTRSNIKKRRSLATLITKIIILVLAPIILGIFIQSYYFSKQIIWQEVDRTKQQTSALI 60
V.parahaem [b]   MTIT-SKLKKRRSLSTLITKIIILVLAPIILGIFVQSYYFSKQIIWQEVDRTKQQTSALI 59
V.vulnific [c]   -MTNEQLQRKHQSLATLITRIIFLVLGLITIGIFIQSYYFSNKIIKQEVMLTKQQTSALV 59
V.cholerae [d]   MNIRPSQIKHKQRIASFITHAVVVVMGVLIVSVLFQSYQISSRLMAQEGQRTSVQTSSLI 60
V.fischeri [e]   ------MNKGKASLADLIFRSIFIIFSILTVGITIHTYQLSVNTINEEVKRNLQQTSGII 54
VIBHAR_01913     ------------------------------------------------------------

V.harveyi [a]    HNIFDSHFAAIQIHHDSNSKSEVIRDFYTDRDTD-VLNFFFLSIDQSDPSHTPEFRFLTD 119
V.parahaem [b]   LNIFESHFAAIQIHHDSNSKSDVILDFYSERNEE-ALNYFFLSIDQSDPSHTPEFRFLTD 118
V.vulnific [c]   KSLFNNHLSILQIHHDSNSKNEAIRRFFLDGDDE-KLEYYFLSMDQADPTHTPEFRFLTT 118
V.cholerae [d]   QSLFDFRLAALRIHQDSTAKNASLINALVSRDSS-RLDEFFSSVDELELSNAPDLRFISS 119
V.fischeri [e]   NNFFEHRLSILQIRQDTDAESLAIYDQYIESDYKNKLNTYFSNIEAKSPYNSPDFRFIEV 114
VIBHAR_019       ------------------------------------MALKKLEMKSQKDLCKFEFEEQ 22
                                                      : .::   .   .  .:.*

V.harveyi [a]    HKGIIWDDGNAHFYGVNDLILDSLANRVSFSNNWYYINVMTSIGSRHMLVRRVPILDPST 179
V.parahaem [b]   HQGIIWDDGNAHFYGINDSMLDGLTSKVTFSNNWYYVTSITSMGARHLLLRRVPVLEPKT 178
V.vulnific [c]   GEGLLWDDGNAHFYGVNEVLLEKISQSVLFGNNWHFMSLHTLMGLRNMLVRRSPVIDTTT 178
V.cholerae [d]   HDNILWDDGNASFYGIAQQELNKLIRRVAISGNWHLVQTPSEGKSVHILMRRSSLIEAGT 179
V.fischeri [e]   NNKLYWDDGNSSFLSLNENCLKIIIKKNLNINTWYYIKMKEPLSEADFLIRKTPIVNNDS 174
VIBHAR_01913     IDATLIKFGRTFLSSTFGESMITSFLEQLTLN-------------SIVLGAYLYLVPENV 69
                   .    . *.: : .     :          .              .*      ::

V.harveyi [a]    GEVLGFSFNAVVLDNNFALMEKLKSESNVDNVVLVANSVPLANSLIGDEPYNVADVLQRK 239
V.parahaem [b]   GEVMGYSYNAVVLDNNFALMEKLKNEGNVDNVVLVANDIPVASSLAGDESYKIFDVLKRK 238
V.vulnific [c]   GEVLGQYYISVVLDNNFPLVEMLESGSNSDNIVMLVGDKVISHSLSGNEPYDLDSLLAMR 238
V.cholerae [d]   GQVVGYLYVGIVLNDNFALLENIRSGSNSENLVLAVDTTPLVSTLKGNEPYSLDYVVHS- 238
V.fischeri [e]   GRIVAFIYNAVMLNNNISFLNKLKKLTNSQDVFIINDNKILASSVSSNTEIYQEVTSILN 234
VIBHAR_01913     SPLRNHQFGYLQLANNRPIQSKPHWRKNCLHLPIIIGSTTFGELIFS------------- 116
                 . :    :  : * :* .: .  .   *  .: :   .   .   : .

V.harveyi [a]    SSDKRLDKLLVIETPIVVNAVTTELCLLTVQDNQSVVTLQIQHILAMLASIIGMIMIALM 299
V.parahaem [b]   ETQKKLDQLLIIQTPIEVNAAITNLKLLTVQDNQSVVTLQIQHFLAMLASVIGMIMIALM 298
V.vulnific [c]   DEPSAFDDCLISQTPIEINSTDTLVSILAIQENSHVASLQRQHYLGLATSVVLMLMLSLA 298
V.cholerae [d]   AKDAMRDSFIVGQTFLEVESVPTYLCVYSIQTNQNVLTLRDNFYFWMAFALISMIGVSIA 298
V.fischeri [e]   GTRKRIPSKILNFTPINIKSMSGEISILSLKNKDNIELLEQRFIYSVVLSFFVLIAIAVL 294
VIBHAR_01913     --------------------------------LELDQVEQEFIFKQMVLVSDLVHGVIS 143
                                                 :  :. ..       .  ::   :
```

```
                SREWIESKVSAQLESLMSYTRSAREEKGFERFGGSDIEEFDHIGSTLESTFEELEAQKKS 359
V.parahaem [b]  TKEWIENRVVEELGSLMSYTRSAREEKGFERFGGSDIEEFDHIGSTLESTFEELEAQKRS 358
V.vulnific [c]  IRSWIQNRVANALESLMAYSRFAGTGEKYERFNGSDILEFAHIGHTLENTFEQLESQRRS 358
V.cholerae [d]  SRWWLQKRIQREIETLMNYTHKLMDLDTKSEFIGSKIYEFDYFGRTLEQSFRRLANKEKQ 358
V.fischeri [e]  IRGLFEHKILLSLNSLLKYTNTVSKNETRSEYQGCGILEFDQIGEKLEQTFSEL------ 348
VIBHAR_01913    RHLSVQR---------------------------------------------------- 150
                 :  .:

V.harveyi [a]   FRDLFNFALSPIMVWSEESVLIQMNPAARKELVIEDD--HEIMHPVFQGFKEKLTPHLKM 417
V.parahaem [b]  FRDLFNFALSPIMVWSEAGVLIQINPAARKELVIEND--IETMHPVFKGFKDKLVPHLRM 416
V.vulnific [c]  FQDLFNFALSPMMVWSESGLLIQMNPAAMKELGIEHASPQDFSNPLFQLFKLKLSPHLKM 418
V.cholerae [d]  FEDLFNFALSPTMLWNTSGRLIRMNPSAQIQFLREDA----QNHFLFEILERQLLPTITN 414
V.fischeri [e]  ------------------------------------------------------------
VIBHAR_01913    ------------------------------------------------------------

V.harveyi [a]   AAQGATLTGVNVPIGNKIYRWNLSPIRVDGDISGIIVQGQDITTLIEAEKQSNIARREAE 477
V.parahaem [b]  AAQGATLTGVNVPIGDKVFRWNLSPIRVDGDISGIIVQGQDITTLIEAEKQSNLARREAE 476
V.vulnific [c]  AAQGATLTGINVPIGEKIFRWNLSPIVVENGISGIIVQGQDITTLIDAEKQSNLARREAE 478
V.cholerae [d]  AAQGNNPSDVTTEVDGRVYRWNLSPIMVEGQIISIITQGQDITTIAEAEKQSQAARREAE 474
V.fischeri [e]  ---------------------------------------------IEAKEDSYKAQRAAE 363
VIBHAR_01913    -------------------------------------------------EVKLRKRAE 159
                                                                .   ::**

                                H box
V.harveyi [a]   KSAQARADFLAKMSHEIRTPINGILGVAQLLKDSVDTQEQKNQIDVLCHSGEHLLAVLND 537
V.parahaem [b]  KSAQARADFLAKMSHEIRTPINGILGVAQLLKDSVEAEEQKNQIDVLRHSGEHLLAVLND 536
V.vulnific [c]  QSAKTRADFLAKMSHEIRTPLNGILGIAQLLKRSVNDAENLKQVDVLCNSGEHLLAVLND 538
V.cholerae [d]  ESARVRAEFLAKMSHELRTPLNGVLGVSQLLKRTPLNDEQREHVAVLCSSGEHLLAVLND 534
V.fischeri [e]  KLAQVKSDFLARMSHEIRTPLNGILGISSLLKSSNLTREQQKQVNILHQGGEHLLAVLND 423
VIBHAR_01913    ASEKATKEFVAMVNHELRTPLNGVLGSADLLDKTLLDDEQQQYLSNLIHSGDLLRVIIND 219
                    :. :*:* :.***:***:**:** :.**. :   *: : :  *  .*: *  .::**

V.harveyi [a]   ILDFSKIEQGKFNIQKHPFSFTDTMRTLENIYRPICTNKGVELVIENELDPNVEIFTDQV 597
V.parahaem [b]  ILDFSKIEQGKFNIQKHPFSFADTMRTLENIYRPICENKGVELVIENQLDGNVEIFTDQV 596
V.vulnific [c]  ILDFSKIEQGKFNIKKRDFNFYDTLNTLENIYRPICREKGVSFEIHNQIPLDCQLHTDQV 598
V.cholerae [d]  ILDFSRLEQGKFRIQKNEFRLKELVCAIDRIYRPLCNEKGLELVVNSNITTAAIVRSDQI 594
V.fischeri [e]  ILDFSQIEQEKLKINSAPFKLSDVLENINAIYFPLCKNKNVLFSIENHVNPDVFINSDQV 483
VIBHAR_01913    LLDFSKMNAGMMEIIDKVFAWKDLENALTGVFAAKAAEKRIHFSIDKKLGIPEFLIGDFE 279
                :****:::   :.* .  *   :   :  :: . . :* : : :...:    :  *
```



LuxQ sequences from different Vibrio species and the corresponding NCBI gene ID: [a]*V. harveyi* – VIBHAR_05352, [b]*V. parahaemolyticus* – VPA1220, [c]*V. vulnificus* – VV_0752, [d]*V. cholerae* – VC_0736, [e]*V. fischeri* – VF_0708.

APPENDIX 1C. Vector Map for HqsK in pET23aHisTev



Quick Change primers to generate mutants:

$HqsK_{H174A}$

Fwd: GTTTAACGGAGTGCGTAATTCGGCGTTAACCATGGCGACAAATTC

Rev: GAATTTGTCGCCATGGTTAACGCCGAATTACGCACTCCGTTAAAC

$HqsK_{D459A}$

Fwd: GTCCATCTCTGGCATCCGGCAGGCCATAAATATCAGTTCGTACTG

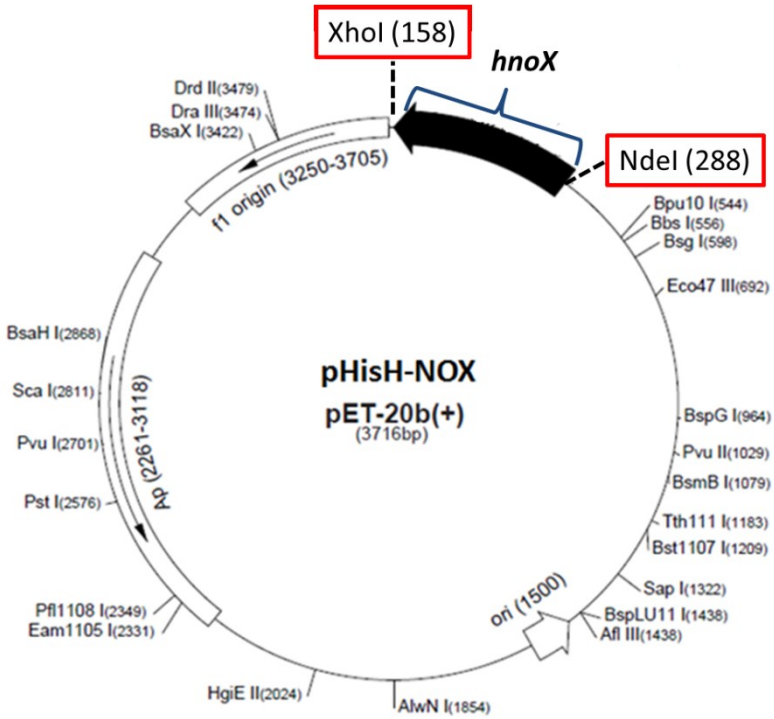
Rev: CAGTACGAACTGATATTTATGGCCTGCCGGATGCCAGAGATGGAC

APPENDIX 2A. Sequence alignment of VIBHAR_01911 with H-NOX from various species using ClustalW.

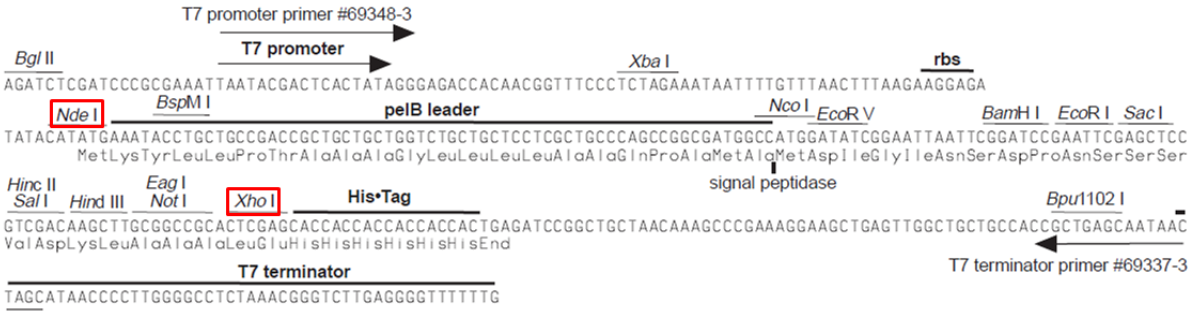
```
HNOX_Vharveyi__1911_      ----------MKGIIFTEFMELVEDKFGLEVLDQVLNLS--NDEGVYTSVGSYDHRDLVK 48
HNOX_Vfischerii           ----------MKGIIFSEFLELVEDKFGLEVCQQMLDEN--NDEGAYTAVGTYDHKHLVK 48
HNOX_Vcholerae            MKTSASEAIQMQGIIYTVLSDMVIEKFGVLFWDQMLEDLKPSSEGVYTSGQQYNDDELLA 60
HNOX_Ttencongenesis       ----------MKGTIVGTWIKTLRDLYGNDVVDESLKSVGWEPDRVITPLEDIDDDEVRR 50
sGC_human_beta            ----------MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYD 50
                                    * * :       :   :*  . ::  .    . :         :. .

HNOX_Vharveyi__1911_      LIVNLSKVSDIPVEKLQEVFGECVFENLLKSIPQSCWHTPNHAGIQQCTNTFQFVRHVEN 108
HNOX_Vfischerii           LIISLSKVTGVSIEDLQQVYGKSVFLTLFQSMP-------ELDG--QALNTFEFIKQVES 99
HNOX_Vcholerae            MVGYLSEKAQIPAPDLVRAYGEYLFTHLFNSLP---------ENYPHKSDLKTFLLSVDK 111
HNOX_Ttencongenesis       IFAKVSEKTGKNVNEIWREVGRQNIKTFSEWFP----------SYFAGRRLVNFLMMMD- 99
sGC_human_beta            LVAAASKVLNLNAGEILQMFGKMFFVFCQESGY-------DTILRVLGSNVREFLQNLD- 102
                          :.   *:       .: .  *.  :    :                        *:  ::

HNOX_Vharveyi__1911_      FIHAEVKKLYPDANPPQFDFISETQSE-IVFDYRSARC-MAHVCLGLIHGCASHFG-ETI 165
HNOX_Vfischerii           YIHLEVKKLYAEANPPRFKFISSTETE-MVMDYISARC-FSHVCFGLILGCAEHFN-EII 156
HNOX_Vcholerae            VIHKEVQRLYPDAYLPQFENR-VEEKT-LTMSYYSKRQ-LCAAAEGLILGAAKQFN-QPV 167
HNOX_Ttencongenesis       EVHLQLTKMIKGATPPRLIAKPVAKDA-IEMEYVSKRK-MYDYFLGLIEGSSKFFK-EEI 156
sGC_human_beta            ALHDHLATIYPGMRAPSFRCTDAEKGKGLILHYYSEREGLQDIVIGIIKTVAQQIHGTEI 162
                           :* .:  :      * :      :   : : * * *  :     *:*   :. :    :

HNOX_Vharveyi__1911_      EVKHQNQSEDGSQVRFWLEAQ---------------------------------------- 186
HNOX_Vfischerii           DIQMTPVLDDGSQVRFTLTKS---------------------------------------- 177
HNOX_Vcholerae            KITQPVCMHCGADHCEIVVEFLPS------------------------------------- 191
HNOX_Ttencongenesis       SVEEVERGEKDGFSRLKVRIKFKNPVFEYKKN---------------------------- 188
sGC_human_beta            DMKVIQQRNEECDHTQFLIEEKESKEEDFYEDLDRFEENGTQESRISPYTFCKAFPFHII 222
                          .:      .       :
```

APPENDIX 2B. Vector Map of *V. harveyi* H-NOX cloned in pET-20b(+)

1. *V. harveyi hnoX* was cloned into pET-20b(+) vector with NdeI and XhoI restriction sites (boxed in red) to give a His6-tagged H-NOX.



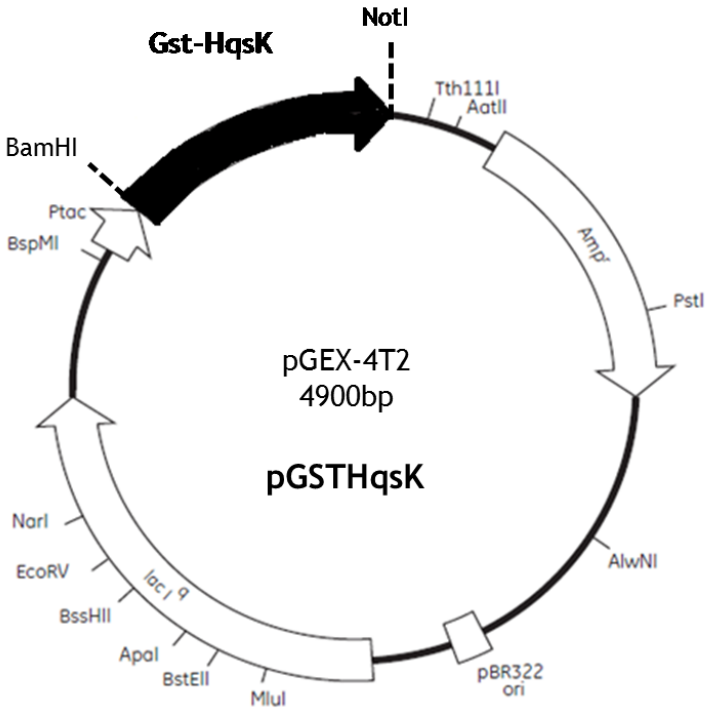
2. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase in the pET-20b(+) vector.



APPENDIX 3A. Vector map of GST-HqsK in pGEX-4T-2

1. *V. harveyi hqsK* was subcloned from pHisHqsK into pGEX-4T-2 with restriction sites at BamHI and NotI to give an N-terminal GST-tagged HqsK

Vector: pGEX-4T2



APPENDIX 4A. Vector map of *ΔhnoX* on pSMV3