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Discovery of a Nitric Oxide Responsive Quorum Sensing Circuit in *Vibrio harveyi*

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Supporting Information

ABSTRACT: Bacteria use small molecules to assess the density and identity of nearby organisms and formulate a response. This process, called quorum sensing (QS), commonly regulates bioluminescence, biofilm formation, and virulence. *Vibrio harveyi* have three described QS circuits. Each involves the synthesis of a molecule that regulates phosphorylation of its cognate receptor kinase. Each receptor exchanges phosphate with a common phosphorelay protein, LuxU, which ultimately regulates bioluminescence. Here, we show that another small molecule, nitric oxide (NO), participates in QS through LuxU. *V. harveyi* display a NO concentration-dependent increase in bioluminescence that is regulated by an *hnoX* gene. We demonstrate that H-NOX is a NO sensor and NO/H-NOX regulates phosphorylation of a kinase that transfers phosphate to LuxU. This study reveals the discovery of a fourth QS pathway in *V. harveyi* and suggests that bacteria use QS to integrate not only the density of bacteria but also other diverse information about their environment into decisions about gene expression.

It is widely accepted that bacteria are social organisms, capable of expressing themselves with the use of chemical signals to signify their presence and gauge their surroundings.[1−4] Quorum sensing (QS) is the regulation of community-wide changes in gene expression, typically regulating biofilm formation, virulence gene production, and bioluminescence, in response to the detection of small molecules called autoinducers (AI). Species-specific QS has intuitive and well-understood advantages for bacteria; 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,[1,2,4] as well as between eukaryotes and bacteria,[1,4,5] has also been well-documented. Cyclic di-GMP signaling pathways, among others, also respond to external signals and affect group activities such as swarming and biofilm formation;[6] it appears that QS reciprocally influences these other pathways, together providing an integrated network for assimilating numerous external stimuli into a community-wide response.[3,7] Furthermore, there is growing evidence that antibiotics serve as signaling molecules at low concentrations, and some AIs appear to function as such.[3]

Thus, the emerging picture is that AIs serve secondary signaling roles when detected by another species (bacterial or eukaryotic) and that molecules other than traditionally defined AIs integrate into QS circuits. Such studies have raised fundamental questions about QS, including whether QS is the appropriate term for this type of cell−cell communication.

Nitric oxide (NO), a potent antibiotic at high concentrations, is a bacterial signaling molecule at nanomolar concentrations. NO produced by the squid *Euprymna scolopes* is detected by *Vibrio fischeri* as a cue for colonization of *E. scolopes*.[8] Low concentrations of NO have been implicated in biofilm dispersal and other community behaviors in many bacterial species,[9] but the molecular mechanisms underlying these behaviors are not understood. We hypothesize that since NO affects bacterial community activities, it may participate in QS directly.

To investigate this possibility, we measured the effect of NO on bioluminescence in *Vibrio harveyi*. This is a good model system because although QS regulates the transcription of at least 50 promoters,[10] light production is a quantitative and direct readout of QS. *V. harveyi* have three described QS pathways (Figure 1a; reviewed in ref 11). These pathways are LuxM/LuxN, LuxS/LuxPQ, and CqsA/CqsS, each named for the AI synthase/receptor pair. LuxM synthesizes HAI-1, an acyl homoserine lactone intraspecies signal recognized by LuxN. LuxS synthesizes AI-2, a furanosyl borate diester, an interspecies AI; in *V. harveyi* it is sensed by LuxP, a periplasmic receptor associated with LuxQ. CqsA synthesizes CAI-1, a genus-specific AI recognized by CqsS. In the absence of its cognate AI, the cytosolic subunit of each receptor is autophosphorylated and transfers phosphate to the common phosphorelay protein LuxU. Phosphorylated LuxU transfers phosphate to LuxO, a transcription factor that when phosphorylated indirectly represses the master regulator of QS, LuxR, through the activation of Quorum Regulatory RNAs (*qrr*s). Because transcription of LuxR is repressed, at low cell

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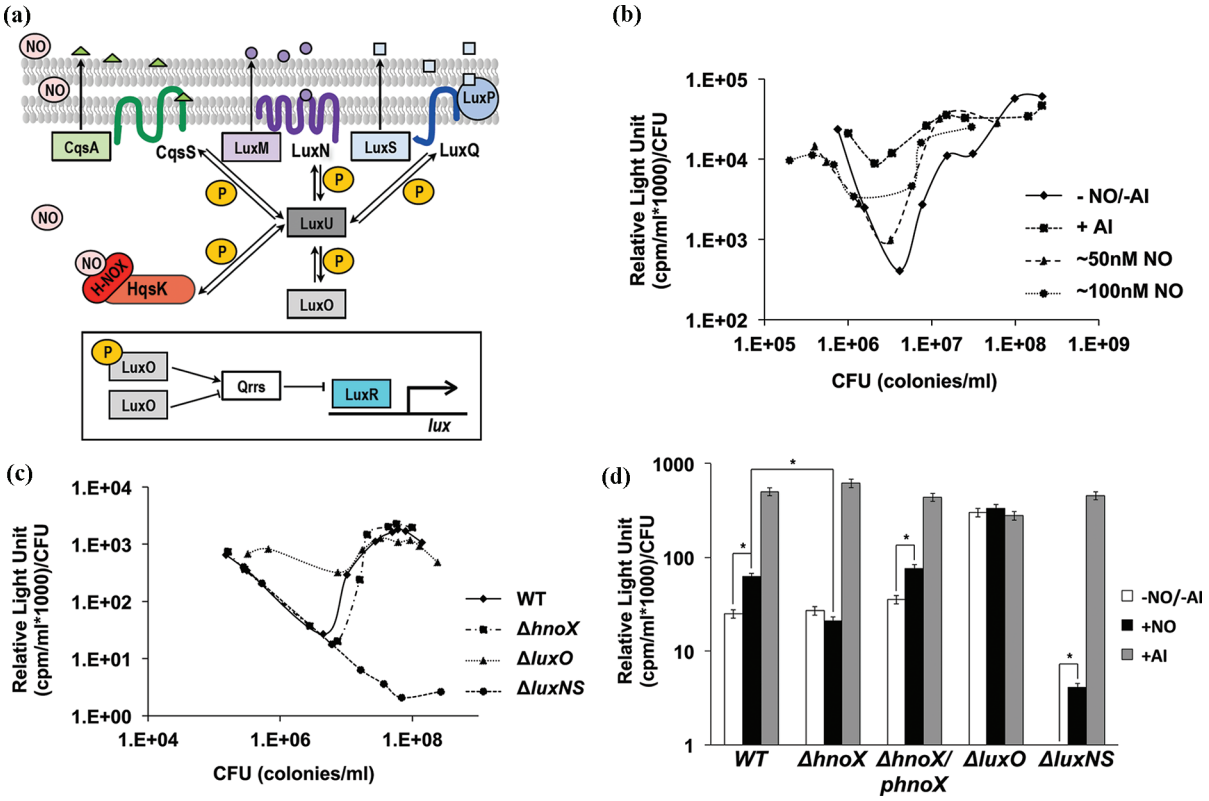

Figure 1. H-NOX is a NO sensor involved in QS in *V. harveyi*. (a) Schematic illustration of *V. harveyi* QS. P represents phosphate; triangles represent CAI-1; circles represent HAI-1; squares represent AI-2. Here, we demonstrate that *V. harveyi* have a fourth quorum sensing pathway responsive to NO. (b) NO causes an earlier onset of light production. Relative light units (RLU) as a function of CFU in the presence of varying NO or WT cell-free supernatant (AIs). (c) Deletion of H-NOX delays light production. RLU as a function of CFU for WT, Δ*hnoX*, Δ*luxO*, Δ*luxNS*. (d) NO/H-NOX contributes to regulation of light production. RLU at a fixed cell density for WT, Δ*hnoX*, Δ*hnoX*/p*hnoX*, Δ*luxO*, and Δ*luxNS* in the presence of no additives, ~50 nM NO, or AIs. Error bars represent one standard deviation from the mean of triplicate experiments (* = $p < 0.001$).

density (low AI) *V. harveyi* are not bioluminescent. At high cell density, however, AI concentrations are elevated, resulting in receptor binding and a switch in receptor function from kinase to phosphatase. The resulting dephosphorylation of LuxO allows translation of LuxR, expression of the *lux* operon, and production of light.

V. harveyi bioluminescence was monitored in the presence of NO. Figure 1b (solid line) illustrates a typical QS bioluminescence curve; upon dilution of a dense, bright culture, bioluminescence decreases until ~5×10^6 cells mL^{-1}, at which point sufficient AIs have accumulated to commence light production. The presence of nanomolar NO (for details see Supplementary Figures 1 and 2) results in advanced light production relative to the absence of NO. This increase in light production at lower cell density is NO dose-dependent and suggests that NO acts analogously to an AI. Earlier bioluminescence implies a decrease in the net phosphate flux from LuxU to LuxO, consistent with decreased kinase activity and/or increased phosphatase activity of a protein capable of exchanging phosphate with LuxU (*i.e.*, a QS kinase). Thus, we reasoned that *V. harveyi* must code for a NO-specific QS kinase.

Upon completion of the *V. harveyi* BB120 genome, we discovered a histidine kinase (VIBHAR_01913) homologous to the cytosolic domain of the QS receptor LuxQ (Supplementary Figure 3). Unlike other QS receptors,[11] VIBHAR_01913 is soluble; however, NO is membrane-permeable and does not require an extracellular sensor. Like LuxQ, VIBHAR_01913 lacks a sensory domain in its primary sequence (AI-2 binds LuxP, LuxP binds LuxQ). Interestingly, the gene coding for VIBHAR_01913 is in the same operon as a gene (VIBHAR_01911) coding for a H-NOX (heme-nitric oxide/oxygen) protein (Supplementary Figure 4). H-NOX proteins are NO sensors discovered by genetic homology with the eukaryotic NO sensor soluble guanylate cyclase.[12] With the discovery of H-NOX, fundamental questions about NO signaling in bacteria are being considered.[13−16] Although H-NOX function is not fully elucidated, regulation of community activities is a strong theme in studies to date. *V. fischeri* H-NOX is involved in sensing the light organ of *E. scolopes* (*i.e.*, early stage symbiosis).[14] In *Shewanella woodyi*[15] and *Legionella pneumophila*[13] H-NOX regulates biofilm formation.

We named VIBHAR_01913 HqsK (H-NOX-associated quorum sensing kinase) and hypothesized that NO/H-NOX regulation of HqsK kinase activity could explain the effect of NO on *V. harveyi* bioluminescence (Figure 1b). In order to test this hypothesis we constructed an in-frame deletion mutant in *hnoX*. In the absence of NO, Δ*hnoX* exhibits delayed bioluminescence compared with wild-type (WT) *V. harveyi*, while control strains (Δ*luxO*, constitutively bright; Δ*luxNS*, dim) display the expected trends (Figure 1c). The effect of NO is most apparent at low cell density, and thus bioluminescence in the presence of ~50 nM NO was monitored at a cell density of ~5×10^6 cells mL^{-1} (where bioluminescence begins). The addition of NO causes a significant increase in WT light production ($p < 0.001$) and no change in Δ*hnoX* bio-

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luminescence (Figure 1d). Plasmid complementation of *hnoX* in the Δ*hnoX* strain restores a WT response. NO causes an increase in Δ*luxNS* bioluminescence, as expected if NO/H-NOX/HqsK operates parallel to LuxN/LuxQ/CqsS and upstream of LuxO. Indeed, NO does not affect Δ*luxO*, indicating NO enters QS upstream of LuxO.

A reasonable mechanism underlying these data is that NO is sensed by H-NOX, NO/H-NOX regulates HqsK activity (like other AI/receptor pairs), and HqsK enters QS circuits by phosphotransfer to LuxU (Figure 1a). To support this hypothesis, we cloned, expressed, and purified H-NOX, HqsK, and LuxU and tested their proposed functions *in vitro*.

Purified H-NOX binds NO without measurable affinity for O_2 (Figure 2a). We measured a dissociation rate constant

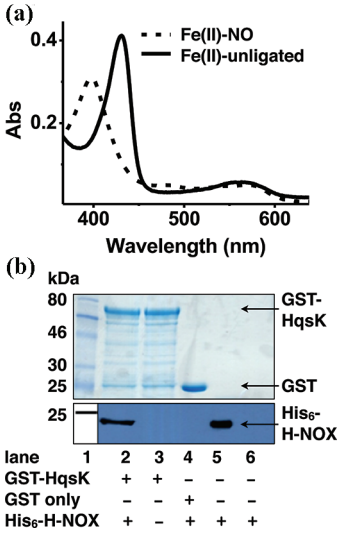

Figure 2. H-NOX binds NO and directly interacts with HqsK. (a) Electronic absorption spectra of H-NOX. H-NOX is purified as the Fe^{2+}-unligated complex (Soret band maximum at 429 nm) and binds NO (Fe^{2+}-NO complex Soret maximum at 399 nm). (b) GST-HqsK bound to glutathione beads, but not GST-glutathione beads only, precipitates His_6-H-NOX from *E. coli* cell lysates as analyzed by SDS-PAGE. Top: protein loading detected by Coomassie 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.

$[k_{off(NO)}]$ of 4.6×10^{-4} s^{-1} for the Fe^{2+}-NO complex (Supplementary Figure 5). The $k_{on(NO)}$ for H-NOX domains is predicted to be ~10^8 M^{-1} s^{-1},[17] indicating a picomolar $K_{D(NO)}$ (Supplementary Table 1). Furthermore, His_6-H-NOX is precipitated from *E. coli* lysate using GST-HqsK-glutathione beads, but not GST-glutathione beads only (Figure 2b). Therefore, H-NOX has the ligand binding properties of an NO sensor and directly interacts with HqsK, consistent with its proposed role in QS.

Purified HqsK exhibits time-dependent, stable autokinase activity (Figure 3a), in contrast to short-lived autokinase activity observed with LuxN.[18] HqsK has two predicted phosphorylation sites, H174 in the transmitter domain and D459 in the receiver domain (Supplementary Figure 3). Upon mutation (Supplementary Figure 6), we found that $HqsK_{H174A}$ has no autokinase activity, whereas $HqsK_{D459A}$ achieves higher

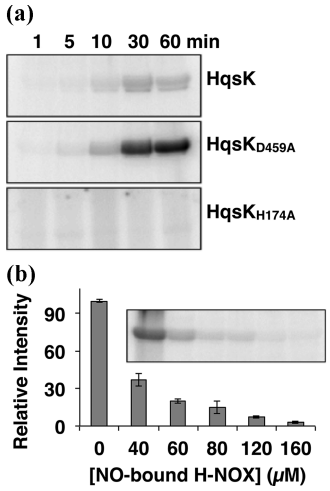

Figure 3. HqsK is a histidine kinase, and H-NOX regulates its activity. (a) *In vitro* autophosphorylation of HqsK with ATP over time. Radiolabeled phosphoproteins were detected by SDS-PAGE and autoradiography. $HqsK_{H174A}$ has no activity, indicating H174 is the phosphorylation site. $HqsK_{D459A}$ accumulates phosphate, presumably because P-His_{174} is more stable to hydrolysis than P-Asp_{459}. (b) NO-bound H-NOX dose-dependent inhibition of HqsK. Intensity of phosphorylated HqsK in the presence of varying NO-bound H-NOX is plotted relative to the intensity in the absence of NO-bound H-NOX. Error bars represent one standard deviation from the mean of triplicate experiments.

levels of phosphorylation than HqsK. The lack of kinase activity in $HqsK_{H174A}$ cannot be attributed to differences in concentration or stability; it is interpreted as the loss of the kinase phosphorylation site. The increased phosphorylation of $HqsK_{D459A}$ is likely due to the higher stability of the phosphoramidate bond (P-His_{174}), relative to the mixed acid ester (P-Asp_{459}), under basic conditions;[19] however, we do not rule out the possibility that $HqsK_{D459A}$ lacks autophosphatase activity. Like the other QS receptors, HqsK has phosphatase activity; in assays using the generic substrate 3-*O*-methylfluorescein, HqsK and $HqsK_{H174A}$ displayed phosphatase activity, whereas $HqsK_{D459A}$ did not (Supplementary Figure 7).

The effect of NO/H-NOX on HqsK activity was determined with $HqsK_{D459A}$ because it yields a more stable product and isolates kinase activity from phosphotransfer/phosphatase activity. NO-bound H-NOX inhibits HqsK activity in a dose-dependent manner (Figure 3b) and H-NOX inhibition of HqsK kinase activity is specific to the NO-bound form (Supplementary Figure 8). Likewise, AI binding inhibits phosphorylation of other QS kinases,[11] and NO/H-NOX inhibits autophosphorylation of an associated kinase in *S. oneidensis*.[20]

Finally, we determined that HqsK participates in QS *via* phosphotransfer to LuxU. Phosphate is transferred from HqsK to LuxU in a time-dependent manner (Figure 4a). We observed a stronger signal for phosphorylated LuxU at the end of the time course than for phosphorylated HqsK at the beginning of the reaction. This cannot be attributed to differences in protein concentration. Rather, as there is excess ATP in solution, HqsK continually autophosphorylates and transfers phosphate to LuxU (phosphate flux is toward LuxU), where it accumulates due to the relative stability of LuxU P-His_{58} compared with HqsK P-Asp_{459}. The LuxU phosphorylation site[21] mutant ($LuxU_{H58R}$) lacks phosphotransfer ability, as does $HqsK_{H174A}$

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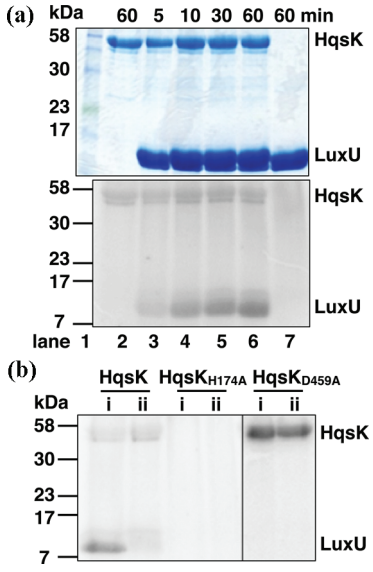

Figure 4. HqsK exchanges phosphate with LuxU. (a) Phosphotransfer from HqsK to LuxU monitored over time and analyzed by SDS-PAGE. Top: protein loading detected by Coomassie staining (His_6-HqsK is ~60 kDa; His_6-LuxU is ~13.5 kDa). Bottom: detection of radiolabeled phosphoproteins by autoradiography. Lane 1, molecular weight marker. Lanes 2–7, the following proteins were incubated with 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) $LuxU_{H58R}$. Phosphotransfer is only observed in the far left lane with wild-type HqsK and LuxU.

and $HqsK_{D459A}$ (Figure 4b). Altogether these results indicate that phosphorelay between HqsK and LuxU takes place as predicted ($HqsK_{H174}$ to $HqsK_{D459}$ to $LuxU_{H58}$), in accordance with the other *V. harveyi* QS receptors.

In summary, HqsK has kinase (Figure 3) and phosphatase (Supplementary Figure 7) activities and can transfer phosphate to LuxU (Figure 4). H-NOX is an NO sensor (Figure 1d, Figure 2) that inhibits HqsK kinase activity (Figure 3b). These biochemical data are consistent with the bioluminescence and genetic data (Figure 1). *V. harveyi* increase their light production in the presence of NO (Figure 1a,d) because HqsK kinase activity is inhibited, contributing to phosphate flow away from LuxU/LuxO and derepression of LuxR. In the absence of NO (Figure 1b) or the absence of H-NOX (Δ*hnoX*; Figure 1c), HqsK acts as a kinase, funneling phosphate toward LuxU, increasing the population of P-LuxO and contributing to a decrease in light production.

Multiple QS circuits with coincidence detectors enable bacteria to respond with high fidelity as fluctuations of signals occur.[11] Here we describe the fourth *V. harveyi* QS circuit. We show that NO enters QS circuits analogously to an AI: it binds its cognate receptor (H-NOX/HqsK) and contributes to the flux of phosphate to and from LuxO. It is likely that *V. harveyi* monitors NO as an environmental cue. The most likely sources of NO are incomplete reduction of NO during anaerobic respiration using nitrate/nitrite[22] and NO produced enzymatically from arginine using NO synthases (NOS).[23] We do not exclude the possibility that NO signals an anaerobic environment.[24] However, we note that NO more acutely affects QS at low cell density, while anaerobic respiration is more likely in dense cultures. Some Gram-positive bacteria code for NOS,[23] and arginine is known to induce luminescence in *V. harveyi*,[25] but there are no predicted NOS genes in the *V. harveyi* genome.

Therefore, we propose that NO is an interkingdom signaling molecule used for eukaryote-to-bacterium communication. In general, bacteria may use NO to detect the presence of eukaryotes during symbiosis or the early stages of pathogenesis (before iNOS produces millimolar NO, obviating need for a picomolar NO sensor). This hypothesis is consistent with the role of NO in establishing symbiosis between *V. fischeri* and *E. scolopes*.[8,14] This hypothesis is also consistent with the lifestyle of *V. harveyi*. *V harveyi* live free-swimming in seawater, adhered to abiotic surfaces, as a constituent of mixed species biofilms in marine animals, and in pathogenic association with marine fish and shrimp.[25]

METHODS

Bacterial Strains and Growth Conditions. See Supplementary Table 2. *V. harveyi* strains WT (BB120), Δ*luxO* (BB721), and Δ*luxNS* (MM30) were purchased from ATCC, maintained in Marine Media (MM; 28 g L^{-1}; 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].[26] *E. coli* strains XL1 and BL21(DE3)-pLysS were used for plasmid amplification and protein purification, respectively. *E. coli* were grown in Luria Broth (LB; 20 g L^{-1}) at 37 °C with agitation at 250 rpm. *E. coli* strain WM3064 was used as a donor for conjugation and was grown in LB with 2,3-diaminopropionic acid (DAP; 0.36 mM) at 37 °C with agitation at 250 rpm.

Construction of Δ*hnoX*. See Supplementary Table 2. PCR was used to amplify *hnoX* (VIBHAR_01911) and 1 kbp regions both up- and downstream of *hnoX* from *V. harveyi* genomic DNA (Pfusion polymerase; NEB). Upstream and downstream primers (koH-NOX-fwd, koH-NOX-rev) contained ApaI and SpeI restriction sites, respectively. The amplified PCR product was cloned into pSMV3[27] (gift from Jeffrey Gralnick). This plasmid was modified to yield pΔ*hnoX* by introducing two *Bam*HI restriction sites (siteA, near the *hnoX* start codon; siteB, near the *hnoX* stop codon) through site-directed mutagenesis (koH-NOX-siteA-fwd, koH-NOX-siteA-rev; koH-NOX-siteB-fwd, koH-NOX-siteB-rev), digestion with *Bam*HI, and ligation with T4 DNA ligase. In pΔ*hnoX*, *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 the *Bam*HI site. pΔ*hnoX* was transformed into *E. coli* WM3064[27] and grown on LM/DAP plates [Luria Marine; 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 20 g L^{-1} NaCl, 15 g L^{-1} Agar] with kanamycin (kan; 100 μg mL^{-1}). WM3064/pΔ*hnoX* was mated with *V. harveyi* in a 1:5 ratio on LM/DAP plates overnight at ambient temperature. *V. harveyi*/pΔ*hnoX* transconjugants were selected on LM/kan plates and verified by colony PCR. Selected colonies were plated on LM plates with 5% sucrose at ambient temperature to select for double recombination events. Colonies were replica plated onto LM/kan and incubated overnight at ambient temperature; kanamycin sensitive colonies were screened by colony PCR for gene deletion.

Construction of p*hnoX*. PCR was used to amplify *hnoX* plus 32 upstream bp from *V. harveyi* genomic DNA. Upstream and downstream primers contained *Eco*RI and *Bam*HI restriction sites, respectively (*hnoX*comp-fwd, *hnoX*comp-rev). The PCR product was cloned into pBBR1MCS-2[28] (gift from Jeffrey Gralnick) and sequenced. The resulting plasmid, p*hnoX*, was introduced into Δ*hnoX* *via* conjugation as previously described.[15]

Bioluminescence Assays. Bioluminescence assays were carried out as described[26] with modifications. Overnight cultures of *V. harveyi* were diluted 2000-fold into fresh AB medium supplemented with 0, 50, or 100 μM DPTA NONOate to yield 0 nM, 80–40 nM, or 300–100 nM NO, respectively. See Supplementary Figures 1– and 2 for detailed NO methods. Cultures were grown at 30 °C with agitation at 250 rpm. The luminescence of 100 μL aliquots were measured in

triplicate (Perkin-Elmer VictorX5 microplate reader using black 96-well Microfluor 1 plates) periodically for 10−12 h. Cell density was expressed as colony forming units (CFU) determined by diluting the same cultures into fresh AB medium and plating on LM. Plates were incubated overnight at 30 °C and then colonies were counted. Light intensity is reported as relative light units (RLU = counts min^{-1} mL^{-1} × 10^3 per CFU mL^{-1}). Cell free supernatant (+AI samples) was prepared as described.[26] For single time-point experiments, cultures were grown overnight at 30 °C and diluted 5000-fold into fresh AB medium containing 50 μM DPTA NONOate prepared to result in ∼50 nM NO at the time of light assessment, at ∼5 × 10^6 cells mL^{-1}. Light intensity and CFU were determined as described above. For full bioluminescence assays, at least three independent trials were performed and representative experiments are shown. For single time-point assays, error bars represent one standard deviation from the mean of triplicate experiments. *t* tests were used to determine two-tailed *p*-values.

Protein Cloning, Expression, and Purification. See Supplementary Table 2. VIBHAR_01911 (*hnoX*), VIBHAR_01913 (*hqsK*), and VIBHAR_02958 (*luxU*) were amplified from *V. harveyi* genomic DNA (Pfu Turbo polymerase) and cloned into pET vectors to yield C-terminal His_6-tagged H-NOX in pET20b and N-terminal His_6-tagged HqsK or LuxU in pET-23a-HisTEV. HqsK and LuxU mutants were generated using QuikChange mutagenesis (Stratagene). The mutated vectors were confirmed by sequencing and MS/MS mass spectrometry. H-NOX expression has been described.[29] HqsK or LuxU transformed BL21(DE3)pLysS were grown in 2X YT (16 g L^{-1} Tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl) with 100 μg mL^{-1} ampicillin, and expression was induced with 100 μM IPTG for 8 h at 18 °C. Purification of His_6-tagged proteins was achieved using Ni^{2+}-NTA affinity chromotography. Protein concentration was determined by bicinchoninic acid assay using BSA as standard.

Electronic Spectroscopy. All electronic spectra were recorded on a Cary 100 spectrophotometer equipped with a constant temperature bath. H-NOX sample preparation and NO dissociation rates have has been described.[29]

Pull-down Assays. Glutathione *S*-transferase (GST) tagged-HqsK was prepared by subcloning HqsK from pHisHqsK into pGEX-4T2 using *Bam*HI and NotI (Supplementary Table 2). GST-HqsK was purified from BL21(DE3)pLysS (500 μM IPTG induction overnight at 18 °C) using glutathione sepharose beads. Captured GST-HqsK was washed with buffer (50 mM Tris/HCl pH 8, 500 mM NaCl, 1 mM PMSF, 2 mM DTT, 0.5% Triton X-100). His_6-H-NOX lysate (200 μL from overexpression in BL21(DE3)pLysS) was incubated with the beads (final volume 1 mL) in buffer for 2 h at 4 °C with gentle rocking. The beads were then washed 3 times with buffer and boiled in 50 μL of SDS loading dye for 3 min. Ten microliters of this was loaded onto a 12.5%T Tris-glycine gel for Western Blot analysis with polyclonal anti-His_6 antibody (Abcam) to detect His_6-H-NOX.

Autophosphorylation Assays. HqsK (15 μM) was incubated with ATP mix (500 μM $MgCl_2$, 1 mM ATP, 10 μCi [γ-^{32}P]ATP) at RT in phosphorylation buffer (50 mM Tris/HCl, 500 mM KCl, 10% (v/v) glycerol, 2 mM DTT). Reactions were terminated at the indicated times by boiling in SDS loading dye for 3 min followed by separation on a 12.5%T Tris-glycine SDS-PAGE. Dried gels were exposed to a phosphoscreen overnight and scanned using a Phosphoimager (Amersham Biosciences). Band intensities were quantified using ImageJ. Protein loading was determined by Coomassie staining. For kinase inhibition assays, $HqsK_{D45A}$ (11 μM) was incubated with varying amounts of NO-bound H-NOX in phosphorylation buffer with ATP mix for 30 min.

Phosphotransfer Assays. HqsK (10 μM) in phosphorylation buffer was preincubated with ATP mix for 1 min prior to the addition of LuxU (100 μM). Phosphotransfer reactions were quenched with SDS loading dye at the indicated time points. Single time point assays with HqsK and LuxU point mutants proceeded for 30 min. Phosphorylated proteins were separated on a 15%T Tris-glycine SDS-PAGE gel and detected by autoradiography.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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