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Nitric oxide regulated two-component signaling in *Pseudoalteromonas atlantica*

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ABSTRACT

Bacteria employ two-component signaling to detect and respond to environmental stimuli. In essence, two-component signaling relies on a protein called a response regulator that can elicit a change in gene expression or protein function in response to phosphoryl transfer from a histidine kinase. Phosphorylation of the associated histidine kinase is regulated by detection of an environmental signal, thus linking sensing to cellular response. Recently, it has been suggested that H-NOX (Heme-nitric oxide/oxygen binding) proteins may act as nitric oxide (NO) sensors in two-component signaling systems. NO/H-NOX regulated histidine kinases have been reported, but their cognate response regulators have yet to be identified. In this work we provide biochemical characterization of a complete NO/H-NOX-regulated two-component signaling pathway in the biofilm-dwelling marine bacterium, *Pseudoalteromonas atlantica*. In *P. atlantica*, as is typical for bacteria that code for H-NOX, an *hnoX* gene is found in the same operon as a gene coding for a two-component signaling histidine kinase (H-NOX-associated histidine kinase; HahK). We find that HahK is capable of autophosphorylation *in vitro* and that NO-bound H-NOX inhibits HahK activity, implicating H-NOX as a selective NO sensor. The cognate response regulator, a protein annotated as a cyclic-di-GMP processing enzyme that we have named HarR (H-NOX-associated response regulator), was identified using bioinformatics tools. Phosphoryl transfer from HahK to HarR has been established. This report reveals the first biochemical characterization of an H-NOX-associated response regulator and contributes to a deeper understanding of NO/H-NOX signaling in bacteria.

1. Introduction

Adaptive response via signaling is essential for the survival of all organisms. Two-component signal transduction (TCS) is the predominant mechanism for stimulus response in bacteria [1]. As the name suggests, the canonical TCS pathway consists of two protein components – a sensor histidine kinase and a response regulator protein. The variable sensor domain of the histidine kinase protein is responsible for detecting an external stimulus in the environment of the organism. Signal detection results in a change in the autophosphorylation state of the kinase. The signal is relayed downstream by means of phosphoryl transfer from a histidine residue in the receptor kinase to an aspartic acid residue in the receiver domain of the response regulator, a process catalyzed by the latter. The activated response regulator, in turn, elicits the appropriate response to the stimulus. The duration of the response is often regulated through autophosphatase activity of the response regulator or through phosphatase activity of the kinase towards its cognate response regulator [2,3]. This simple, rapid, and reversible form of signaling is widespread in bacteria and is involved in diverse processes such as chemotaxis, light perception, osmoregulation, and quorum sensing [4–7].

Many TCS systems deviate from the simple system (two proteins, one phosphoryl transfer step) described above, however. For example, multiprotein, multistep phosphorelay events between signal detection and cellular response are common. In some systems, the sensor domain of histidine kinases is replaced by an accessory protein, which directly detects the signal and conveys it to the kinase via a protein–protein interaction [8,9]. These modified TCS systems are sometimes referred to as three-component signaling systems [10].

Heme-nitric oxide/oxygen binding (H-NOX) proteins are a recently discovered family of proteins that are homologous to the nitric oxide (NO) sensing domain of the well-characterized eukaryotic NO sensor soluble guanylate cyclase (sGC) [11]. Bacterial genome-wide analyses predict *hnoX* to be present in a variety of system architectures, usually found upstream of genes coding for signaling proteins. The most common arrangement is for *hnoX* to be found in the same putative operon as a two-component signaling kinase (H-NOX-associated histidine kinase; HahK), usually without a response regulator in the same operon. It has been shown in *Shewanella oneidensis* MR-1 that H-NOX has an inhibitory effect on the autophosphorylation activity of HahK, but the downstream players in the phosphorelay pathway remain unknown [12].

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In this paper we report the discovery of an H-NOX regulated response regulator (HarR; H-NOX-associated response regulator) in the marine bacterium *Pseudoalteromonas atlantica*. The full *in vitro* characterization of this three-component signaling pathway is described. NO/H-NOX regulation of the autophosphorylation activity of a HahK, as well as phosphoryl transfer from HahK to HarR is described.

2. Materials and methods

2.1. Materials

All reagents were purchased in their highest available purity and used as received.

2.2. Bacterial strains and growth conditions

The *P. atlantica* strain used in this study was wild-type T6c (ATCC #BAA-1087). *P. atlantica* T6c was grown in filtered Marine Broth (Difco, 28 g/L) at room temperature. *Escherichia coli* strains XL1-blue or DH5a were used for cloning and BL21(DE3)pLysS or Tuner(DE3)pLysS were used for protein expression.

2.3. Protein cloning, expression, and purification

H-NOX (PATL1532) was amplified from *P. atlantica* T6c genomic DNA (primers: 5′-GGAATTCCATATGAAAGGCATTATTTTT-3′, 5′-CCGCTCGAGTTAGCCAACTTTGGTAACC-3′) and cloned into pET20b (Novagen) with Nde1 and Xho1. The stop codon was removed by site directed mutagenesis to glycine to allow for a C-terminal hexa-histidine tag. The site-directed mutagenesis took place following Stratagene's QuikChange protocol (primers: 5′-GTTACCAA AGTTGGCGGACTCGAGCACCACCAC-3′, 5′-GTGGTGGTGCTCGAGTCC GCCAACTTTGGTAAC-3′). HahK (PATL1533) was amplified (primers: 5′-CCAAGGGGTACCATGGCTAAATCGAACG-3′, 5′-ATAAGAATG CGGCCGCTTACTGAGGTAG-3′) and cloned into pET23-His-Tev with Kpn1 and Not1. HarR (PATL1534) was amplified (primers: 5′-ATTA-CGAATTCATGAACACGTTATCAGAAAAC-3′, 5′-GGTAACTCGAGTTAG TTTTCCTCTACCTCAG-3′) and cloned into pET23-His-Tev with EcoR1 and Xho1. HahK and HarR were cloned with N-terminal hexa-histidine tags. Each construct was propagated in *E. coli* XL1-blue cells at 37 °C in Luria–Bertani broth (RPI) overnight and purified using a Zyppy™ Plasmid Miniprep Kit (Zymo Research). Purified plasmids were sequenced (Stony Brook University Sequencing).

Mutations in HahK and HarR were carried out using Stratagene QuikChange. HahK H257A primers were 5′-GCCGCTGGCGTTGC CGCTGAAATTAATAACCC-3′ and 5′-GGGTTATTAATTTCAGCGGCAA CGAAAGCGGC-3′. HarR D62A primers were 5′-CAGGTCATCGT TTCAGCTATGAGAATGCCAAAT-3′ and 5′-ATTTGGCATTCTCATAGCT GAAACGATGACCTG-3′. Successful mutations were verified by sequencing.

Cell culture procedures for H-NOX were carried out as previously described [13]. Cell culture procedures for HahK and HarR were the same with these exceptions: BL21(DE3)pLysS in 2XYT medium (16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl) were used and expression was induced with 200 μM IPTG. Purification of each protein took advantage of C-terminal or N-terminal hexa-histidine tag. Proteins were purified by metal affinity chromatography (Ni–NTA) followed by gel filtration.

2.4. Electronic spectroscopy and NO dissociation kinetics

Electronic spectra were measured using a Varian Cary 100 Bio UV–vis spectrophotometer equipped with a constant temperature bath set to 20 °C. H-NOX complexes were prepared in an oxygen-free glove bag as previously described [13]. NO dissociation kinetics were measured as previously described [13].

2.5. HahK autophosphorylation activity assays

Kinase autophosphorylation was detected using γ-P^{32} labeled ATP. HahK (8 μM) in reaction buffer (50 mM Tris base, 300 mM NaCl, pH 7.5) was incubated with 5 mM $MgCl_2$, 0.5 mM ATP, and 5 μCi γ-P^{32}-ATP. Reactions were carried out at ambient temperature. Ten microliter aliquots were removed at time points (0.5, 2, 15, 30 and 60 min) and quenched with SDS–PAGE loading buffer (3 μl). Proteins were separated by 12.5% SDS–PAGE and dried (Bio-Rad Model 583) before exposure to an autoradiography screen for 12–16 h. Gel images were obtained using a Typhoon Imager and quantified using ImageJ software.

To determine the effect of H-NOX on HahK activity, HahK (5 μM) was incubated with Fe^{2+}–NO H-NOX (0, 20, 40, 80 and 100 μM) in reaction buffer for 30 min before addition of 5 mM $MgCl_2$, 0.5 mM ATP, and 5 μCi γ-P^{32}-ATP to initiate HahK autophosphorylation. To compare the extent of HahK inhibition for all H-NOX complexes, H-NOX complexes were prepared (100 μM) and incubated with 5 μM HahK for 30 min before HahK autophosphorylation was initiated. All reactions were analyzed on the same SDS–PAGE gel to allow for comparison. Experiments were performed in triplicate.

2.6. Identification of the H-NOX-associated response regulator

The H-NOX-associated response regulator was identified using Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). The protein sequence of HahK (PATL1533) in FASTA format was input into the tool to generate identities of conserved domains and predict protein interaction partners. Similarly the sequences of HarR (PATL1534) and H-NOX (PATL1532) were also input into the tool for cross-comparison. The results of these computations were used to predict HarR as the cognate response regulator for HahK.

2.7. Phosphoryl transfer assays

Phosphoryl transfer was demonstrated by incubating 8 μM autophosphorylated HahK (HahK autophosphorylation was carried out as described above for 30 min before addition of HarR) with varying amounts of HarR (5, 10, 15, 20, 25 and 30 μM) for 0.5 min. Alternatively, 8 μM autophosphorylated HahK was incubated with 30 μM HarR for varying amounts of time (0.5, 1, 2, 5, 15 and 30 min). Reactions were quenched by addition of 3 μl SDS–PAGE gel loading buffer. Proteins were separated by 12.5% SDS–PAGE and dried before exposure to an autoradiography screen for 12–16 h. Gel images were obtained using a Typhoon Imager. Autoradiography gels were accompanied by identical Coomassie stained gels (autophosphorylated without γ-P^{32} labeled ATP) for the purpose of comparing protein loading.

3. Results and discussion

3.1. H-NOX has ligand binding properties consistent with a selective NO sensor

We hypothesized that H-NOX is an NO sensor that regulates the autophosphorylation of HahK and that phosphorylated HahK transfers phosphate to its response regulator in order to control the cellular response of *P. atlantica* to low concentrations of NO. The first step in analyzing this hypothesis was to confirm that

H-NOX (PATL1532) exhibits NO binding consistent with a role as an NO sensor.

H-NOX proteins are histidine-ligated protoporphyrin IX hemoproteins that bind ligands at a ferrous iron center. Ligand binding is easily monitored using electronic spectroscopy. UV–visible spectra of H-NOX in various oxidation states and ligand complexes are shown in Fig. S1 and compared to sGC, and other histidyl-ligated heme proteins in Table S1. Like many other members of the H-NOX family, H-NOX from *P. atlantica* does not bind molecular oxygen, but sensitively binds NO. We measured an NO dissociation rate constant [k_{off} (NO)] of $8.9 \pm 3.6 \times 10^{-4}\ s^{-1}$ for this H-NOX. Assuming a nearly diffusion limited k_{on} of $\sim 10^{8}\ M^{-1}\ s^{-1}$, as has been observed for other H-NOX proteins [14,15], the K_D (NO) for H-NOX is in the picomolar range. From this study, we conclude that H-NOX has ligand binding properties consistent with a sensitive and selective NO sensor.

3.2. HahK is a histidine kinase with autophosphorylation properties

Having established H-NOX as a potential NO sensor, the next step in the characterization of this putative TCS pathway required us to confirm that the H-NOX-associated histidine kinase HahK (PATL1533) does indeed exhibit autophosphorylation activity. Fig 1A shows that the purified 55 KDa protein exhibits increasing accumulation of radiolabeled phosphate with time and is active for at least 60 min at room temperature. Confirmation of the predicted phosphorylation site, H257, was carried out by using a H257A point mutant of HahK. As shown in Fig 1B, HahK H257A does not exhibit any autophosphorylation activity, confirming H257 to be the site of autophosphorylation.

3.3. H-NOX inhibits HahK autophosphorylation

We hypothesized that H-NOX regulates the autophosphorylation activity of HahK in *trans*, as part of a three-component signaling system. Consistent with this, we demonstrated using RT-PCR (Fig. S2) that H-NOX and HahK are in the same operon, thus they are likely functional partners.

To further test this part of our hypothesis, we investigated the effect of H-NOX on HahK activity. Incubation of HahK with varying concentrations of NO-bound H-NOX resulted in a dose-dependent decrease in autophosphorylation activity of HahK (Fig 2A). Incubation of HahK with equal concentrations of various H-NOX complexes (Fig 2B) indicated that, although all H-NOX complexes inhibit HahK autophosphorylation, the extent of inhibition by NO-bound H-NOX was at least twofold greater than any other H-NOX complex (Fig 2B). At physiological concentrations, it is likely that only NO-bound H-NOX has a significant effect on HahK autophosphorylation suggesting that H-NOX is a selective NO sensor.

H-NOX inhibition of histidine kinase activity has previously been observed in *S. oneidensis* MR-1 [12], although *So*H-NOX in the ferric oxidation state inhibited *So*HahK to the same extent as NO-bound *So*H-NOX, while the reduced form of *So*H-NOX did not have any effect on *So*HahK activity.

3.4. HarR is a cognate response regulator to HahK

If NO/H-NOX regulation of HahK autophosphorylation is biologically significant, there must be a response regulator to translate the NO signal into a cellular response. As is often the case, the cognate response regulator for HahK is not in the same predicted operon (Fig. S2). There is known to be significant crosstalk in bacterial protein phosphorylation networks; very often multiples kinases act upon the same response regulator, and vice versa. We used Simple Modular Architecture Research Tool (SMART), a web-based alignment tool that predicts common signaling domains within protein sequences [16] to predict a response regulator partner for HahK. The tool predicts an interaction network based on criteria derived from multiple-sequence alignments of the protein of interest with a database of sequenced genomes. Criteria include neighborhood, co-occurrence, co-expression and text mining. Every protein hit is assigned a confidence score between 0 and 1. The results from the predicted interaction network of HahK (PATL1533)

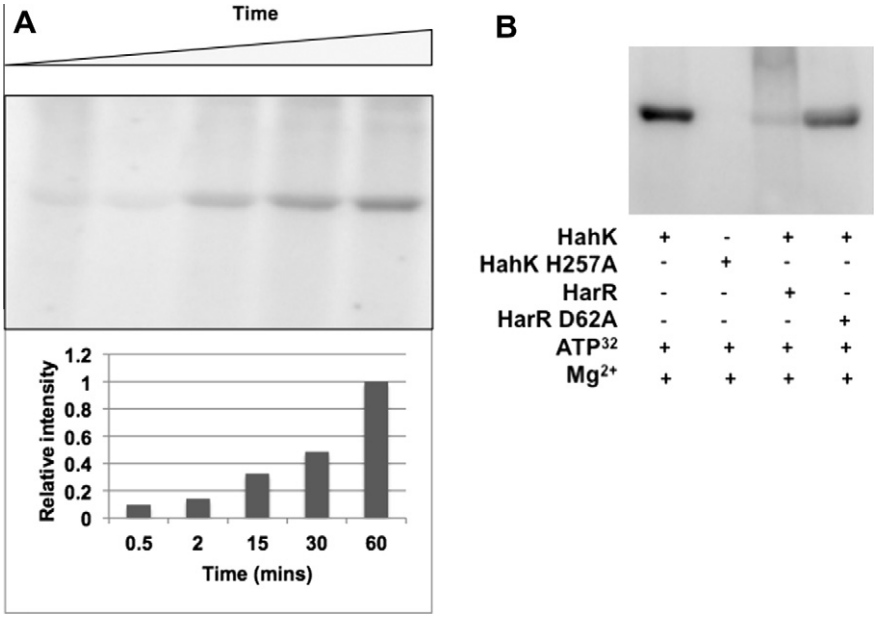


Fig. 1. HahK is a histidine kinase that transfers phosphate to HarR. Radiolabeled HahK was detected by SDS–PAGE and autoradiography. (A) *In vitro* autophosphorylation of HahK with ATP over time. Time points from left to right are 30 s, 2, 15, 30 and 60 min. The intensity of phosphorylated HahK as a function of time is plotted relative to the intensity at 60 min. (B) The phosphotransfer path is HahK H257 to HarR D62. HahK H257 has no autophosphorylation activity, indicating H257 is the phosphorylation site. HarR D62A has no phosphoryl transfer activity, indicating D62 is the receiving residue. The contents of each lane are indicated below the gel.

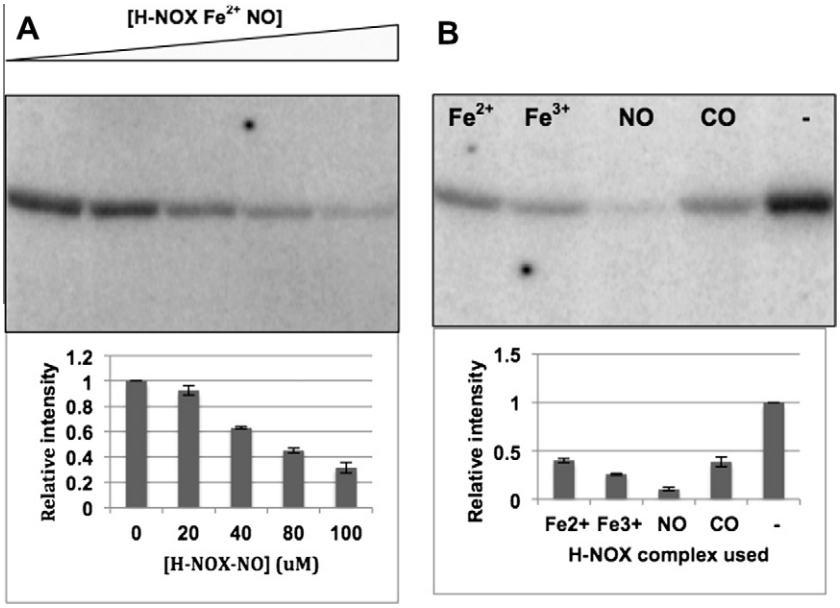


Fig. 2. The autophosphorylation activity of HahR is inhibited by NO/H-NOX. Radiolabeled HahK was detected by SDS–PAGE and autoradiography. (A) Dose-dependent HahK inhibition by NO-bound H-NOX. Intensity of phosphorylated HahK in the presence of varying NO-bound H-NOX concentration is plotted relative to the intensity in the absence of NO-bound H-NOX. Error bars represent one standard deviation from the mean of triplicate experiments. (B) HahK is inhibited by all forms of H-NOX, but NO-bound H-NOX exhibits the most potent inhibition. Intensity of phosphorylated HahK in the presence of varying H-NOX complexes is plotted relative to the intensity in the absence of H-NOX. Error bars represent one standard deviation from the mean of triplicate experiments. Fe^{2+} refers to ferrous-unligated H-NOX, Fe^{3+} refers to oxidized H-NOX, NO refers to the Fe^{2+}–NO complex, and CO refers to the Fe^{2+}–CO complex.

and H-NOX (PATL1532) are summarized in Table S2. Based on these results we selected PATL1534, in the operon just upstream of H-NOX and HahK, as a potential response regulator for HahK. We named PATL1534 HarR for H-NOX-associated response regulator.

HarR was cloned, expressed, and purified. Phosphoryl transfer of radiolabeled phosphate from HahK to HarR was then carried out *in vitro*. The results of this experiment are illustrated in Fig 3. Transfer of radiolabeled phosphate from HahK to HarR was indeed successful, as predicted. HahK and HarR are very close in molecular weight (55 KDa and 50 KDa respectively) and thus clean separation could not be achieved by SDS–PAGE. Instead, phosphoryl transfer was demonstrated by incubating autophosphorylated HahK with increasing concentrations of HarR for 30 s. Fig 3A reports an autoradiogram of an SDS–PAGE illustrating that the band corresponding to radiolabeled HahK is disappearing and a band corresponding to HarR is accumulating. An otherwise identical experiment (with only unlabeled ATP as HahK substrate) was analyzed by Coomassie brilliant blue staining to specify protein loading. These data indicate that radiolabeled phosphate is being transferred from HahK to HarR as a function of HarR concentration.

Residue D62 of HarR was predicted to be the site of phosphorylation by sequence homology to other response regulators. Indeed, as shown in Fig 1B, mutation of D62 to Ala eliminates phosphoryl transfer from HahK to HarR. Wild-type HarR affects a rapid decrease in the band for phosphorylated HahK, while the D62A mutant shows no effect on the intensity of HahK phosphorylation even after 20 min. The reason we do not see a band corresponding to HarR in this experiment is because this experiment was performed for 20 min, which is much longer than the half-life of phosphorylated HarR.

It is not known if HahK is the only kinase that associates with HarR (or if HarR is the only response regulator that associates with HahK), although it seems likely that HarR also associates with predicted kinase PATL1535, which is in the same operon as HarR (Fig. S2). This is consistent with the crosstalk that is common in bacterial phosphorylation networks.

3.5. HarR exhibits autophosphatase activity

Fig. 3B illustrates the time dependence of phosphoryl transfer from HahK to HarR. When autophosphorylated HahK is incubated with HarR for extended periods of time (greater than 5 min), the band corresponding to phosphorylated HarR disappears completely. In two-component signaling systems, the duration of response regulator phosphorylation determines the duration of the stimulus response [17]. As such, most response regulators have autophosphatase activity to control response time. In *E. coli*, the response regulator CheY, which is involved in chemotaxis, is seen to be phosphorylated for only a few seconds [18]. In contrast, Spo0F, the sporulation regulator from *Bacillus subtilis*, remains phosphorylated for up to 3 h [2].

In order to determine if the loss of radiolabeled phosphate from the HahK/HarR system was due to HarR phosphatase activity, we examined its phosphatase activity towards an external generic substrate, 3-*O*-methylfluorescein phosphate (OMFP). As illustrated in Fig. S3, HarR does indicate phosphatase activity towards OMFP, and this activity is inhibited by the phosphatase inhibitor sodium orthovanadate. In contrast, HahK does not exhibit any phosphatase activity towards OMFP. Thus, we conclude that HarR has intrinsic phosphatase activity as a means to control stimulus response duration. Furthermore, we can conclude that the response duration for HarR is on the order of minutes. This is of intermediate length compared with other characterized response regulators.

3.6. The H-NOX/HahK/HarR three-component signaling pathway: implications for NO mediated signal transduction

In this report we have shown the identification and biochemical characterization of a novel nitric oxide–responsive TCS system in the marine bacteria *P. atlantica*. We find that NO has an inhibitory effect on phosphorelay in this pathway. To our knowledge this is the first report of a response regulator protein whose phosphorylation state is regulated by NO/H-NOX.

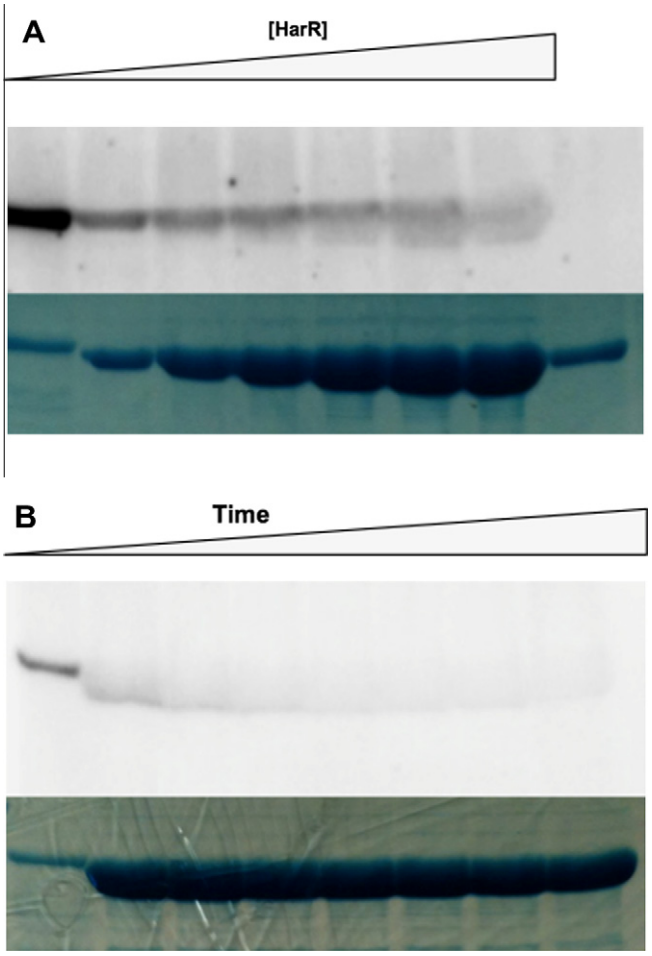


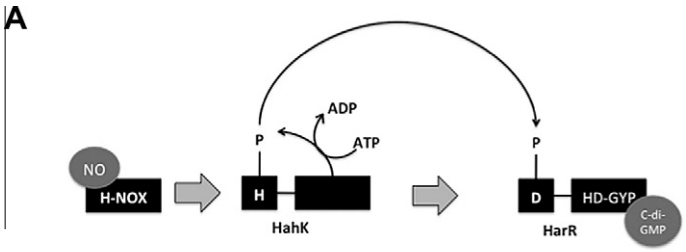
Fig. 3. HarR is the cognate response regulator for HahK and the signal duration is modulated by HarR phosphatase activity. Phosphotransfer from HahK to HarR analyzed by SDS–PAGE. Top: detection of radiolabeled phosphoproteins by autoradiography. Bottom: protein loading detected by Coomassie staining. (A) Phosphotransfer from HahK to HarR as a function of HarR concentration. Increasing concentrations of HarR were incubated with 8 μM HahK. The first lane on the left contains HahK only, followed by HahK incubated with 5, 10, 15, 20, 25 and 30 μM HarR. The last lane contains only 5 μM HarR, indicating HarR has no intrinsic autophosphorylation activity. (B) Phosphotransfer from HahK to HarR as a function of time. Phosphoryl transfer at increasing time points shows a disappearance of phosphorylated HahK. The first lane on the left contains HahK alone, followed by kinase incubated with 30 μM HarR for 0.5, 1, 2, 5, 15 and 30 min.

The response regulator HarR is annotated by NCBI as a response regulator protein with a CheY like receiver domain and an HD-GYP domain. HD-GYP proteins are not as well understood. The first CheY like HD-GYP proteins discovered are from *Synechocystis* sp. and *Thermotoga maritima* [19,20], but the best-characterized representative is RpfG from *Xanthomonas campestris* [21]. CheY like response regulator proteins containing HD-GYP domains are phosphodiesterases that hydrolyze the bacterial second messenger cyclic-di-GMP [bis-(3′-5′)-cyclic dimeric guanosine monophosphate; c-di-GMP] to its linearized form, pGpG [5′-phosphoguanylyl-(3′-5′)-guanosine], and eventually GMP. This class of proteins is very important because they are implicated in regulating c-di-GMP levels; c-di-GMP levels are strongly correlated with the transition between motility and biofilm formation in bacteria [22,23].

The response regulator HarR, while predicted to have an HD-GYP domain, only shares weak homology with characterized HD-GYP phosphodiesterases. In fact, the conserved HD-GYP motif is replaced by CE-GP and we were not able to observe hydrolytic activity towards c-di-GMP (Fig. S4) with HarR. However, unconventional HD-GYP proteins are not uncommon. Bd1817 from *Bdellovibrio bacteriovorus* is an 'HD-GYP' protein without the conserved motif, but structural studies have suggested a role for Bd1817 in c-di-GMP binding and regulation of proteins that synthesize c-di-GMP [24]. Pa2572 from *Pseudomonas aeruginosa* is another ill-conserved HD-GYP protein that does not seem to have an effect on the absolute concentration of intracellular c-di-GMP levels, but affects swarming motility when deleted [25].

Based on available information, we propose that HarR is indirectly involved in c-di-GMP metabolism in *P. atlantica*. Interestingly, H-NOX has previously been implicated in the regulation of intracellular c-di-GMP levels and biofilm formation. In *Shewanella woodyi*, NO-bound H-NOX enhances the degradation of c-di-GMP by inhibiting c-di-GMP synthesis and enhancing c-di-GMP hydrolysis [26]. In *Legionella pneumophila*, an *hnoX* gene deletion results in a hyper biofilm phenotype [27].

P. atlantica is not the only organism with an HD-GYP-like response regulator associated with an H-NOX protein. SMART also predicts an HD-GYP response regulator for the H-NOX/HahK pairs from *S. oneidensis*, *Vibrio cholerae*, *Colwellia psychrerythraea* and *Saccharophagus degradans*. In fact, in *S. degradans*, the HD-GYP-like response regulator is in the same predicted operon as the H-NOX and HahK proteins. Fig 4 summarizes the gene IDs of H-NOX, HahK and response regulators in these bacteria. Based on this informa-



B

Organism	H-NOX	Histidine Kinase	Response Regulator
Pseudoalteromonas atlantica	Patl_1532	Patl_1533	Patl_1534
Shewanella oneidensis	So_2144	So_2145	So_2541
Vibrio cholerae	Vc_0720	Vc_0719	Vc_1087
Colwellia psychrerythraea	Cps_3609	Cps_3608	Cps_3241
Saccharophagus degradans	Sde_3804	Sde_3806	Sde_3805

Fig. 4. Schematic illustration of NO regulated TCS. (A) H-NOX is a selective NO sensor that regulates autophosphorylation of HahK. Phosphorylated HahK transfers phosphate to the response regulator HarR. We propose that HarR indirectly affects intracellular cyclic-di-GMP levels. (B) Other bacteria are predicted to have homologous NO/H-NOX regulated TCS systems. The predicted members of each predicted NO/H-NOX regulated TCS system are indicated.

tion we propose that we have identified not only an NO-responsive pathway in *P. atlantica*, but an NO-responsive signaling *motif* found in a variety of marine bacteria, and insight into the function of this signaling pathway will be applicable to all of these species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.04.037.

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