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Discovery of a Novel Nitric Oxide Binding Protein and Nitric-Oxide-Responsive Signaling Pathway in *Pseudomonas aeruginosa*

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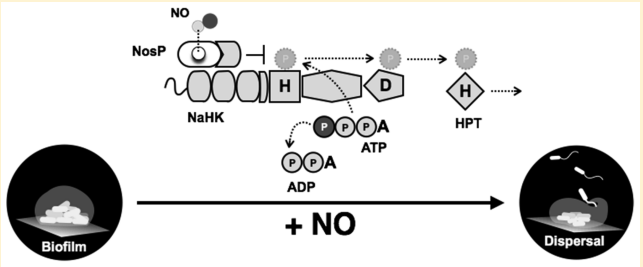
Supporting Information

ABSTRACT: Nitric oxide (NO) is a radical diatomic gas molecule that, at low concentrations, plays important signaling roles in both eukaryotes and bacteria. In recent years, it has become evident that bacteria respond to low levels of NO in order to modulate their group behavior. Many bacteria respond via NO ligation to a well-established NO sensor called H-NOX (heme-nitric oxide/oxygen binding domain). Many others, such as *Pseudomonas aeruginosa*, lack an annotated *hnoX* gene in their genome yet are able to respond to low levels of NO to disperse their biofilms. This suggests the existence of a previously uncharacterized NO sensor. In this study, we describe the discovery of a novel nitric oxide binding protein (NosP; NO-sensing protein), which is much more widely conserved in bacteria than H-NOX, as well as a novel NO-responsive pathway in *P. aeruginosa*. We demonstrate that biofilms of a *P. aeruginosa* mutant lacking components of the NosP pathway lose the ability to disperse in response to NO. Upon cloning, expressing, and purifying NosP, we find it binds heme and ligates to NO with a dissociation rate constant that is comparable to that of other well-established NO-sensing proteins. Moreover, we show that NO-bound NosP is able to regulate the phosphorelay activity of a hybrid histidine kinase that is involved in biofilm regulation in *P. aeruginosa*. Thus, here, we present evidence of a novel NO-responsive pathway that regulates biofilm in *P. aeruginosa*.



KEYWORDS: *nitric oxide, nitric oxide signaling, biofilm, nitric oxide sensor, NosP, nitric oxide-responsive kinase*

Bacterial biofilm formation occurs when free swimming bacteria aggregate in a community, usually on a solid surface, within a self-secreted exopolysaccharide matrix. Biofilming bacteria are responsible for many chronic human infections as well as nosocomial diseases; they also pose a significant threat to food and water safety, civilian and military naval operations, irrigation, and more.[1−6] Bacteria residing in biofilms are recalcitrant to conventional therapeutics because they are highly resistant to antibiotics, host defenses, and even some harsh chemical treatments.[7−10]

The opportunistic pathogen *Pseudomonas aeruginosa* has drawn special attention in microbiology because it readily forms biofilms and, as such, is a major cause of hospital-acquired infection.[11,12] *P. aeruginosa* biofilm infections in the lung are the leading cause of death in cystic fibrosis patients.[13] Although *P. aeruginosa* is a model biofilming organism, assembly and dispersal of biofilm in *P. aeruginosa* is still poorly understood.

The diatomic gas nitric oxide (NO) is well-documented as a signaling molecule that directs *P. aeruginosa* to disperse from biofilms; as low as picomolar concentrations of NO have been shown to cause *P. aeruginosa* to leave biofilms.[14] The details underlying this phenomenon are not well-understood, but some aspects of NO signaling in *P. aeruginosa* have been reported. It has been documented that NO-mediated biofilm dispersal is correlated with increased cyclic-di-GMP phosphodiesterase activity, resulting in decreased cyclic-di-GMP levels.[15] This is expected because decreased levels of cyclic-di-GMP are tightly correlated with biofilm dispersal in many bacterial species.[14−18] The chemotaxis transducer BdlA has been implicated in cyclic-di-GMP degradation and biofilm dispersal upon NO detection, through a currently unknown mechanism.[19] A domain of BdlA called PASa can bind heme, which likely binds NO.[20] However, BdlA appears also to respond to many environmental cues in addition to NO, including succinate, Ag^{+}, Hg^{2+}, and As^{3+}. The cyclic-di-GMP synthase GcbA has also been implicated in NO-induced biofilm dispersal because it contributes to the activation of BdlA, but it does not appear to bind NO itself. The cyclic-di-GMP phosphodiesterases DipA[21] and NbdA[22] have been linked to decreasing cyclic-di-GMP concentrations upon exposure to NO. Further, bioinformatics data suggest that NbdA could coordinate copper, a potential NO binding site.[23] However, the mechanism of action for both is NO-induced upregulation of *dipA* and *nbdA* expression, suggesting action downstream of initial NO sensing. DNR (dissimilative nitrate respiration regulators), a transcription factor in *P. aeruginosa*, was hypothesized to be the primary NO sensor in *P. aeruginosa*.[24] However, its affinity for NO was found to be in the range of 88−350 μM, which is inconsistent with the pico- to nanomolar

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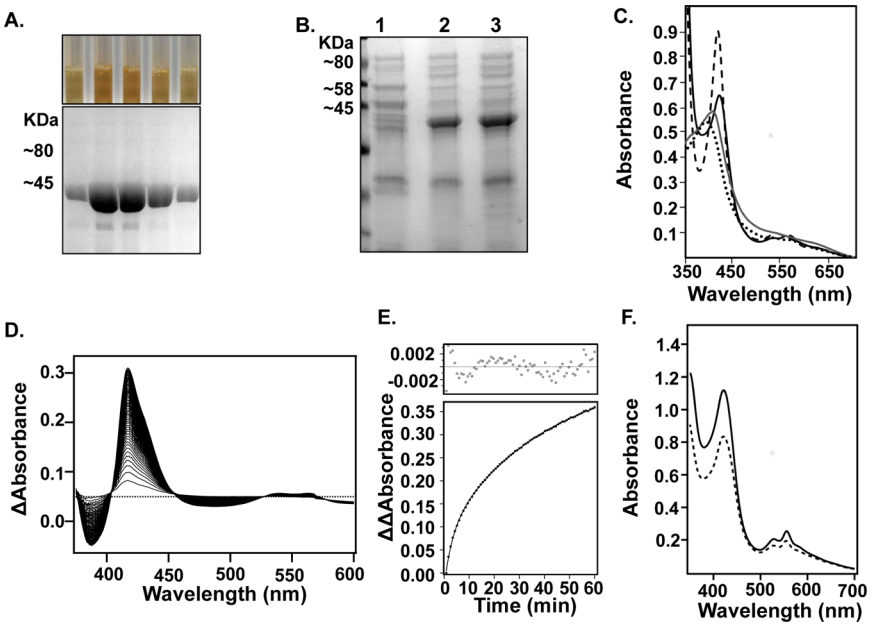

Figure 1. NosP is a hemoprotein that ligates NO. (A) Purified hexaHis-tagged NosP is yellow-orange in color. Top: NosP fractions as eluted from IMAC. Bottom: Coomassie-stained SDS-PAGE of the same purified fractions. (B) *E. coli* lysate with overexpressed NosP (lanes 2 and 3), but not lysate from cells transformed with NosP but without expression induction (lane 1), contains a 42 kDa protein that binds heme-agarose. Lane 3 shows the presence of the same protein band with more stringent wash steps. (C) NosP has the ligand binding specificity of a NO sensor. UV/vis absorption spectra at 20 °C of ferric NosP (solid gray line) with a λ_{max} at 410 nm, ferrous NosP (solid black line) with a λ_{max} at 420 nm, CO-ligated NosP (dashed line) with a λ_{max} at 416 nm, and NO-ligated NosP (dotted line) with a λ_{max} at 396 nm. (D,E) Example of an experiment to measure the dissociation rate for the Fe^{II}–NO complex of NosP at 20 °C measured by UV/vis absorption spectroscopy with saturating CO and 30 mM dithionite as a trap for the released NO. Measured rates and amplitudes [$k_1 = k_{off} = (1.8 \pm 0.5) \times 10^{-4}$ s^{-1}, $k_2 = (13 \pm 2) \times 10^{-4}$ s^{-1}] were independent of CO and dithionite at all concentrations tested (3–300 mM). (D) Absorbance difference spectrum (the spectrum at time = 0 min is subtracted from the spectrum at each subsequent time point) of the Fe^{II}–CO complex growing in over time is shown as well as (E) a plot of the change in absorbance at 417 nm minus 387 nm (the maximum and the minimum in the difference spectrum) versus time along with the exponential fit of those data. (F) N-terminal domain of NosP is sufficient to bind heme. UV/vis absorption spectra of ferrous NosP (black line) and ferrous NosP-NT (dashed line), with a λ_{max} at 420 nm.

concentrations of NO shown to cause biofilm regulation in *P. aeruginosa*. To date, a primary sensitive NO sensor in *P. aeruginosa* has yet to be established.

The molecular basis for NO-mediated biofilm regulation has been demonstrated in some bacteria, including *Legionella pneumophila*,[25] *Shewanella oneidensis*,[26] *Shewanella woodyi*,[27] *Vibrio harveyi*,[28,29] and *Silicibacter* sp. strain TrichCH4B.[30] In these bacteria, the NO sensor H-NOX (heme-nitric oxide/oxygen binding protein) affects biofilm formation by regulating intracellular cyclic-di-GMP concentrations or quorum sensing.[31,32] *P. aeruginosa* does not encode an *hnoX* gene, however.

Here, we describe the discovery of a new family of heme-based NO biniding proteins in bacteria called NosP (NO-sensing protein). In *P. aeruginosa*, NosP binds heme and, upon ligating to NO at the heme iron, modulates the activity of a cocistronic kinase, which subsequently controls the phosphorylation of a histidine-containing phosphotransfer domain that ultimately contributes to NO-responsive biofilm regulation.

RESULTS AND DISCUSSION

Discovery of NosP. The primary NO sensor involved in *Pseudomonas aeruginosa* biofilm regulation has not been identified. We became interested in an uncharacterized protein domain, sometimes called FIST (F-box and intracellular signal transduction proteins), that is widely distributed in bacteria [found in about 620 independent sequenced species (i.e., if multiple strains of the same species have been sequenced, the species was only counted once in this analysis); see supplemental Figure 1A]. This domain is found in some eukaryotic genomes and a few archaeal species, but it is predominately found in bacterial genomes.

This domain was previously predicted to be a sensory domain by Borziak et al.,[33] due to its appearance of the N-terminal to MCP (methyl-accepting chemotaxis protein) domains in some proteins. Upon a more detailed look into the genomes of bacteria coding for these domains, however, it is evident that they are most commonly encoded in bacterial genomes in operons with signaling proteins like histidine kinases, diguanylate cyclases, and cyclic-di-GMP phosphodiesterases (supplementary Figure 1B). Interestingly, the signaling proteins cocistronic with these FIST domains generally lack an annotated sensory domain, suggesting an alternate regulatory domain could function *in trans*. In fact, this genomic arrangement is highly reminiscent of the H-NOX family of NO-sensing proteins. Thus, we hypothesized that FIST could be an uncharacterized bacterial-sensing protein, perhaps an NO sensor involved in biofilm formation.

In support of this hypothesis, in *Vibrio cholerae*, a FIST domain is N-terminal to a cyclic-di-GMP phosphodiesterase (Vc0130) that has been shown to be involved in cyclic-di-GMP-mediated biofilm regulation.[34] In *Shewanella oneidensis*, this domain (SO_2542) is upstream of a histidine kinase that is involved in NO-mediated biofilm regulation.[26] In addition, the FIST domain (lpg0279) in *Legionella pneumophila* is coded for in the same operon with a histidine kinase (lpg0278) and a cyclic-di-GMP metabolizing enzyme (lpg0277) with a receiver

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domain at its N-terminus. In a recent publication, it was demonstrated that deletion of the homologue of lpg0277 in the *Legionella pneumophila* Lens strain (lpl1054) results in a hyperbiofilm phenotype,[35] suggesting involvement of FIST in biofilm regulation. Most relevant to this study, in *Pseudomonas aeruginosa*, a FIST domain (Pa1975) is cocistronic with the hybrid histidine kinase Pa1976 (supplementary Figure 1B). Notably, Pa1976 has been implicated in biofilm regulation in previous studies.[36] The specific stimulus for this kinase has not yet been determined, but as cocistronic proteins often function together in the same pathway in bacteria, we hypothesized that Pa1975 might interact with Pa1976 and thereby be involved in biofilm regulation in *P. aeruginosa*. Interestingly, Pa1976 is predicted to be soluble, which is consistent with a role in NO signaling: NO is a membrane-permeable gas; indeed, most known NO sensors are soluble.[26−29,31,32] Therefore, we hypothesized this uncharacterized protein domain could be a missing primary NO sensor in *P. aeruginosa*.

Purified NosP Shows Ligand-Binding Properties That Are Consistent with NO Sensing. In order to test our hypothesis that NosP is a NO-sensing protein, we cloned and expressed *P. aeruginosa* NosP (Pa1975; 42 kDa) in *Escherichia coli*. Upon purification, we found that it has the yellow-orange color common for hemoproteins (Figure 1A). In order to confirm that NosP is a heme protein, we performed a heme pulldown assay. As illustrated in Figure 1B, *E. coli* lysate containing overexpressed NosP, but not lysate without NosP, contains a 42 kDa protein that binds tightly to heme-agarose. These data are consistent with heme affinity for NosP.

The π electrons of the tetrapyrrole in the porphyrin ring of heme-bound proteins are known to absorb energy in the UV/vis range, resulting in a $\pi \rightarrow \pi^*$ transition. This UV/vis transition gives rise to a characteristic absorbance peak known as the Soret band.[37] Depending on the oxidation and ligation state of the iron at the heme core, the Soret band can appear between ∼350 and ∼450 nm. UV/vis spectra of NosP as the Fe^{II}, Fe^{II}−CO, and Fe^{II}−NO complexes at room temperature are shown in Figure 1 and are compared with those of H-NOX and other histidyl-ligated heme proteins in Table 1.

NosP is purified with a Soret maximum of 413 nm, which is presumably a mixture of ferrous and ferric complexes. Treatment of purified NosP with ferricyanide to form the ferric state results in a complex that is indicative of a histidine-ligated, high-spin, five-coordinate complex with a Soret maximum at 410 nm. Anaerobic treatment of ferric NosP with sodium dithionite reduces the protein and shifts the Soret maximum to 422 nm with split α/β bands at 554 and 524 nm (Figure 1C). This spectrum is similar to those of hexacoordinated hemoproteins, such as CooA, cytochrome c', and the truncated globins, where the iron is ligated to two axial ligands, usually histidine and an additional amino acid (see Table 1).[38−42] These spectra differ from H-NOX proteins, which form high-spin, five-coordinate complexes in their ferrous state with a single broad α/β around 555 nm, consistent with one axial histidine ligand.[43−45] When carbon monoxide (CO) is added to the Fe^{II} NosP protein, the Soret maximum shifts to 416 nm, suggestive of a histidine- and CO-ligated, low-spin, six-coordinate complex. Binding of NO to the Fe^{II} protein shifts the Soret maximum to 396 nm, indicative of a high-spin, five-coordinate complex with NO. The CO and NO complexes are similar to other histidine-ligated hemoproteins, including the H-NOX and globin families. Therefore, the data suggest that ferrous NosP ligates histidine as well as an additional ligand, probably an amino acid side chain or water. This additional ligand is displaced upon binding CO or NO.

Table 1. UV/Vis Peak Positions and NO Disassociation Kinetics

protein	Soret (nm)	β (nm)	α (nm)	ref
Fe^{II}				
sGC	431	555		46
VcH-NOX	429	568		44
CooA	425.5	529.5	559.5	38, 39, 41
cyt *c*	420	526	556	40, 42
NosP	420	524	554	this article
NosP-NT	420	524	554	this article
Fe^{II}−CO				
sGC	423	541	567	46
VcH-NOX	429	541	566	44
CooA	422	539.5	569	37−41
NosP	416	538	565	this article
Fe^{II}−NO				
sGC	398	537	571	46
VcH-NOX	398	540	573	44
NosP	396	534	574	this article
k_{off} NO				
sGC	$(3.6 \pm 0.8) \times 10^{-4}$ s^{-1}			46
SwH-NOX	$(15.2 \pm 3.5) \times 10^{-4}$ s^{-1}			27
VhH-NOX	$(4.6 \pm 0.9) \times 10^{-4}$ s^{-1}			28
NosP	$(1.8 \pm 0.5) \times 10^{-4}$ s^{-1}			this article

NosP N-Terminal Domain Is Sufficient for Heme Binding. *P. aeruginosa* NosP is annotated to contain an N-terminal domain and a C-terminal domain. In efforts to understand whether both domains are needed for heme binding, we made a truncated mutant of NosP (NosP-NT) that contains the first 235 residues (the N-terminal domain) with a C-terminal hexaHis-tag. When this mutant was purified, it retained its yellow-orange color, indicating that it is bound to heme, similar to the full-length protein. The UV/vis spectroscopy of this mutant is consistent with the full-length protein, indicating that heme binding is contained within the N-terminus of NosP (Figure 1E and Table 1).

NosP NO Dissociation Rate Is Slow. We investigated the NO dissociation rate of NosP using a standard CO and dithionite trap[43,45] for released NO, consisting of saturating CO and 30 mM dithionite, to minimize rebinding of dissociated NO. The NO dissociation rate was followed by the formation of the Fe^{II}−CO complex at 416 nm. This rate was independent of CO and dithionite at all concentrations tested (3, 30, and 300 mM dithionite). Representative data are shown in Figure 1D, and Table 1 compares these data with other Fe^{II}−NO heme proteins.

Figure 1E shows the data fit with two parallel exponentials ($k_1 = (1.8 \pm 0.5) \times 10^{-4}$ s^{-1}, $k_2 = (13 \pm 2) \times 10^{-4}$ s^{-1}) of the form $f(x) = Ax(1 - e^{-kx})$. We used two exponential functions because a single exponential fit resulted in very high residuals. The second rate is possibly due to association of the unknown second axial ligand to the ferrous-unligated form of NosP after NO dissociation. This additional rate cannot be CO association, as our measured rate is independent of CO addition to the dithionite trap. We report the NO dissociation rate as the slower of the two exponentials because this is the overall slowest step in the NO dissociation mechanism, although at this time, we cannot assign that rate to the molecular step of NO dissociation. Our reported NO_{off} rate of

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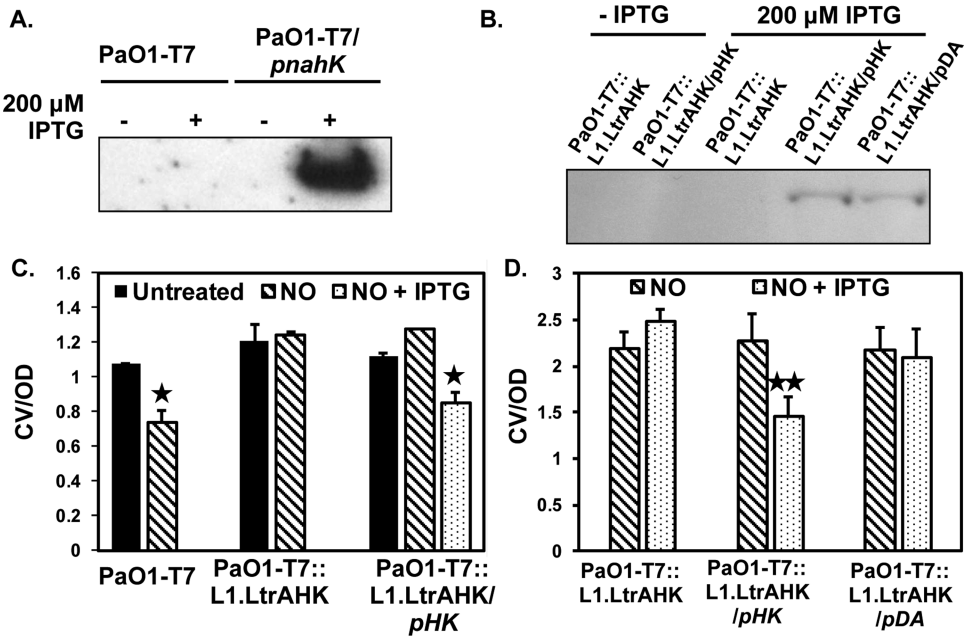

Figure 2. NO-mediated biofilm dispersal requires NahK. (A,B) Western blot analyses with an anti-hexaHis-tag antibody (HRP). (A) NahK can be expressed in *P. aeruginosa* strain PaO1-T7. Anti-His Western blot of NahK in PaO1-T7 and PaO1-T7 that is transformed with an IPTG-inducible plasmid expressing NahK (PaO1-T7/*pnahK*), with and without 200 μM IPTG added to the media for induction of expression of NahK. (B) NahK expression was disrupted in *P. aeruginosa* strain PaO1-T7. Anti-His Western blot of uninduced and induced (+200 μM IPTG) NahK in strains PaO1-T7::L1.LtrAHK (*nahK*-disrupted), PaO1-T7::L1.LtrA/*pHK* (*nahK*-disrupted and complemented back with wild-type *nahK*), and PaO1-T7::L1.LtrA/*pDA* (*nahK*-disrupted and complemented back with inactive *nahK*). A band for NahK is only visible in the complemented strains in the presence of IPTG. (C,D) Biofilms of wild-type and *nahK*-disrupted *P. aeruginosa* formed at the liquid−air interface of PVC plates after 24 h of growth, in the presence and absence of NO, as quantified by crystal violet staining. (C) Disruption of the *nosP* operon in *P. aeruginosa* results in loss of the NO phenotype compared with the wild-type strain. NO (∼5 nM) causes a decrease in PAO1-T7 biofilm thickness (left set of columns). This NO-dependent decrease in biofilm is not seen in a *pa1976*-disrupted mutant (PaO1-T7::L1.LtrAHK; second set of columns). The decrease in biofilm thickness in the presence of NO is dependent on *pa1976* expression. Biofilm formation in the *pa1976*-disrupted strain transformed with *pa1976* on an IPTG-inducible plasmid (PaO1-T7::L1.LtrAHK/*pHK*) depends upon the addition of IPTG and NO (third group of columns); i.e., IPTG-induced expression of *pa1976* from plasmid pJLQ restores a wild-type-like response to NO. (D) When the *pa1976*-disrupted strain is transformed with an inactive mutant of *pa1976* (D809A) on an IPTG-inducible plasmid (PaO1-T7::L1.LtrA/*pDA*), NO sensitivity is lost, independent of IPTG addition, indicating that NO signaling requires PA1976 activity. Error bars are one standard deviation from the mean of triplicate experiments; ★ = $p \leq 0.005$ compared to wild-type PaO1-T7; ★★ = $p \leq 0.005$ compared to PaO1-T7::L1LtraHK/*pHK* before IPTG addition.

1.8×10^{-4} s^{-1} for NosP is very similar to that of sGC (3.6×10^{-4} s^{-1})[46] and other H-NOX domains (Table 1), indicating NosP has ligand binding properties consistent with an NO sensor.[27,28,44,45] We are currently measuring the NO association rate constant in order to determine the thermodynamic dissociation binding constant for NO to NosP. Although we have not yet measured the NO association rate constant, we expect it will fall between 10^4 and 10^8 M^{-1} s^{-1}, as is typical for histidine-ligated hemoproteins.[47] Therefore, the NO thermodynamic dissociation binding constant is likely to be low nanomolar to picomolar.

NO-Mediated Biofilm Dispersal Requires NahK. NO is well-understood to regulate biofilm formation in *P. aeruginosa*. In order to determine whether NosP regulates biofilm formation, we sought to generate a NosP mutant and study its effect on biofilm formation. We chose to generate strains of *P. aeruginosa* PAO1-T7 that were defective in *nosP* or its cocistronic kinase (Pa1976; named *nahK* for NosP-associated histidine kinase), using targeted type II intron disruption (TargeTron).[48] In these studies, we employed a strain of *P. aeruginosa* PAO1 that stably expresses the T7 polymerase (*P. aeruginosa* strain PAO1-T7), in order to be able to induce expression of proteins from recombinant plasmids using isopropyl β-D-1-thiogalactopyranoside (IPTG) in wild-type and mutant backgrounds.

Therefore, we identified potential insertion sites for both *nosP* and *nahK* and engineered retargeted L1.LtrB introns to disrupt these genes. Unfortunately, we were unsuccessful in generating the *nosP*-disrupted mutant. Interestingly, the commercially available library of *P. aeruginosa* PAO1 mutants (http://www.gs.washington.edu/labs/manoil/libraryindex.htm) also does not contain a disruption of *pa1975*. At present, however, we do not believe *nosP* is essential in *P. aeruginosa*. Many nonessential genes are not represented in transposon libraries due to gene length, GC content, etc. Furthermore, *nosP* has never been found in any essential gene data sets derived from *P. aeruginosa* mutant libraries.[49] Finally, the *nosP*-disrupted PA14 mutant is available in a commercial library; to our knowledge, there are no examples of genes that are essential in only PA14 or PAO1. It is possible, however, that there are suppressor mutations in the commercial *nosP*-disrupted PA14 mutant. Nonetheless, to address this issue, in future studies, we plan to make and complement clean deletions of *nosP* and *nahK*.

We were able to target *nahK* (*pa1976*) with L1.LtrA to generate the strain PaO1-T7::L1.LtrA*HK*. We complemented PaO1-T7::L1.LtrA*HK* with an IPTG-inducible vector that

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expresses NahK (PaO1-T7::L1.LtrAHK/*pHK*) with a C-terminal hexaHis-tag. We characterized these constructs by evaluating the induction of *nahK* with IPTG in both PaO1-T7 and in the PaO1-T7::L1.LtrA*HK* strains. The results indicate that the induction and expression of NahK takes place only when IPTG is added to the growth media (Figure 2A lane 4 and Figure 2B lane 4).

To characterize the biofilming characteristics of these strains, we conducted a static biofilm assay. As illustrated in Figure 2C, wild-type PaO1-T7 forms less biofilm in the presence of NO (from 500 nM DETA NONOate, ~5 nM NO). This is expected and has been observed many times in studies of *P. aeruginosa* biofilm formation.[13,50] PaO1-T7::L1.LtrA*HK*, the kinase-disrupted mutant, is able to form biofilm, but it does not display an NO phenotype, as demonstrated in Figure 2C. The NahK kinase-complemented strain recovers the NO phenotype but only once IPTG is added to the medium to induce *pa1976* expression (Figure 2C). Additionally, when PaO1-T7::L1.LtrA is complemented with a Pa1976 construct with a mutation of the conserved aspartate (D809A) in its receiver domain (PaO1-T7::L1.LtrA/*pDA*), so as to prevent downstream signaling, it fails to recover the NO phenotype, despite addition of IPTG (illustrated in Figure 2D). These data confirm that expressed and active NahK is required for NO-mediated biofilm regulation in *P. aeruginosa*.

Pa1976 Is a NosP-Associated Histidine Kinase. NosP is in a putative gene operon with a hybrid histidine kinase (Pa1976; named NahK for NosP-associated histidine kinase). Frequently cocistronic proteins (proteins encoded within the same gene cluster) function together, thus we decided to study the kinase activity of NahK to determine if it is regulated by NosP. First we cloned Pa1976 with a C-terminal hexaHis-tag. However, the full-length protein expressed and purified extremely poorly from *E. coli*. A previous study of Pa1976 had indicated that a truncated version of Pa1976 can be purified to study phosphorelay,[36] but this truncated mutant lacks the PAS (per-arnt-sim) and PAC (motif C-terminal to PAS) domains, which are likely protein−protein interaction domains (Figure 3A). Thus, we decided to clone a truncated variant of the kinase with all three PAS/PAC domains but lacking the first 84 amino acids on the N-terminus, which are predicted to be mostly unstructured with a coiled-coil motif. We named this variant NahKΔN84. This truncated construct did not express or purify well, but sufficient quantities were obtained to continue with the study.

Figure 3B shows the autokinase activity of NahKΔN84 over time. NahK is a hybrid histidine kinase with a receiver domain at its C-terminus. Hybrid histidine kinases usually contain dual activities: autophosphorylation of a conserved histidine catalyzed by the kinase domain and dephosphorylation and phosphotransfer from this histidine residue to a conserved aspartate within the receiver domain catalyzed by the receiver domain.[51,52] Due to phosphatase activity,[53] phosphotranferase activity, and/or the intrinsic chemical instability of phosphorylated aspartate, it is often difficult to detect phosphorylated hybrid histidine kinases in typical biochemical assays. The autophosphorylation assay in Figure 3C, however, demonstrates stable phosphorylation of NahKΔN84 over 30 min.

Hybrid histidine kinases typically transfer phosphate from the aspartate in the receiver domain to a histidine-containing phosphotransfer protein (HPT) in order to continue in signal transduction.[52] *P. aeruginosa* has three annotated HPTs, of which Pa3345 (HptB) has been shown to accept phosphate

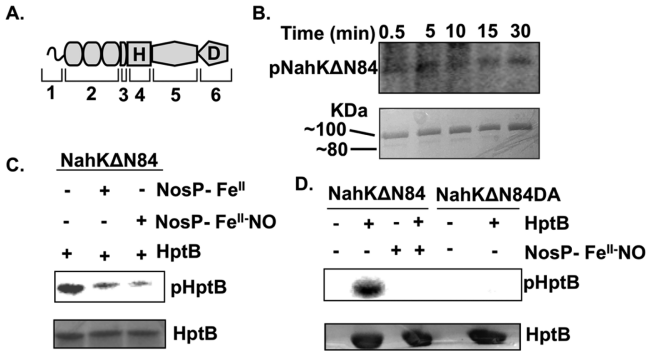

Figure 3. NO/NosP regulates NahK. (A) Schematic of predicted domain organization of NahK (pa1976). The N-terminus is predicted to be coiled-coil followed by three PAS domains, a PAC domain, a HisKA domain [His kinase A (phosphoacceptor) domain], a HATPase_c domain (histidine kinase-like ATPases), and a REC domain. (B) In vitro autophosphorylation of NahKΔN84 over time. Radiolabeled phosphoproteins were detected by SDS-PAGE (bottom) and autoradiography (top). (C,D) NahKΔN84 transfers phosphate to HptB. (C) Phosphotransfer from NahKΔN84 to HptB, analyzed by SDS-PAGE, is inhibited by NosP. Bottom: Protein loading detected by Coomassie staining. Top: Detection of radiolabeled HptB by autoradiography. Lanes 1−3, the following proteins were incubated with ^{32}P-labeled ATP and monitored over time: lane 1, NahKΔN84 + HptB; lane 2, NahKΔN84 + HptB + ferrous NosP; lane 3, NahKΔN84 + HptB + NO-bound NosP. (D) NahKΔN84DA cannot transfer phosphate to HptB, indicating Asp809 is necessary for phosphotransfer from NahKΔN84 to HptB. Top: Protein loading detected by Coomassie staining. Bottom: Detection of radiolabeled HptB by autoradiography. Lanes 1−6, the following proteins were incubated with ^{32}P-labeled ATP and monitored over time: lane 1, NahKΔN84; lane 2, NahKΔN84 + HptB; lane 3, NahKΔN84 + NO-bound NosP; lane 4, NahKΔN84 + HptB + NO-bound NosP; lane 5, NahKΔN84DA; lane 6, NahKΔN84DA + HptB.

from NahK.[36] Thus, we cloned, expressed, and purified HptB in order to study phosphotransfer from NahK to HptB. Upon incubation of purified HptB with phosphorylated NahK, phosphotransfer is evident, as illustrated in Figure 3C, lane 1. We made a mutant of NahKΔN84, NahKΔN84DA, in which the conserved aspartate (D809) in the receiver domain is mutated to alanine to prevent phosphotransfer from the histidine residue, thus trapping phosphate on the histidine residue. As expected, no phosphotransfer to HptB was observed when NahKΔN84DA was used instead of NahKΔN84 in the phosphorelay assay (Figure 3D).

NosP/NahK Signaling Is NO-Sensitive. We hypothesized that NO/NosP might regulate the kinase and signal transduction activities of NahK. To evaluate this hypothesis, ferrous and NO-bound NosP were added to the phosphorelay assay described above (Figure 3C). Inhibition of NahKΔN84 autophosphorylation was observed when excess NosP, as either the Fe^{II} or the Fe^{II}−NO complex, was added to the phosphorelay assay, but the greatest inhibition of NahKΔN84 was observed in the presence of NO-bound NosP (Figure 3C, lane 3). Indeed, excess (~30-fold) NO-bound NosP is able to completely inhibit phosphotransfer to HptB (Figure 3D, lane 3).

Interestingly, this pattern of kinase inhibition is similar to the inhibition of histidine kinase activity observed in H-NOX/HahK (H-NOX and H-NOX-associated histidine kinase) signaling in *Vibrio harveyi*,[28] *Pseudalteromonas atlantica*,[54] *Shewanella oneidensis*,[26] and *Silicibacter* sp. strain TrichCH4B.[30]

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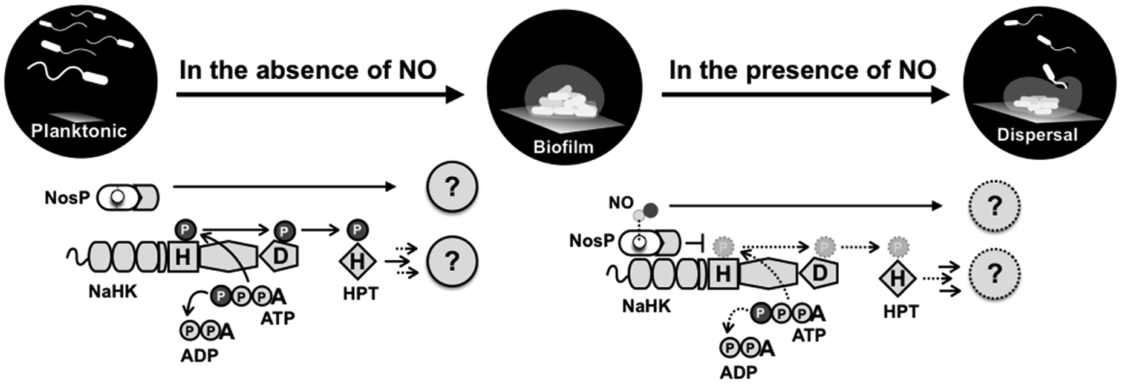

Figure 4. Schematic representation of NO-mediated biofilm dispersal through HptB. In the absence of NO, NosP has no effect on NahK activity. As a result, NahK can autophosphorylate its conserved histidine residue, transfer phosphate intramolecularly to its receiver domain, and then engage in phosphotransfer with the HptB protein (Pa3345). We hypothesize that NosP, HptB, and NahK can interact with effector proteins that are yet to be identified, but their activity leads to biofilm formation. When NO is present, NO/NosP inhibits NahK activity, ultimately yielding a decrease in phosphate flow through the pathway and resulting in biofilm dispersal.

In all of these systems, Fe^{II} H-NOX inhibits HahK activity somewhat and Fe^{II}–NO inhibits it more. This is possibly due to the fact that, in all of these systems, the sensor (H-NOX or NosP) is encoded on a separate polypeptide from its associated kinase. Perhaps, the act of sensor/kinase binding results in some inhibition that is enhanced upon ligation of NO. We hypothesize that in cells the sensor may always be bound to its associated kinase, such that the activity of the Fe^{II} complex is the physiologically relevant baseline kinase activity, which is then inhibited upon NO binding.

We are currently investigating the possibility that rather than inhibiting the kinase activity of NahK, NosP enhances the phosphatase activity of the receiver domain of NahK in the presence of NO-bound NosP. HptB receives phosphate from NahK as well as at least three other kinases [Pa1611, Pa2824 (SagS; surface attachment and growth sensor histidine kinase), and Pa4856] that are modulated by stimuli yet to be identified.[36] Enhanced phosphatase activity of NahK might result in more dramatic modulation of the phosphorylated state of not only HptB but also Pa1611, Pa2824, and/or PA4856 when NO is present in the environment, thus leading to an amplified effect on HptB signaling in the presence of NO.

The accepted signaling mechanism downstream of HptB has not been tied to cyclic-di-GMP regulation, although NO-mediated biofilm dispersal has been linked to cyclic-di-GMP levels.[15] In short, the SagS, NahK, Pa1611, and Pa4856 kinases initiate a phosphorelay cascade in *P. aeruginosa* through HptB to the bifunctional protein Pa3346. Pa3346 possesses both kinase and phosphatase activities, depending on the phosphorylation state of the protein. It has been demonstrated that HptB, through its interaction with Pa3347, can modulate flagella-related gene expression and thus is able to regulate biofilm. According to Hsu et al.,[36] biofilm formation is dependent on HptB and biofilm dispersal is correlated with phosphorylated HptB, which at first seems to be inconsistent with our data.

However, in a recent publication, Xu et al.[55] demonstrated HptB-mediated biofilm regulation in *P. aeruginosa* to be more complicated. The authors showed that a PilZ protein [Pa2799 or HapZ (histidine kinase associated PilZ)] could down-regulate phosphotransfer from SagS (Pa2824) to HptB by directly interacting with the SagS receiver domain. This down-regulation of phosphotransfer was further inhibited in the presence of cyclic-di-GMP. Essentially, SagS phosphorylation of HptB in vitro is significantly reduced in the presence of HapZ or cyclic-di-GMP/HapZ. Based on these results, deletion of HapZ, in principle, should result in increased phospho-HptB, which should result in biofilm formation. Regardless, the Δ*hapZ* mutant was shown to be deficient in biofilm formation, due to an early attachment defect, as demonstrated by a flow cell biofilm assay.[55] Therefore, despite the phosphorylation state of HptB, upon deleting HapZ, a lack of biofilm formation is observed.

Similarly, in our study, we find NO/NosP reduces phosphorelay from NahK to HptB, yet the phenotype we observe is less biofilm formation. Evidently, more proteins must be involved in this biofilm regulation pathway. It is possible that NahK is involved in protein–protein interactions with partners aside from NosP, similar to the situation with SagS described above. It is also possible that NosP or NahK might interact with other kinases and/or effector proteins to modulate total cyclic-di-GMP concentrations downstream of NO sensing. We are currently investigating the possibility of HptB-mediated regulation of receiver domain-containing diguanylate cyclases or cyclic-di-GMP phosphodiesterases, as well as possible NosP interactions with these types of proteins or orphan kinases in *P. aeruginosa*.

With the data presented here, we propose that NosP is a hemoprotein that ligates NO. In the presence of NO, NO/NosP is able to suppress NahK-mediated phosphorelay to HptB, which ultimately leads to modulation of biofilm in *P. aeruginosa* (Figure 4). In conclusion, we have identified a novel family of bacterial primary NO binding proteins and a NO-responsive signaling pathway in *P. aeruginosa* that regulates biofilm formation. Although a role for NO/NosP has yet to be established in organisms other than *P. aeruginosa*, we speculate a possible role for NO/NosP in biofilm regulation in *S. oneidensis*,[26] where the histidine kinase cocistronic with NosP is directly involved in regulating *S. oneidensis* biofilm. Furthermore, NosP is clearly involved in biofilm regulation in *V. cholerae* and in *L. pneumophila*: in *V. cholerae*, a NosP-fused cyclic-di-GMP phosphodiesterase protein has been shown to be involved in *V. cholerae* biofilm formation by regulating cyclic-di-GMP concentrations,[34] and a NosP-*co*-cistronic bifunctional diguanylate cyclase/cyclic-di-GMP phosphodiesterase enzyme has been shown to be involved in *L. pneumophila*[35] biofilm formation. We are currently working to evaluate the role for NO/NosP is these and other bacterial systems.

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METHODS

More detailed methods can be found in the Supporting Information.

Cloning and Genetics. See Table S1. *nosP*, *nosP-NT*, *nahK*, *nahKΔN84*, and *hptB* were each cloned into pET20(b) vectors (Novagen) via the NdeI and XhoI restriction sites. Mutants were generated by site-directed mutagenesis. The PaO1-T7::L1LtrAHK gene disruption strain was constructed according to Yao et al.[56] *nahK* was subcloned into the broad-host-range vector pLJQhis[57] via NdeI and XhoI restriction sites and introduced into PaO1-T7::L1LtrA with the Benchmarks BioTechnique protocol[58] to make the PaO1-T7::L1LtrAHK/pHK strain. All plasmids were confirmed by DNA sequencing.

Protein Expression and Analysis. NosP expression and purification was adapted from Boon et al.,[44] and NahK purification was adapted from Hsu et al.[36] The Anti-6X His-tag antibody (HRP) from Abcam (ab1187) was used according to the manufacturer's specifications for Western blot analysis. Kinase assay conditions were adapted from Hsu et al.[36] Autoradiographs were analyzed with ImageJ software. All UV/vis spectra were recorded on a Cary 100 spectrophotometer equipped with a constant temperature bath. NosP complexes were prepared in an oxygen-free glovebag, and NO dissociation kinetics were measured as previously described.[44]

Heme Agarose Pulldown Assay. Hemin-agarose (Sigma; 40 μL) was equilibrated with 500 μL of assay buffer (20 mM Tris·HCl, 300 mM NaCl, 1 mM PMSF, 1% Triton X-100, pH 8.0). Cleared lysate (1 mL) from an induced, nonspecific protein was used as a control along with lysate from induced NosP. Microcentrifuge tubes containing lysate with beads were then incubated with rocking for 1 h at 4 °C. The beads were collected by centrifugation. The lysate supernatant fraction was discarded, and the beads were washed three times with 1 mL of the assay buffer containing either 10 or 100 mM imidazole. Following the wash steps, 50 μL of SDS loading dye was added directly to the beads and they were boiled for 3 min at 95 °C. Samples were centrifuged again to settle the beads, and 10 μL of the supernatant fraction was analyzed via SDS-PAGE.

Biofilm Analysis. PaO1-T7 wild-type and mutants were analyzed using the microtiter dish assay described elsewhere.[59]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00027.

Additional methods; supplemental figure illustrating the distribution of H-NOX and NosP; and a table listing the strains, plasmids, and primers used in this study (PDF)

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Author Contributions

E.M.B. and S.H. designed the experiments; S.H. performed the experiments, and E.M.B. and S.H. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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