Published in final edited form as:

Acc Chem Res. 2017 July 18; 50(7): 1633–1639. doi:10.1021/acs.accounts.7b00095.

Discovery of Two Bacterial Nitric Oxide-Responsive Proteins and Their Roles in Bacterial Biofilm Regulation

Sajjad Hossain[‡], Lisa-Marie Nisbett[†], and Elizabeth M. Boon^{*,†,‡,§}

[†]Graduate Program in Biochemistry and Structural Biology, Stony Brook University, Stony Brook, New York 11794-5215, United States

[‡]Department of Chemistry, Stony Brook University, Stony Brook, New York 11794-3400, United States

[§]Institute of Chemical Biology & Drug Discovery, Stony Brook University, Stony Brook, New York 11794-3400, United States

CONSPECTUS

Bacterial biofilms form when bacteria adhere to a surface and produce an exopolysaccharide matrix (Costerton et al. *Science* **1999**, *284*, 1318; Davies et al. *Science* **1998**, *280*, 295; Flemming et al. *Nat. Rev. Microbiol.* **2010**, *8*, 623). Because biofilms are resistant to antibiotics, they are problematic in many aspects of human health and welfare, causing, for instance, persistent fouling of medical implants such as catheters and artificial joints (Brunetto et al. *Chimia* **2008**, *62*, 249). They are responsible for chronic infections in the lungs of cystic fibrosis patients and in open wounds, such as those associated with burns and diabetes. They are also a major contributor to hospital-acquired infections (Sievert et al. *Infec. Control Hosp. Epidemiol.* **2013**, *34*, 1; Tatterson et al. *Front. Biosci.* **2001**, *6*, D890).

It has been hypothesized that effective methods of biofilm control will have widespread application (Landini et al. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 813). A promising strategy is to target the mechanisms that drive biofilm dispersal, because dispersal results in biofilm removal and in the restoration of antibiotic sensitivity. First documented in *Nitrosomonas europaea* (Schmidt et al. *J. Bacteriol.* **2004**, *186*, 2781) and the cystic fibrosis-associated pathogen *Pseudomonas aeruginosa* (Barraud et al. *J. Bacteriol.* **2006**, *188*, 7344; *J. Bacteriol.* **2009**, *191*, 7333), regulation of biofilm formation by nanomolar levels of the diatomic gas nitric oxide (NO) has now been documented in numerous bacteria (Barraud et al. *Microb. Biotechnol.* **2009**, *2*, 370; McDougald et al. *Nat. Rev. Microbiol.* **2012**, *10*, 39; Arora et al. *Biochemistry* **2015**, *54*, 3717; Barraud et al. *Curr. Pharm. Des.* **2015**, *21*, 31). NO-mediated pathways are, therefore, promising candidates for biofilm regulation. Characterization of the NO sensors and NO-regulated signaling pathways should allow for rational manipulation of these pathways for therapeutic applications.

Several laboratories, including our own, have shown that a class of NO sensors called H-NOX (heme-nitric oxide or oxygen binding domain) affects biofilm formation by regulating intracellular

*Corresponding Author Elizabeth M. Boon. Phone: (631) 632-7945. Fax: (631) 632-7960. elizabeth.boon@stonybrook.edu.

Notes

The authors declare no competing financial interest.

cyclic di-GMP concentrations and quorum sensing (Arora et al. *Biochemistry* **2015**, *54*, 3717; Plate et al. *Trends Biochem. Sci.* **2013**, *38*, 566; Nisbett et al. *Biochemistry* **2016**, *55*, 4873). Many bacteria that respond to NO do not encode an *hnoX* gene, however. My laboratory has now discovered an additional family of bacterial NO sensors, called NosP (nitric oxide sensing protein). Importantly, NosP domains are widely conserved in bacteria, especially Gram-negative bacteria, where they are encoded as fusions with or in close chromosomal proximity to histidine kinases or cyclic di-GMP synthesis or phosphodiesterase enzyme, consistent with signaling. In this Account, we briefly review NO and HNOX signaling in bacterial biofilms, describe our discovery of the NosP family, and provide support for its role in biofilm regulation in *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Legionella pneumophila*, and *Shewanella oneidensis*.

NITRIC OXIDE SIGNALING IN BACTERIAL BIOFILMS

Bacterial Biofilms

Most bacteria, most of the time, live in a multicellular community called a biofilm.¹⁻⁵ Biofilms are frequently formed on surfaces (e.g., medical implants, tissues, metals, soil, plastics) at the solid–liquid interface, but they can also form at liquid–air interfaces. Bacteria in a biofilm are encased within a selfsecreted exopolysaccharide matrix.⁶ In part due to this protective coating, biofilms are extremely persistent, effective, and tolerant.⁷⁻¹³ For example, biofilms coating human intestines contribute to health through the production of vitamins and digestion of food¹⁴ but also harbor pathogens that lead to inflammatory diseases.¹⁵ Furthermore, biofilm communities are extremely tolerant of antibiotics (~1000-fold more resistant than planktonic cells); thus pathogenic biofilms represent a significant threat to human health and welfare.^{16,17}

Nitric Oxide and Bacterial Biofilms

Nitric oxide (NO) has well documented concentrationdependent functions in eukaryotes. NO is produced by the enzyme NO synthase. When produced at low concentrations, NO acts as a signaling molecule, regulating processes like vasodilation and neurotransmission upon ligation to its sensor, soluble guanylyl cyclase (sGC).¹⁸ Eukaryotes also produce NO at high concentrations to, for example, combat invading pathogens.^{19,20} At these elevated concentrations, eukaryotic cells must detect and respond to NO to prevent host cell damage.¹⁹⁻²²

Growing evidence suggests that bacteria also have concentration-dependent responses to NO. Bacteria experience high concentrations of NO during denitrification, a process by which bacteria respire nitrate or nitrite under oxygen-limiting conditions,²³ as well as during infection of a eukaryotic host, as suggested above. Therefore, characterization of

NO-responsive bacterial proteins such as FNR-like transcription factors (fumarate and nitrate regulatory proteins),²⁴ the NO-responsive transcriptional activator NorR (regulator of NO reductase),²⁵ and the NO-sensitive repressor NsrR (repressor of nitrosative stress)²⁶ have been pursued to understand bacterial responses to elevated levels of NO.

More recently, it has been documented that bacteria also respond to low concentrations of NO. In particular, low levels of NO are linked to regulation of biofilm formation in bacteria.²⁷ NO is implicated in establishing symbiosis between *Euprymna scolopes* and *Vibrio fischeri*,²⁸ and in *Nitrosomonas europaea*²⁹ and *Pseudomonas aeruginosa*,³⁰⁻³³ nanomolar concentrations of NO cause biofilm dispersal. Materials that slowly release NO are linked to antibiofilm properties in model systems.³⁴⁻⁴³ Furthermore, a *P. aeruginosa* mutant that lacks nitrite reductase (*nirS*), which generates NO from nitrite in anaerobic conditions, does not disperse from biofilms, whereas a mutant deficient in NO reductase (*norCB*) exhibits enhanced dispersal.³⁰ Indeed, NO has been shown to affect biofilm formation in a wide variety of both Gram-negative and Gram-positive bacteria.^{27,44-46} Therefore, NO-mediated pathways are promising candidates for biofilm regulation.

NITRIC OXIDE AND H-NOX SIGNALING IN BACTERIAL BIOFILMS

H-NOX (heme-nitric oxide or oxygen binding domain) proteins have now been established as NO sensors in bacteria.^{27,47,48} Our laboratory has been working to characterize the H-NOX family of bacterial NO sensors for 11 years.⁴⁹⁻⁶⁰ H-NOX proteins were discovered based on homology to the mammalian NO sensor, soluble guanylate cyclase,⁶¹ and they are found in the genomes of ~200 species of bacteria. In obligate anaerobes, H-NOX proteins appear to primarily function as molecular oxygen sensors⁶² and are encoded as N- or C-terminal fusions with methyl-accepting chemotaxis proteins.⁶¹ In facultative anaerobes, H-NOX proteins primarily function as NO sensors^{47,48} and are encoded near genes coding for cyclic di-GMP synthases or cyclic di-GMP phosphodiesterases or histidine kinases.⁶¹ Because NO regulates biofilm formation in many bacteria and H-NOX genes are encoded in bacterial genomes adjacent to signaling proteins, we hypothesized that two well established biofilm regulatory mechanisms, intracellular cyclic di-GMP signaling and intercellular quorum sensing, might be regulated by NO binding to *hnoX*-encoded proteins, that is, H-NOXs (Figure 1). Our findings are briefly summarized below.

NO-Bound H-NOX Regulated Cyclic Di-GMP Signaling

It is widely recognized that bis(3'-5')-cyclic-dimeric-guanosine monophosphate (cyclic di-GMP) is a ubiquitous bacterial signaling molecule^{63,64} that balances motility and virulence in individual cells vs cell adhesion and persistence in biofilms. It appears that, at least in a broad sense, the total concentration of intracellular cyclic di-GMP is tightly correlated with biofilm development: as cyclic di-GMP concentrations go up, bacteria enter biofilm, and as cyclic di-GMP concentrations drop, bacteria leave biofilm. The total concentration of intracellular cyclic di-GMP is tightly regulated by a variety of enzymes that both synthesize and degrade cyclic di-GMP.⁶⁵ Cyclic di-GMP is synthesized by proteins with diguanylate cyclase (DGC) activity, identified by a GGDEF motif (a five amino acid conserved sequence) and often called a GGDEF domain.⁶⁶ Proteins with phosphodiesterase (PDE)

activity degrade cyclic di-GMP. These are identified by a conserved EAL⁶⁷ or HDGYP⁶⁸ amino acid motif and referred to as EAL or HD-GYP domains. Although the details of cyclic di-GMP regulation in bacteria are far from completely understood, the current hypothesis is that the activity of GGDEF, EAL, and HD-GYP domains is regulated by specific stimuli.⁶⁹⁻⁷²

In the genomes of many bacteria, especially environmental species, *hnoX* (heme-nitric oxide or oxygen binding domain) genes are located near genes coding for cyclic di-GMP cyclases or phosphodiesterases, leading to the hypothesis that the NO-ligation of H-NOX could regulate biofilm formation through cyclic di-GMP signaling. Indeed, several of these H-NOX/cyclic di-GMP systems have been biochemically characterized, and to varying extents, also biologically characterized, including those from the bacteria *Legionella pneumophila*,⁷³ *Shewanella woodyi*,^{57,58,60} *Shewanella oneidensis*,⁷⁴ and *Agrobacterium vitis*.⁴⁸ Interestingly, in some of these species, NO-bound H-NOX appears to promote biofilm formation, while in other species, it appears to suppress biofilm formation. Specifically, in *L. pneumophila* and *S. oneidensis*, NO signaling through H-NOX is reported to enhance biofilm formation, while in *S. woodyi* and *A. vitis*, NO signaling through H-NOX disperses biofilms. Although the exact NO phenotype in these species vary, it is very clear that in all cases, H-NOX is able to regulate cyclic di-GMP concentrations in response to NO ligation and that these molecular events ultimately affect biofilm formation, as briefly described below.

In *L. pneumophila*,⁷³ *S. woodyi*,^{57,58,60} and *A. vitis*,⁴⁸ NO-bound H-NOX binds to and directly regulates a bifunctional enzyme that affects cyclic di-GMP synthesis and cyclic di-GMP hydrolysis, but again, the details vary. In *L. pneumophila*, the cyclic di-GMP phosphodiesterase domain is degenerate and NO-bound H-NOX inhibits cyclic di-GMP synthesis. In *S. woodyi* and *A. vitis*, both the cyclic di-GMP cyclase and phosphodiesterase domains are active, and in both organisms, NO-bound H-NOX appears to strongly inhibit cyclic di-GMP cyclase activity and substantially increase phosphodiesterase activity.

Finally, in *S. oneidensis*,⁷⁴ as well as *Vibrio cholerae*⁷⁴ and *Pseudoalteromonas atlantica*,⁴⁹ NO-ligated H-NOX binds to and inhibits the activity of a histidine kinase, which subsequently regulates cyclic di-GMP levels through a phosphotransfer signaling pathway. All of these NO and H-NOX-mediated cyclic di-GMP signaling pathways have been previously reviewed,^{27,47,48} so we direct the readers to those reviews for more detailed information on the studies described here.

Discovery and Characterization of NO and H-NOX Regulated Quorum Sensing

Quorum sensing regulates community-wide processes like biofilm formation, virulence gene production, and bioluminescence.⁷⁵ *Vibrio harveyi* has three described quorum sensing circuits. Each involves the synthesis, secretion, and detection of a small molecule called an autoinducer. Autoinducer detection regulates phosphorylation of its cognate receptor-hybrid histidine kinase. Each receptor-kinase exchanges phosphate with a common phosphorelay protein, LuxU, which ultimately regulates bioluminescence through transcription factors named LuxO and LuxR. We have demonstrated that NO participates in quorum sensing through LuxU. In essence, NO enters quorum sensing circuits analogously to an

autoinducer: it binds its cognate receptor (the H-NOX/HqsK complex; HqsK = HNOX-associated quorum sensing kinase) and contributes to the flux of phosphate to and from LuxO (Figure 1). In a subsequent study, we found that H-NOX and HqsK quorum sensing also regulate biofilm and flagella formation in a NO concentration dependent manner.⁵⁵ Furthermore, we have also recently found that a similar NO-regulated quorum sensing circuit is also operational in *Vibrio parahaemolyticus*.⁴⁸

Interestingly, similar H-NOX-regulated histidine kinase signaling pathways have been described in *Vibrio fischeri*⁷⁶ and *Silicibacter* sp. strain *Trich4B*.⁷⁷ In these H-NOX signaling pathways, NO-bound H-NOX appears to regulate the activity of a hybrid histidine kinase that exchanges phosphate with LuxU-like phosphorelay proteins to ultimately control, not quorum sensing, but bacterial/eukaryotic symbiosis. These mechanisms of NO and H-NOX-mediated hybrid histidine kinase signaling pathways have also been reviewed previously,^{27,47,48} so please refer to those manuscripts for more details.

NITRIC OXIDE AND NosP SIGNALING IN BACTERIAL BIOFILMS

As described above, NO has been found to affect biofilm regulation in many bacteria,^{27,44-46} and some bacteria respond to NO via ligation with a H-NOX domain.^{27,47,48} However, there are many examples of organisms that lack an annotated *hnoX* gene in their genome, yet are able to respond to low levels of NO to disperse their biofilms, suggesting the existence of an alternative NO sensor. My laboratory has now discovered an additional family of putative bacterial NO sensors, called NosP (nitric oxide sensing protein). The discovery and preliminary characterization of NosP signaling pathways is described below.

Discovery of NosP and NO and NosP-Mediated Biofilm Formation in *P. aeruginosa*

The opportunistic pathogen *Pseudomonas aeruginosa* has drawn special attention in microbiology because it readily forms biofilms and is the leading cause of death in cystic fibrosis patients as well as a leading cause of hospital-acquired infections.⁷⁸ 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.^{30,31,33} Very little is known about the mechanism underlying NO-mediated biofilm dispersal in *P. aeruginosa*, however. Not surprisingly, it is correlated with increased cyclic di-GMP phosphodiesterase activity, resulting in decreased cyclic di-GMP levels;³¹ as described above, decreased levels of cyclic di-GMP are tightly correlated with biofilm dispersal in many bacterial species.^{64,79-81} Prior to our work, several proteins, including BdlA,^{31,82} GcbA,⁸³ DipA,⁸⁴ and NbdA⁸⁵ had been associated with NO-mediated biofilm dispersal, but none of them had been demonstrated to be a primary NO sensor. Therefore, we set out to discover the primary NO sensor in *P. aeruginosa*.

Using various bioinformatics and biochemical analyses, as well as analysis of published literature,^{32,74,86-90} we identified a novel family of hemoproteins that we termed NosP.⁹¹ NosP domains are widely distributed in bacterial genomes (found in ~800 sequenced genomes) and encoded in putative operons with kinases or cyclic di-GMP synthesis/phosphodiesterase enzymes, suggesting signaling. NosP shares no significant sequence homology with H-NOX.

In *P. aeruginosa*, NosP (PA1975) is in the same operon as kinase PA1976. Prior to our work, PA1976 had previously been implicated in biofilm regulation,³² although the specific stimulus regulating PA1976 activity was not known. We demonstrated that biofilms of a *P. aeruginosa* mutant lacking components of the NosP signaling pathway lose the ability to disperse in response to NO (Figure 2).⁹¹ Upon cloning, expressing, and purifying NosP, we found it binds heme and ligates to NO with a dissociation rate constant that is comparable to other well-established NO-sensing proteins. Moreover, we showed that NO-bound NosP is able to regulate the phosphorelay activity of a hybrid histidine kinase that was previously shown to be involved in biofilm regulation in *P. aeruginosa*.³² Thus, we submit that we have discovered a novel NO-responsive pathway that regulates biofilm in *P. aeruginosa*.

The Role of NO and NosP in Biofilm Formation in Other Organisms

Based on its wide conservation in bacteria, we hypothesize that NosP may be a global biofilm regulator. We have found support of our hypothesis in the literature and preliminary data from our laboratory (Figure 3). We are currently investigating the role of NosP in several organisms in order to test our hypothesis. Below we briefly discuss some of our initial findings.

In *V. cholerae*, a stand-alone NosP domain (VC1444) is predicted to be cocistronic with a histidine kinase (VC1445) that has recently been annotated as a quorum sensing hybrid histidine kinase.^{86,87} Overexpression of this kinase results in a hyperbiofilm phenotype⁸⁹ and a lack of this hybrid histidine kinase results in a phenotype that is identical to quorum sensing mutants.⁸⁶ In our laboratory, we have found that, when ligated to NO, NosP from *V. cholerae* inhibits the autophosphorylation activity of this quorum sensing histidine kinase, suggesting a potential molecular mechanism by which NO may regulate quorum sensing (and thus biofilm formation) in *V. cholerae*.⁹² Interestingly, this would be analogous to the NO and H-NOX/HqsK mediated quorum sensing pathway we discovered in *V. harveyi*.⁵⁴

In *S. oneidensis*, a NosP domain (SO_2542) is predicted to be upstream of a histidine kinase (SO_2543) and cognate response regulator protein (SO_2541) that has been shown to be involved in NO-mediated biofilm regulation through a multicomponent cyclic di-GMP signaling network.⁷⁴ As in *P. aeruginosa*, we have found that NosP from *S. oneidensis* can undergo heme-dependent NO ligation.⁹³ Further, we have found that *S. oneidensis* NosP can regulate the autophosphorylation activity of its cocistronic kinase in a NO dependent manner and that mutant *S. oneidensis* strains that lack either *nosP* or its associated histidine kinase produce immature biofilms. Consequently, we hypothesize that NO and NosP may regulate biofilm formation in *S. oneidensis* by modulating the autokinase activity of this histidine kinase (SO_2543), which in turn modulates the previously described cyclic di-GMP signaling network.⁷⁴

A NosP domain (lpg0279) in *Legionella pneumophila* is coded for in the same operon as a histidine kinase (lpg0278) and a cyclic di-GMP metabolizing enzyme (lpg0277) with a receiver 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,⁸⁸ suggesting involvement of NosP in biofilm regulation.

Similar to the results described above for NosP in *P. aeruginosa* and *V. cholerae*, we have found that NO-ligated NosP from *L. pneumophila* inhibits the autokinase activity of its cocistronic histidine kinase. This kinase normally phosphorylates a bifunctional cyclic di-GMP processing enzyme that is also in the same putative operon. We hypothesize that this change will ultimately affect the intracellular cyclic di-GMP, and thus the biofilm, levels of this bacterium in the presence of NO.

PERSPECTIVES AND FUTURE DIRECTIONS

Our lab is making the case that NosP is a newly discovered NO binding protein involved in bacterial biofilm regulation. To date, we have found that NosP domains exhibit slow NO dissociation kinetics and selectively bind NO and CO, but not molecular oxygen. Additionally, we have also observed that NO bound NosP domains are effective regulators of the activities of their cocistronic partner proteins, thus underscoring a role for these domains as physiological NO sensing hemoproteins.

Interestingly, although the NosP and H-NOX domains are generally not coded for in the same bacteria, there are a few exceptions to this generalization, which raises the questions of if and how they both function as NO sensors. In *S. oneidensis*, remarkably, not only does this bacterium possess both HNOX and NosP domains but also the respective H-NOX- and NosP-associated signaling pathways have been demonstrated to participate in the same multicomponent cyclic di-GMP signaling network, which has been shown to regulate biofilm formation in this bacterium.⁷⁴ We are currently investigating the interaction between NosP and H-NOX in *S. oneidensis*, as well as in *V. cholerae* and *L. pneumophila*.

Finally, although we have identified two separate families of bacterial NO responsive proteins, H-NOX and NosP, there are still bacteria, specifically many Gram-positive bacteria, for which there are known NO-mediated biofilm regulation phenotypes but neither H-NOX nor NosP domains are encoded in their genomes. Consequently, the identity of the putative primary NO sensor(s) and the associated NO-responsive signaling pathway(s), as well as the molecular mechanism of NO regulation in these bacteria, are all currently unknown. Since the field of NO signaling in bacteria is rapidly expanding, however, it is our hope that these gaps in our understanding of NO signaling in bacteria will be addressed in the near future.

Acknowledgments

This work was supported by the Stony Wold-Herbert Fund, the National Science Foundation, (Grant CHE-1607532 to E.M.B.), and the National Institutes of Health (Grant GM118894-01A1 to E.M.B.). We also thank Dr. Roger Johnson and the Boon Group for helpful discussions.

References

1. Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev. 2000; 64:847–867. [PubMed: 11104821]
2. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. Nat Rev Microbiol. 2004; 2:95–108. [PubMed: 15040259]
3. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annu Rev Microbiol. 2002; 56:187–209. [PubMed: 12142477]

4. Webb JS, Givskov M, Kjelleberg S. Bacterial biofilms: prokaryotic adventures in multicellularity. *Curr Opin Microbiol.* 2003; 6:578–585. [PubMed: 14662353]
5. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Annu Rev Microbiol.* 2000; 54:49–79. [PubMed: 11018124]
6. Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol.* 2010; 8:623–633. [PubMed: 20676145]
7. Brunetto PS, Fromm KM. Antimicrobial coatings for implant surfaces. *Chimia.* 2008; 62:249–252.
8. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, Fridkin S. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. *Infect Control Hosp Epidemiol.* 2013; 34:1–14. [PubMed: 23221186]
9. Landini P, Antoniani D, Burgess JG, Nijland R. Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Appl Microbiol Biotechnol.* 2010; 86:813–823. [PubMed: 20165945]
10. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science.* 1998; 280:295–298. [PubMed: 9535661]
11. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science.* 1999; 284:1318–1322. [PubMed: 10334980]
12. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev.* 2002; 15:167–193. [PubMed: 11932229]
13. Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. *Trends Microbiol.* 2005; 13:34–40. [PubMed: 15639630]
14. Van Wey AS, Cookson AL, Roy NC, McNabb WC, Soboleva TK, Shorten PR. Bacterial biofilms associated with food particles in the human large bowel. *Mol Nutr Food Res.* 2011; 55:969–978. [PubMed: 21638777]
15. Swidsinski A, Weber J, Loening-Baucke V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol.* 2005; 43:3380–3389. [PubMed: 16000463]
16. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet.* 2001; 358:135–138. [PubMed: 11463434]
17. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents.* 2010; 35:322–332. [PubMed: 20149602]
18. Denninger JW, Marletta MA. Guanylate cyclase and the NO/cGMP signaling pathway. *Biochim Biophys Acta, Bioenerg.* 1999; 1411:334–350.
19. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol.* 1997; 15:323–350. [PubMed: 9143691]
20. Bogdan C. Nitric oxide and the immune response. *Nat Immunol.* 2001; 2:907–916. [PubMed: 11577346]
21. Connelly L, Palacios-Callender M, Ameixa C, Moncada S, Hobbs AJ. Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. *J Immunol.* 2001; 166:3873–3881. [PubMed: 11238631]
22. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev.* 2007; 87:315–424. [PubMed: 17237348]
23. Poole RK. Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem Soc Trans.* 2005; 33:176–180. [PubMed: 15667299]
24. Cruz-Ramos H, Crack J, Wu G, Hughes MN, Scott C, Thomson AJ, Green J, Poole RK. NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. *EMBO J.* 2002; 21:3235–3244. [PubMed: 12093725]
25. D'Autreaux B, Tucker NP, Dixon R, Spiro S. A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature.* 2005; 437:769–772. [PubMed: 16193057]
26. Bodenmiller DM, Spiro S. The yjeB (nsrR) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. *J Bacteriol.* 2006; 188:874–881. [PubMed: 16428390]

27. Arora DP, Hossain S, Xu Y, Boon EM. Nitric oxide regulation of bacterial biofilms. *Biochemistry*. 2015; 54:3717–3728. [PubMed: 25996573]
28. Davidson S, Koropatnick T, Kossmehl R, Sycuro L, McFall-Ngai M. NO means ‘yes’ in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell Microbiol*. 2004; 6:1139–1151. [PubMed: 15527494]
29. Schmidt I, Steenbakkers P, op den Camp H, Schmidt K, Jetten M. Physiologic and proteomic evidence for a role of nitric oxide in biofilm formation by *Nitrosomonas europaea* and other ammonia oxidizers. *J Bacteriol*. 2004; 186:2781–2788. [PubMed: 15090520]
30. Barraud N, Hassett DJ, Hwang S-H, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol*. 2006; 188:7344–7353. [PubMed: 17050922]
31. Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, Kjelleberg S. Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic diguanosine-5'-monophosphate levels and enhanced dispersal. *J Bacteriol*. 2009; 191:7333–7342. [PubMed: 19801410]
32. Hsu JL, Chen HC, Peng HL, Chang HY. Characterization of the histidine-containing phosphotransfer protein B-mediated multistep phosphorelay system in *Pseudomonas aeruginosa* PAO1. *J Biol Chem*. 2008; 283:9933–9944. [PubMed: 18256026]
33. Tattersall LE, Poschet JF, Firoved A, Skidmore J, Deretic V. CFTR and pseudomonas infections in cystic fibrosis. *Front Biosci Landmark Ed*. 2001; 6:D890.
34. Carpenter AW, Schoenfisch MH. Nitric oxide release: Part II. Therapeutic applications. *Chem Soc Rev*. 2012; 41:3742–3752. [PubMed: 22362384]
35. Barraud N, Kardak BG, Yepuri NR, Howlin RP, Webb JS, Faust SN, Kjelleberg S, Rice SA, Kelso MJ. Cephalosporin-3'-diazoniumdiolates: targeted NO-donor prodrugs for dispersing bacterial biofilms. *Angew Chem, Int Ed*. 2012; 51:9057–9060.
36. Duong HTT, Adnan NNM, Barraud N, Basuki JS, Kutty SK, Jung K, Kumar N, Davis TP, Boyer C. Functional gold nanoparticles for the storage and controlled release of nitric oxide: applications in biofilm dispersal and intracellular delivery. *J Mater Chem B*. 2014; 2:5003–5011.
37. Duong HTT, Jung K, Kutty SK, Agustina S, Adnan NNM, Basuki JS, Kumar N, Davis TP, Barraud N, Boyer C. Nanoparticle (Star Polymer) Delivery of Nitric Oxide Effectively Negates *Pseudomonas aeruginosa* Biofilm Formation. *Biomacromolecules*. 2014; 15:2583–2589. [PubMed: 24915286]
38. Engelsman AF, Krom BP, Busscher HJ, van Dam GM, Ploeg RJ, van der Mei HC. Antimicrobial effects of an NO-releasing poly(ethylene vinylacetate) coating on soft-tissue implants in vitro and in a murine model. *Acta Biomater*. 2009; 5:1905–1910. [PubMed: 19251498]
39. Hetrick EM, Shin JH, Paul HS, Schoenfisch MH. Antibiofilm efficacy of nitric oxide-releasing silica nanoparticles. *Biomaterials*. 2009; 30:2782–2789. [PubMed: 19233464]
40. Lu Y, Slomberg DL, Schoenfisch MH. Nitric oxide-releasing chitosan oligosaccharides as antibacterial agents. *Biomaterials*. 2014; 35:1716–1724. [PubMed: 24268196]
41. Lu Y, Slomberg DL, Shah A, Schoenfisch MH. Nitric Oxide-Releasing Amphiphilic Poly(amidoamine) (PAMAM) Dendrimers as Antibacterial Agents. *Biomacromolecules*. 2013; 14:3589–3598. [PubMed: 23962307]
42. Parzuchowski PG, Frost MC, Meyerhoff ME. Synthesis and Characterization of Polymethacrylate-Based Nitric Oxide Donors. *J Am Chem Soc*. 2002; 124:12182–12191. [PubMed: 12371858]
43. Slomberg DL, Lu Y, Broadnax AD, Hunter RA, Carpenter AW, Schoenfisch MH. Role of Size and Shape on Biofilm Eradication for Nitric Oxide-Releasing Silica Nanoparticles. *ACS Appl Mater Interfaces*. 2013; 5:9322–9329. [PubMed: 24006838]
44. Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, Kjelleberg S. Nitric oxide-mediated dispersal in single- and multispecies biofilms of clinically and industrially relevant microorganisms. *Microb Biotechnol*. 2009; 2:370–378. [PubMed: 21261931]
45. McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol*. 2012; 10:39–50.

46. Barraud N, Kelso MJ, Rice SA, Kjelleberg S. Nitric oxide: a key mediator of biofilm dispersal with applications in infectious diseases. *Curr Pharm Des.* 2015; 21:31–42. [PubMed: 25189865]
47. Plate L, Marletta MA. Nitric oxide-sensing H-NOX proteins govern bacterial communal behavior. *Trends Biochem Sci.* 2013; 38:566–575. [PubMed: 24113192]
48. Nisbett LM, Boon EM. Nitric Oxide Regulation of H-NOX Signaling Pathways in Bacteria. *Biochemistry.* 2016; 55:4873–4884. [PubMed: 27479081]
49. Arora DP, Boon EM. Nitric oxide regulated two-component signaling in *Pseudoalteromonas atlantica*. *Biochem Biophys Res Commun.* 2012; 421:521–526. [PubMed: 22521885]
50. Arora DP, Boon EM. Unexpected biotinylation using ATPgamma- Biotin-LC-PEO-amine as a kinase substrate. *Biochem Biophys Res Commun.* 2013; 432:287–290. [PubMed: 23399564]
51. Dai Z, Boon EM. Engineering of the heme pocket of an HNOX domain for direct cyanide detection and quantification. *J Am Chem Soc.* 2010; 132:11496–11503. [PubMed: 20684546]
52. Dai Z, Boon EM. Probing the local electronic and geometric properties of the heme iron center in a H-NOX domain. *J Inorg Biochem.* 2011; 105:784–792. [PubMed: 21497576]
53. Dai Z, Farquhar ER, Arora DP, Boon EM. Is histidine dissociation a critical component of the NO/H-NOX signaling mechanism? Insights from X-ray absorption spectroscopy. *Dalton Trans.* 2012; 41:7984–7993. [PubMed: 22430114]
54. Henares BM, Higgins KE, Boon EM. Discovery of a Nitric Oxide Responsive Quorum Sensing Circuit in *Vibrio harveyi*. *ACS Chem Biol.* 2012; 7:1331–1336. [PubMed: 22606970]
55. Henares BM, Xu Y, Boon EM. A Nitric Oxide-Responsive Quorum Sensing Circuit in *Vibrio harveyi* Regulates Flagella Production and Biofilm Formation. *Int J Mol Sci.* 2013; 14:16473–16484. [PubMed: 23965964]
56. Kosowicz JG, Boon EM. Insights into the distal heme pocket of H-NOX using fluoride as a probe for H-bonding interactions. *J Inorg Biochem.* 2013; 126:91–95. [PubMed: 23792914]
57. Liu N, Pak T, Boon EM. Characterization of a diguanylate cyclase from *Shewanella woodyi* with cyclase and phosphodiesterase activities. *Mol Biosyst.* 2010; 6:1561–1564. [PubMed: 20467666]
58. Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA, Boon EM. Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry.* 2012; 51:2087–2099. [PubMed: 22360279]
59. Muralidharan S, Boon EM. Heme flattening is sufficient for signal transduction in the H-NOX family. *J Am Chem Soc.* 2012; 134:2044–2046. [PubMed: 22257139]
60. Lahiri T, Luan B, Raleigh DP, Boon EM. A structural basis for the regulation of an H-NOX-associated cyclic-di-GMP synthase/phosphodiesterase enzyme by nitric oxide-bound H-NOX. *Biochemistry.* 2014; 53:2126–2135. [PubMed: 24628400]
61. Iyer L, Anantharaman V, Aravind L. Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins. *BMC Genomics.* 2003; 4:5. [PubMed: 12590654]
62. Hespen CW, Bruegger JJ, Phillips-Piro CM, Marletta MA. Structural and Functional Evidence Indicates Selective Oxygen Signaling in *Caldanaerobacter subterraneus* H-NOX. *ACS Chem Biol.* 2016; 11:2337–2346. [PubMed: 27328180]
63. Romling U. Cyclic di-GMP, an established secondary messenger still speeding up. *Environ Microbiol.* 2012; 14:1817. [PubMed: 22040037]
64. Romling U, Galperin MY, Gomelsky M. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev.* 2013; 77:1–52. [PubMed: 23471616]
65. Simm R, Morr M, Kader A, Nimtz M, Romling U. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol.* 2004; 53:1123–1134. [PubMed: 15306016]
66. Ausmees N, Mayer R, Weinhouse H, Volman G, Amikam D, Benziman M, Lindberg M. Genetic data indicate that proteins containing the GGDEF domain possess diguanylate cyclase activity. *FEMS Microbiol Lett.* 2001; 204:163–167. [PubMed: 11682196]
67. Tischler AD, Camilli A. Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. *Mol Microbiol.* 2004; 53:857–869. [PubMed: 15255898]

68. Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He YW, Zhang LH, Heeb S, Camara M, Williams P, Dow JM. Cell-cell signaling in *Xanthomonas campestris* involves an HDGYP domain protein that functions in cyclic di-GMP turnover. *Proc Natl Acad Sci U S A*. 2006; 103:6712–6717. [PubMed: 16611728]
69. Rajagopal S, Key JM, Purcell EB, Boerema DJ, Moffat K. Purification and initial characterization of a putative blue lightregulated phosphodiesterase from *Escherichia coli*. *Photochem Photobiol*. 2004; 80:542–547. [PubMed: 15453820]
70. Karatan E, Duncan TR, Watnick PI. NspS, a predicted polyamine sensor, mediates activation of *Vibrio cholerae* biofilm formation by norspermidine. *J Bacteriol*. 2005; 187:7434–7443. [PubMed: 16237027]
71. Chang AL, Tuckerman JR, Gonzalez G, Mayer R, Weinhouse H, Volman G, Amikam D, Benziman M, Gilles-Gonzalez MA. Phosphodiesterase A1, a regulator of cellulose synthesis in *Acetobacter xylinum*, is a heme-based sensor. *Biochemistry*. 2001; 40:3420–3426. [PubMed: 11297407]
72. Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. Involvement of Nitric Oxide in Biofilm Dispersal of *Pseudomonas aeruginosa*. *J Bacteriol*. 2006; 188:7344–7353. [PubMed: 17050922]
73. Carlson HK, Vance RE, Marletta MA. H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Mol Microbiol*. 2010; 77:930–942. [PubMed: 20572940]
74. Plate L, Marletta MA. Nitric Oxide Modulates Bacterial Biofilm Formation through a Multicomponent Cyclic-di-GMP Signaling Network. *Mol Cell*. 2012; 46:449–460. [PubMed: 22542454]
75. Ng WL, Bassler BL. Bacterial quorum-sensing network architectures. *Annu Rev Genet*. 2009; 43:197–222. [PubMed: 19686078]
76. Wang Y, Dufour YS, Carlson HK, Donohue TJ, Marletta MA, Ruby EG. H-NOX-mediated nitric oxide sensing modulates symbiotic colonization by *Vibrio fischeri*. *Proc Natl Acad Sci U S A*. 2010; 107:8375–8380. [PubMed: 20404170]
77. Rao M, Smith BC, Marletta MA. Nitric Oxide Mediates Biofilm Formation and Symbiosis in *Silicibacter* sp. Strain TrichCH4B. *mBio*. 2015; 6:e00206–15. [PubMed: 25944856]
78. Pierce GE. *Pseudomonas aeruginosa*, *Candida albicans*, and device-related nosocomial infections: implications, trends, and potential approaches for control. *J Ind Microbiol Biotechnol*. 2005; 32:309–318. [PubMed: 15868157]
79. Hengge R. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol*. 2009; 7:263–273. [PubMed: 19287449]
80. Jenal U, Malone J. Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet*. 2006; 40:385–407. [PubMed: 16895465]
81. Romling U, Gomelsky M, Galperin MY. C-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol*. 2005; 57:629–639. [PubMed: 16045609]
82. Morgan R, Kohn S, Hwang SH, Hassett DJ, Sauer K. BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *J Bacteriol*. 2006; 188:7335–7343. [PubMed: 17050921]
83. Petrova OE, Cherny KE, Sauer K. The diguanylate cyclase GcbA facilitates *Pseudomonas aeruginosa* biofilm dispersion by activating BdlA. *J Bacteriol*. 2015; 197:174–187. [PubMed: 25331436]
84. Roy AB, Petrova OE, Sauer K. The phosphodiesterase DipA (PA5017) is essential for *Pseudomonas aeruginosa* biofilm dispersion. *J Bacteriol*. 2012; 194:2904–2915. [PubMed: 22493016]
85. Li Y, Heine S, Entian M, Sauer K, Frankenberg-Dinkel N. NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. *J Bacteriol*. 2013; 195:3531–3542. [PubMed: 23729646]
86. Jung SA, Chapman CA, Ng WL. Quadruple Quorum- Sensing Inputs Control *Vibrio cholerae* Virulence and Maintain System Robustness. *PLoS Pathog*. 2015; 11:e1004837. [PubMed: 25874462]
87. Jung SA, Hawver LA, Ng WL. Parallel quorum sensing signaling pathways in *Vibrio cholerae*. *Curr Genet*. 2016; 62:255–260. [PubMed: 26545759]

88. Pecastaings S, Allombert J, Lajoie B, Doublet P, Roques C, Vianney A. New insights into *Legionella pneumophila* biofilm regulation by c-di-GMP signaling. *Biofouling*. 2016; 32:935–948. [PubMed: 27494738]
89. Shikuma NJ, Fong JCN, Odell LS, Perchuk BS, Laub MT, Yildiz FH. Overexpression of VpsS, a Hybrid Sensor Kinase, Enhances Biofilm Formation in *Vibrio cholerae*. *J Bacteriol*. 2009; 191:5147–5158. [PubMed: 19525342]
90. Borziak K, Zhulin IB. FIST: a sensory domain for diverse signal transduction pathways in prokaryotes and ubiquitin signaling in eukaryotes. *Bioinformatics*. 2007; 23:2518–2521. [PubMed: 17855421]
91. Hossain S, Boon EM. Discovery of a Novel Nitric Oxide Binding Protein and Nitric-Oxide-Responsive Signaling Pathway in *Pseudomonas aeruginosa*. *ACS Infect Dis*. 2017; 3:454. [PubMed: 28238256]
92. Hossain S, Heckler I, Boon EM. Discovery of a Nitric Oxide-Responsive Quorum Sensing Circuit in *Vibrio cholerae*. 2017 manuscript in preparation.
93. Binnenkade L, Nisbett L-M, Bacon B, Hossain S, Arora DP, Muralidharan S, Thormann K, Gralnick JA, Boon EM. Elucidating the role of a NosP signaling pathway in regulating c-di-GMP concentration and biofilm formation in *Shewanella oneidensis*. 2017 manuscript in preparation.

Biographies

Sajjad Hossain obtained his Bachelor's in Biology and Master's in Biotechnology from Hunter College in 2003 and 2004, respectively. He worked as a research assistant on testicular cancer at Lindsley F. Kimball Research Institute New York from 2004 to 2007 and recently received his Ph.D. in Molecular and Cellular Biology from Stony Brook University under the guidance of Elizabeth Boon. He is currently a Postdoctoral Fellow in Dr. Elizabeth Boon's lab and is working to discover novel bacterial signal transduction pathways that are responsive to nitric oxide.

Lisa-Marie Nisbett received both her B.S. and M.S. degrees in Biology from the LIU Post Campus (formerly known as the C.W. Post Campus) of Long Island University in 2009 and 2012, respectively. For her M.S., she worked on assessing the antiviral efficacy of DABCO-hydrocarbon modified cloths against influenza virus in MDCK cells. Currently, she is a Ph.D. candidate working on the biochemical characterization of NosP signaling pathways in various bacteria.

Elizabeth M. Boon received her A.B. with Highest Honors in Chemistry from Kenyon College in 1997 and her Ph.D. from the California Institute of Technology in 2003. She was a NIH Postdoctoral Fellow at the University of California, Berkeley, before starting at Stony Brook University in 2006. She has received several awards including the Presidential Early Career Award for Scientists and Engineers (PECASE), the NYSTAR Watson Young Investigator Award, the Office of Naval Research Young Investigator Award, and the Rising Star Award from the Research Foundation of the State University of New York. In 2011, the Kavli Foundation and the National Academy of Sciences elected Liz a Kavli Fellow.

Figure 1.

NO ligated H-NOX has been shown to regulate biofilm formation through several biochemical mechanisms. (A) NO-bound H-NOX in *V. harveyi* inhibits the autokinase activity of a cocistronic hybrid quorum sensing histidine kinase. (B) NO-bound H-NOX in *S. woodyi* regulates the cyclic di-GMP output of a cocistronic bifunctional cyclic di-GMP synthase and cyclic di-GMP phosphodiesterase. (C) NO-bound H-NOX in *S. oneidensis* inhibits the autokinase activity of its cocistronic histidine kinase, which results in a change in cyclic di-GMP levels due to a decrease in the phosphorylation levels of the cognate response regulators HnoB, HnoC, and HnoD.

Figure 2.

NO ligated NosP has been shown to regulate biofilm formation in *P. aeruginosa* through regulation of a phosphorelay pathway. NO-bound NosP in *P. aeruginosa* inhibits the autokinase activity of a cocistronic hybrid histidine kinase, which alters the phosphate flux in a downstream signaling pathway, ultimately affecting biofilm formation in this bacterium.

Figure 3.

NO ligated NosP has been predicted to regulate biofilm formation through several biochemical mechanisms. (A) NO-bound NosP in *V. cholerae* inhibits the autokinase activity of its cocistronic histidine kinase. (B) NO-bound NosP in *L. pneumophila* inhibits the autokinase activity of its cocistronic histidine kinase. (C) NO-bound NosP in *S. oneidensis* inhibits the autokinase activity of its cocistronic histidine kinase, which also should result in a change in the cyclic di- GMP levels due to a decrease in phosphorylation levels of the cognate response regulators HnoB, HnoC, and HnoD.