

Stony Brook University



OFFICIAL COPY

The official electronic file of this thesis or dissertation is maintained by the University Libraries on behalf of The Graduate School at Stony Brook University.

© All Rights Reserved by Author.

Discovery of a Novel Nitric Oxide Sensing Protein, NosP

A Dissertation Presented

by

Sajjad Hossain

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Molecular and Cellular Biology

Stony Brook University

December 2016

Stony Brook University

The Graduate School

Sajjad Hossain

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Elizabeth M. Boon, PhD – Dissertation Advisor

Associate Professor, Department of Chemistry

Jessica C. Seeliger, PhD - Chairperson of Defense

Assistant Professor, Department of Chemistry

Erwin London, PhD – Committee Member of Defense

Professor, Department of Biochemistry and Cell Biology

David Green, PhD – Committee Member of Defense

Associate Professor, Department of Applied Mathematics and Statistics

Roger Johnson, PhD – Outside Committee Member of Defense

Professor Emeritus, Department of Physiology and Biophysics

This dissertation is accepted by the Graduate School

Charles Taber

Dean of the Graduate School

Abstract of Dissertation

Discovery of a Novel Nitric Oxide Sensing Protein, NosP

by

Sajjad Hossain

Doctor of Philosophy

in

Molecular and Cellular Biology

Stony Brook University

2016

In nature, most bacteria live in surface attached communities called bacterial biofilms. Community living is beneficial to the bacteria, as it offers heightened resistance to environmental stresses due to the production of a protective exopolymeric matrix. When the growth of a bacterial community reaches a certain threshold, some bacteria disperse to find new frontiers to occupy and repopulate. Bacterial biofilms can be beneficial as is the case with those found in our gastrointestinal tracks that help us digest food particles. However, biofilms can pose a major threat to human health when they are composed of pathogenic bacteria that cause chronic infections. In order for bacteria to switch between a free-swimming and biofilm state, they respond to various environmental stimuli, including nitric oxide (NO), a diatomic gas molecule that has been shown to modulate biofilm formation in many bacteria. In some bacteria, NO is sensed by the H-NOX (heme nitric oxide/oxygen binding protein) protein, a homologue of a mammalian nitric oxide sensor, the heme containing soluble guanylate cyclase (sGC). However, many bacteria that respond to NO, including the opportunistic

pathogen *Pseudomonas aeruginosa*, lack an *hnoX* gene; therefore, suggesting the presence of an alternative NO sensing protein.

In this dissertation, I present the discovery of a novel NO sensing protein (NosP). NosPs, like H-NOX proteins, are typically organized within bacterial genomes in operons with signaling proteins that lack known sensing modules. We demonstrate that *Pseudomonas aeruginosa* NosP is able to ligate to NO via the ferrous iron of a heme cofactor. By disrupting a NosP associated histidine kinase, I illustrate a defect in NO-mediated biofilm dispersal in *Pseudomonas aeruginosa*. Further, I investigated the pattern of NosP and NosP associated effector protein signaling biochemically within *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Legionella pneumophila*. Finally, I characterized NosPs from *Vibrio harveyi* and *Shewanella woodyi* spectroscopically to identify similarities of their ligand binding properties. Considering all the findings, I propose that NosP is a primary NO sensor that shares no sequence homology with the H-NOX proteins. Thus, for the first time, I highlight a novel NO signaling pathway in bacteria, providing a strong foundation for future research.

Because you are, I am.

To my parents.

Table of Contents

List of Figures	X
List of Tables	XII
List of Abbreviations	XIII
List of Symbols	XIV
Acknowledgements	XV
Publications	XVI
Chapter 1- Nitric Oxide and Bacterial Biofilm	1
Abstract	1
Nitric oxide as a signaling molecule	2
Bacterial biofilms	5
Two-component signal transduction	7
Quorum sensing	7
Cyclic-di-GMP signaling	8
Nitric oxide regulation of biofilm formation	10
NO regulation of a biofilm in <i>Nitrosomonas europaea</i>	10
NO regulation of a biofilm in <i>Pseudomonas aeruginosa</i>	12
Other examples of clinically relevant NO-regulated biofilm formation	14
A primary NO sensor in bacteria: the H-NOX family	17
NO/H-NOX regulation of biofilm formation in <i>L. pneumophila</i>	18
NO/H-NOX regulation of biofilm formation in <i>S. woodyi</i>	19
NO/H-NOX regulation of biofilm formation in <i>S. oneidensis</i>	19
NO/H-NOX regulation of quorum sensing and biofilm formation in <i>V. harveyi</i>	21
NO/H-NOX regulation of symbiosis in <i>Vibrio fischeri</i>	22
Synthesis of nitric oxide in bacteria	23
NOS and NO/H-NOX/HaHK signaling and bacterial symbiosis	25
References	27

Chapter 2- NosP: Discovery of a novel nitric oxide sensor and nitric oxide-responsive signaling pathway in <i>Pseudomonas aeruginosa</i>	40
Abstract	40
Introduction	41
Results and discussion	43
Discovery of NosP	43
Purified NosP shows ligand-binding properties that are consistent with NO sensing	45
The NosP N-terminal domain is sufficient for heme binding	48
The NosP NO dissociation rate is slow	48
NO-mediated biofilm dispersal requires NaHK	50
Pa1976 is a NosP-associated histidine kinase	52
NosP/NaHK signaling is NO sensitive	55
Conclusion	58
Materials and methods	58
Cloning genes for heterogenous expression and protein purification from <i>E. coli</i>	58
Generation of PaO1-T7::L1LtrAHK	59
Generation of PaO1-T7::L1LtrAHK/pHK	59
Protein expression and purification	61
Expression and purification of other proteins	62
Western blot	63
Kinase assay and autoradiography	63
Electronic spectroscopy	64
Heme agarose pulldown assay	64
Biofilm assay	64
References	66
Chapter 3- Discovery of a nitric oxide sensitive quorum sensing circuit in <i>Vibrio cholerae</i>	73
Abstract	73
Introduction	74

Results and discussion	78
Purified VcNosP exhibits ligand-binding properties that are consistent with NO sensing	78
Vc1445 transfers phosphate to VcLuxU	81
NosP/NqsK signaling is NO sensitive	83
Quorum sensing and cyclic di-GMP in <i>V. cholerae</i>	85
Vc0130 is a hemoprotein	86
Vc0130 is an active PDE <i>in vitro</i>	86
Conclusion	88
Materials and methods	89
Cloning	89
Protein expression and purification	89
Expression and purification of NosPs	89
Expression and purification of other proteins	89
UV-Visible spectroscopy	91
Kinase assay	91
Phosphodiesterase assays	91
References	93
Chapter 4- NosP in <i>Legionella pneumophila</i> is involved in cyclic di-GMP metabolism	98
Abstract	98
Introduction	99
Results and discussion	101
LpgNosP ligates to NO	101
Lpg0278 is an active Kinase	102
Lpg0278 transfers phosphate to Lpg0277	102
NO ligated LpgNosP (Lpg0279) inhibits autophosphorylation of Lpg0278	103
Conclusion	103
Materials and methods	108
Cloning	108

Protein expression and purification	109
Expression and purification of NosP	109
Expression and purification of other proteins	109
UV-Visible spectroscopy	109
Kinase assay	110
Cyclase/phosphodiesterase assay	110
References	112
Chapter 5- Final thoughts	114
Abstract	114
A novel nitric oxide sensor in bacteria: NosP	115
Do all NosP domains sense NO?	116
Is there a need for multiple NO sensors?	117
What of other NO sensors?	118
Science and I	118

List of Figures

Chapter 1

- Figure 1.1: Generation of nitric oxide in mammalian system. 2
- Figure 1.2: Schematic of bacterial biofilm. 6
- Figure 1.3: NO regulates biofilm formation through ligation to H-NOX. 18

Chapter 2

- Figure 2.1: Distribution of H-NOX and NosP. 44
- Figure 2.2: NosP is a hemoprotein that ligates NO. 47
- Figure 2.3: NO mediated biofilm dispersal requires NaHK. 51
- Figure 2.4. NO/NosP regulates NaHK. 54
- Figure 2.5. Schematic representation of NO-mediated biofilm dispersal through HptB. 57

Chapter 3

- Figure 3.1: Schematic of QS circuit in *V. cholerae*. 75
- Figure 3.2: UV-Visible spectra of VcNosP at unlighted and ligated states. 79
- Figure 3.3: NO/VcNosP inhibits autophosphorylation of NqsK. 82
- Figure 3.4: Operon organization. 84
- Figure 3.5: Alignment of DGC domains. 85
- Figure 3.6: Cyclic di-GMP hydrolysis assay. 87

Chapter 4

- Figure 4.1: H-NOX and NosP in *Legionella pneumophila*. 100
- Figure 4.2: Spectroscopic characterization of LpgNosP. 102
- Figure 4.3: NO/LpgNosP suppresses NaHK phosphorylation. 104
- Figure 4.4: Cyclase and phosphodiesterase activity of NarR. 105
- Figure 4.5: Protein alignment. 107

Chapter 5

Figure 5.1: General mechanism of NO ligated NosP. 115

Figure 5.2: Comparison of *V. cholerae* and *V. harveyi* . 117

List of tables

Chapter 1

Table 1.1. The effect of nitric oxide on bacterial biofilms	11
---	----

Chapter 2

Table 2.1. UV/Vis Peak Positions and NO disassociation kinetics	49
---	----

Table 2.2. Strains, Plasmids, and Primers Used	60
--	----

Chapter 3

Table 3.1. UV/Vis Peak Positions and NO disassociation kinetics	80
---	----

Table 3.2. Strains, Plasmids, and Primers Used	90
--	----

Chapter 4

Table 4.1. UV-Visible of NosPs	103
--------------------------------	-----


















Table 4.2. Strains, Plasmids, and Primers Used	108
--	-----

Table 4.3. Reactions involved in Malachite Green Assay	110
--	-----

List of Abbreviations

ALA – β -aminolevulinic acid
ATP – Adenosine-5'-triphosphate
ATP-biotin - ATP- γ -Biotin-LC-PEO-amine
c-di-GMP, cyclic-di-GMP – bis-(3',5')-cyclic dimeric guanosine monophosphate
cGMP – Guanosine-3',5'-cyclic phosphate
CV – Crystal violet
DGC – Diguanylate Cyclase
DNA – Deoxyribonucleic acid
DETA – Diethylenetriamine
EPS – Extracellular polysaccharide
Fe(II) – Ferrous
Fe(III) – Ferric
FMM – Filtered marine media
GTP – Guanosine-5'-triphosphate
H-NOX – Heme nitric oxide/oxygen binding domain
Hace – H-NOX associated cyclic di-GMP processing enzyme
HaHK – H-NOX associated histidine kinase
HarR – H-NOX associated response regulator
HK – Histidine kinase
Hpt – Histidine containing phosphotransfer protein
HqsK – H-NOX associated quorum sensing kinase
HRP – Horseradish peroxidase
HTH – Helix-turn-helix
IPTG – Isopropyl β -D-1-thiogalactopyranoside
LB – Luria broth
NaHK – NosP associated histidine kinase
NaRR – NosP associated response regulator
NOS – Nitric oxide synthase
NosP – Nitric oxide sensing protein
NqsK – NosP associated quorum sensing kinase
OD – Optical density
PAS – Per-ARNT-Sim domain
PDE – Phosphodiesterase
pGpG – 5'-phosphoguananylyl-(3',5')-guanosine
RNA – Ribonucleic acid
RR – Response regulator
SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGC – Soluble guanylate cyclase
TCS – Two-component signaling

List of Symbols

Symbol	Name	Description
	H-NOX	Heme oxygen/nitric oxide binding protein
	NosP-N	N-terminal domain of nitric oxide sensor protein
	NosP-C	C-terminal domain of nitric oxide sensor protein
	MCP	Methyl accepting chemotaxis protein
	DGC	Diguanylate cyclase with GGDEF motif
	Degenerate DGC	Inactive Diguanylate cyclase
	PDE	Cyclic di-GMP hydrolyzing phosphodiesterase with ExL motif
	Inactive PDE	Inactive phosphodiesterase
	HD-GYP	Phosphodiesterase of HD superfamily with GYP motif
	Degenerate HD-GYP	Inactive HD-GYP
	PAS	Per-Arnt-Sim domain
	PAC	PAS associated domain
	HisKA	His Kinase A (phosphoacceptor) domain
	Atypical HisKA	Atypical His Kinase A domain
	HATPase_e	Histidine kinase-like ATPase
	REC	CheY-homologous receiver domain
	HPT	Histidine phosphotransfer domain

Acknowledgements

I would like to acknowledge everyone who have helped me along the way.

Prof. Roger Johnson for helpful advice and guidance.

Dr. Leszek Kotula for his guidance throughout. Dr. Stephen Walker for allowing *Pseudomonas aeruginosa* related work. Dr. Peter Greenberg for providing us with the PaO1-T7 strain of *P. aeruginosa* and the pLJQ-His vector. We thank Dr. Alan M. Lambowitz for providing us with the pBL1 vector.

Dr. James Truglio for stimulating scientific conversations.

Current members of the Boon Lab. Especially, Lisa-Marie Nisbett for helping me around the lab and with editing.

Lu Bai, from Dr. Jessica Seeliger lab for helping me with many experiments. Most importantly, for tolerating me and helping me in any whatsoever way imaginable.

None of this would have been possible without my advisor, Dr. Elizabeth Boon. Thank you, Liz, for allowing me in your lab.

Publications

1. Arora DP, Hossain S, Xu Y, & Boon EM (2015). Nitric Oxide Regulation of Bacterial Biofilms. *Biochemistry* 54(24):3717-3728.
2. Hossain S, Boon EM (2016). NosP: Discovery of a novel nitric oxide sensor and nitric oxide-responsive signaling pathway in *Pseudomonas aeruginosa*. PNAS (submitted)

CHAPTER 1

Nitric Oxide and Bacterial Biofilm¹

Abstract

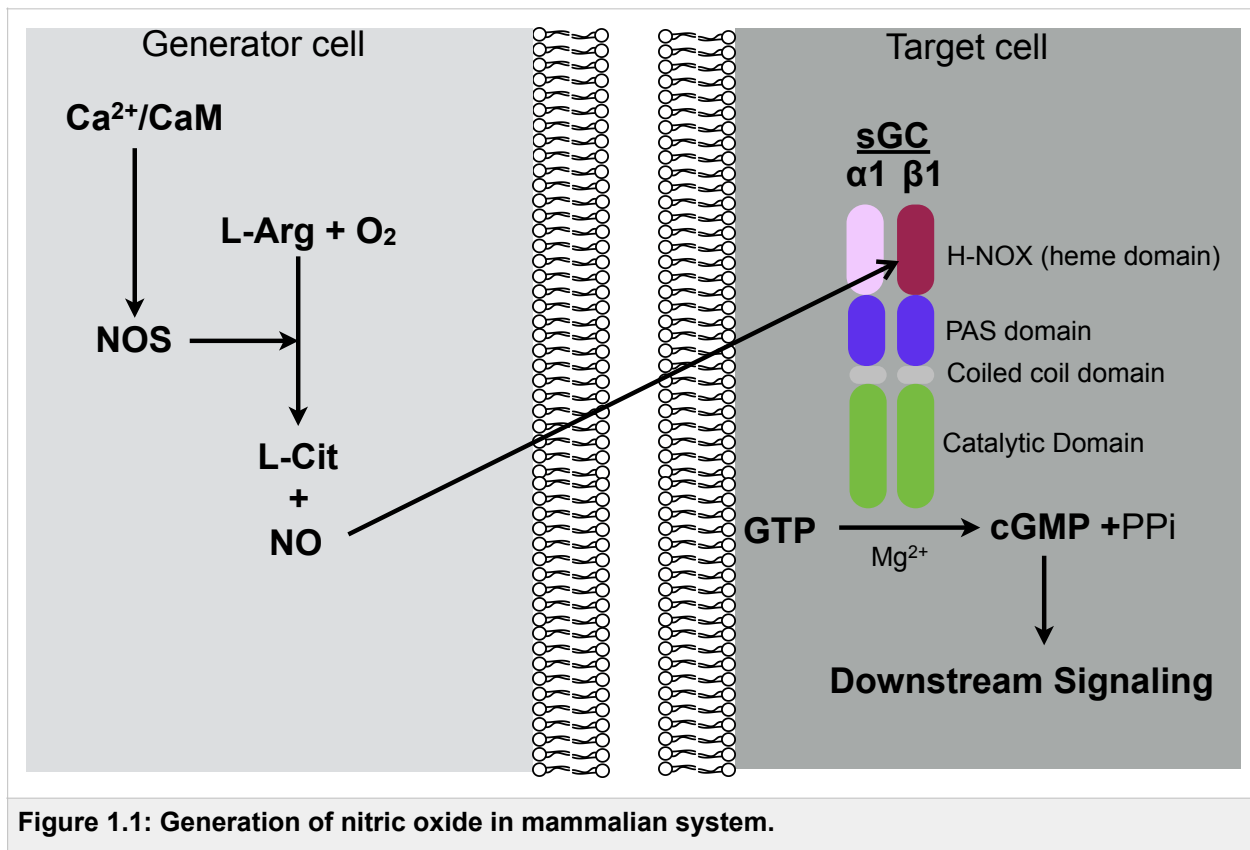
Nitric oxide (NO) is a diatomic, uncharged radical gas molecule. At high concentrations, NO is a poisonous gas. At low concentrations, however, NO has come to be known as a vital signaling molecule in eukaryotic biology, for which it was selected in 1992 as Science Magazine's Molecule of the Year. In recent years, the role of NO in bacterial biology has become prevalent. NO, as we know now, plays crucial role in bacterial biofilm regulation. The purpose of this chapter is to familiarize the reader with our understanding of NO signaling in general as well as NO signaling in bacteria. Where it has been investigated, the underlying NO sensors or signaling pathways are also discussed. Most of the examples of NO-mediated biofilm regulation have been documented with exogenously applied NO, but we also survey possible natural sources of NO in biofilm regulation, including endogenously generated NO.

¹This chapter is adapted from the following published review:
Arora, D. P., Hossain S., Xu Y., and Boon, E. M. (2012) Nitric Oxide Regulation of Bacterial Biofilms, *Biochemistry*, 2015, 54 (24): p. 930-942.

Nitric Oxide and Bacterial Biofilm

■ NITRIC OXIDE AS A SIGNALING MOLECULE

In mammals, NO is produced from L-arginine by nitric oxide synthases (NOSs) (1-3) (Figure 1.1). As a highly diffusible gas molecule, NO is ideal for intracellular signaling and is able to act in adjacent cells where it binds its specific protein receptor, an enzyme called soluble guanylyl cyclase (sGC) (Figure 1.1). sGC binds NO at the ferrous iron



center of a protoporphyrin IX heme cofactor; upon ligation of NO, the enzymatic conversion of guanosine triphosphate (GTP) to second-messenger cyclic guanosine 3', 5'-monophosphate (cGMP) is increased several hundred-fold above basal levels (4). cGMP has an array of downstream targets, and its increased level of production results

in the regulation of calcium channels and protein phosphorylation cascades to ultimately regulate essential physiological processes such as blood flow, platelet aggregation, myocardial function, tissue formation, and angiogenesis, to name a few (1, 5-8). As a testament to the importance of NO signaling in human health, impaired NO signaling can play a role in a wide variety of ailments, including cardiovascular disease, diabetes, erectile dysfunction, and cancer.

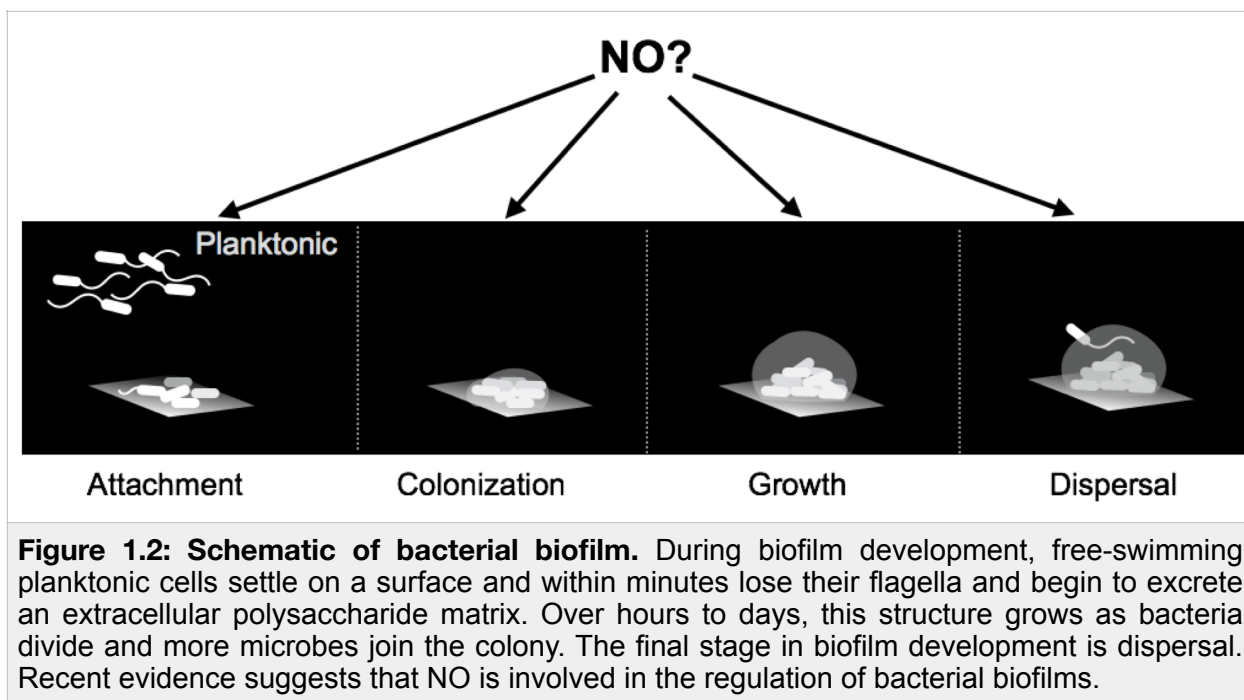
Because of the clinical implications of NO signaling, most of the research on the biological function of NO has focused on its role in eukaryotes. In the past several decades, however, bacterial detection of NO has emerged as a widespread phenomenon and fascinating area of study. NO is toxic to bacteria at high concentrations (approximately micromolar). This is, in fact, one line of defense that NO-producing eukaryotes have against bacterial infection (9, 10). Bacteria may also encounter relatively high concentrations of NO during denitrification, a process by which some bacteria can respire on nitrate or nitrite under oxygen-limiting conditions. Many NO-responsive bacterial proteins involved in the elimination of NO through denitrification and detoxification pathways have been characterized, including FNR-like transcription factors (11), the NO-responsive transcriptional activator NorR (12), and the nitrite-sensitive repressor NsrR (13).

Our laboratory has been fascinated by an increasing number of reports in which bacteria apparently respond to relatively low, nontoxic, concentrations of NO (approximately nanomolar to micromolar) to elicit physiological responses other than

those involved in denitrification and detoxification, i.e., other than processes primarily aimed at the elimination of NO from the cell (14-20).

■ BACTERIAL BIOFILMS

Most bacteria have a strong predisposition for growth in a sessile multicellular community called a biofilm (21-25). Biofilms are matrix-encapsulated microbial colonies that adhere to all kinds of surfaces, including soil, medical implants, tissue, metals, and plastics; biofilms form on just about any surface in a moist environment. Biofilms can be formed by a single bacterial species, but more often than not, they consist of many bacterial species as well as other microbes such as fungi, algae, and protozoa. These slimy films are formed when bacteria attach themselves to a surface and secrete a hydrated polymeric substance, often called the exopolysaccharide (EPS) matrix (26). This matrix serves many purposes: it cements the colony to the surface, attracts new members, helps to capture and concentrate nutrients, and offers protection from the surrounding environment. Biofilms constitute a widespread and extremely persistent form of bacterial growth (27-29). The biofilm community is able to conduct diverse functions, some harmful to and some beneficial to humans. These functions are distinct from those conducted by planktonic cells. In the environment, biofilms promote remediation of contaminated groundwater and soil; however, they also cause persistent biofouling of ship hulls, underwater sensors, and oil and water transport and storage containers. Of clinical interest, biofilms in the human body, including those lining surfaces in the mouth and the gastrointestinal tract, can both promote and harm human health. For example, biofilms coating the intestinal lining contribute to the production of vitamins and digestion of food (30), but they can also harbor pathogens and contribute to inflammatory diseases such as irritable bowel syndrome (31). Other examples of clinically relevant biofilms include dental plaque, responsible for tooth



decay and gum disease, and bacterial biofilms that form on medical equipment, which are a major cause of secondary infections contracted in medical facilities. Disease-causing biofilms are particularly problematic because host immune systems are, typically, unable to respond to biofilm infections. In addition, current antibiotic solutions are largely ineffective against these compact and well-protected structures; cells in a biofilm are 10–1000-fold more protected from antibiotics than planktonic cells (32, 33). Although biofilms are the most abundant form of bacterial growth, they are currently poorly understood. Significant effort is being spent to improve our understanding of the fundamental processes involved in the biofilm life cycle and to develop new antibiofilm agents for practical and therapeutic use. Biofilm regulation is likely multifactorial and complex, and understanding discrete contributing factors is expected to have a significant impact (34). NO has emerged as an important factor in regulating biofilm formation (Figure 1.2). The molecular details of these NO-regulated signal transduction

pathways are also not well understood, but current evidence indicates that NO likely mediates changes in the biofilm through two-component signaling, cyclic-di-GMP signaling (cyclic diguanylate; 3',5'-cyclic diguanylic acid; c-di-GMP), and/or quorum sensing. These are briefly described below.

Two-Component Signal Transduction. Two-component signal transduction is an important mechanism for stimulus response in bacteria (35, 36). In its simplest form, a two-component signaling pathway consists of a sensor histidine kinase and a response regulator. The variable sensor domain of the histidine kinase is responsible for detecting a stimulus, which results in a change in the autophosphorylation state of the histidine kinase (37). The signal is relayed downstream by means of the transfer of phosphoryl from the histidine in the kinase domain to an aspartic acid in the receiver domain of the response regulator. The activated response regulator then elicits a response to the initial stimulus. However, many two-component systems deviate from this canonical system (37, 38). For example, in three-component systems, an accessory protein that detects a signal and regulates kinase autophosphorylation *in trans* replaces the kinase sensor domain. In hybrid signaling, the histidine kinase has a receiver domain; thus, a His-to-Asp phosphotransfer takes place within the hybrid kinase, and then a histidine-containing phosphotransfer protein is required to relay the signaling phosphate from the histidine kinase to the appropriate response regulator in two additional phosphotransfer steps (39).

Quorum Sensing. Biofilm formation is one of many behaviors bacteria coordinate as a group. Key to understanding the group behavior of bacteria is understanding the ability of bacteria to communicate with each other, as well as their environment, by exchanging

chemical signals. Quorum sensing (QS), as the phenomenon is termed, involves the production and detection of small molecules known as autoinducers. Detection of autoinducers in the environment allows cells to gauge cell density. Arrival at a critical population size or “quorum” triggers a signaling cascade that ultimately regulates gene expression (40-42). In many organisms, this signaling cascade involves His-to-Asp phosphotransfer similar to what is described above. QS regulates changes in global gene expression of entire bacterial communities affecting behaviors such as virulence, biofilm formation, bioluminescence, and antibiotic resistance, all activities that are best served by large population sizes.

Cyclic-di-GMP Signaling. It has become apparent that a wide variety of bacteria use c-di-GMP to regulate biofilm formation (43-46). Indeed, many of the histidine kinase pathways mentioned above ultimately regulate c-di-GMP concentrations. Generally speaking, as the intracellular concentration of c-di-GMP increases, a higher percentage of bacteria enter biofilm modes; as the intracellular concentration of c-di-GMP decreases, bacteria become motile or more virulent. Although this is a relatively new area of inquiry, the emerging theme is that the total concentration of intracellular c-di-GMP is tightly regulated by a variety of enzymes that both synthesize and degrade c-di-GMP. c-di-GMP is synthesized by proteins with diguanylate cyclase activity, which can be identified by a conserved GGDEF (or GGEEF) amino acid sequence (47). Proteins with phosphodiesterase activity degrade c-di-GMP. These are identified by a conserved EAL or HD-GYP amino acid motif (48, 49). The details of c-di-GMP regulation in bacteria are under investigation, and the current evidence indicates that c-di-GMP synthase and phosphodiesterase activities are controlled by signal transduction.

GGDEF-, EAL-, and HD-GYP-containing domains are invariably found to be associated with sensing domains specific for a certain stimulus. Most of the downstream targets of c-di-GMP discovered to date include proteins involved in production and maintenance of the exopolysachharide (EPS) matrix and proteins involved in motility. These mechanisms have recently been reviewed in great detail (46).

■ NITRIC OXIDE REGULATION OF BIOFILM FORMATION

A summary of documented NO-mediated biofilm regulation responses is listed in Table 1.1. As noted above, in several species, distinct concentration-dependent biofilm-related responses to NO have been documented. The better understood examples of NO-dependent biofilm phenotypes are described in more detail below. In several cases, it is difficult to definitively separate NO detoxification and denitrification responses from NO signaling responses. For example, an increase in the level of biofilm formation may be attributed to a defensive bacterial growth mode. Likewise, it is also possible that at high NO concentrations, an observed reduction in biofilm biomass is due to cell death. Nonetheless, in many bacteria, NO is observed to cause biofilm dispersal at low NO concentrations, which is not consistent with a defense mechanism.

NO Regulation of a Biofilm in *Nitrosomonas europaea*. One of the first documented observations of the effect of NO on biofilm formation was documented in *N. europaea*, a bacillus-shaped, Gram-negative, obligate chemolithoauxotroph. Because *N. europaea* oxidize ammonia for energy production, they are commonly found in sewage and untreated water. In *N. europaea* it has been shown that NO levels above 30 ppm (~1 mM) result in biofilm formation and below 5 ppm (<200 μ M) in biofilm dispersal, consistent with a separate, non-stress-related response to NO at lower concentrations (15). The bacteria were grown in a chemostat laboratory reactor so that the gas mixtures in the chamber could be precisely defined. NO was applied to the bacteria as the pure gas, and its concentration was monitored with a nitric oxide analyzer (Eco

Table 1.1-The effect of nitric oxide on bacterial biofilms

Species	NO source	NO donor concentration	Approximate NO concentration	Ref.	Effect on biofilm
Gram Negative					
<i>Pseudomonas aeruginosa</i>	SNP	25 nM - 2.5 mM	0.025-2,500 nM ^a	25	Decreases
<i>Pseudomonas aeruginosa</i>	SNP	>25 mM	>25,000 nM ^a	25	Enhances
<i>Vibrio cholerae</i>	SNP	25-500 nM	0.025-0.5 nM ^a	31	Decreases
<i>Serratia marcescens</i>	SNP	25-500 nM	0.025-0.5 nM ^a	31	Decreases
<i>Escherichia coli</i>	SNP	500 nM	0.5 nM ^a	31	Decreases
<i>Escherichia coli</i>	DETA NONOate	100 µM	~100-300 nM	32	Decreases
<i>Fusobacterium nucleatum</i>	SNP	1-10 µM	1-10 nM ^a	31	Decreases
<i>Acinetobacter baumannii</i>	gaseous NO	200 ppm	~7,000,000 nM ^b	135	Decreases
<i>Nitrosomonas europaea</i>	gaseous NO	>30 ppm	>1,000,000 nM ^b	30	Decreases
<i>Nitrosomonas europaea</i>	gaseous NO	<5 ppm	<170,000 nM ^b	30	Enhances
<i>Shewanella woodyi</i>	DETA NONOate	200 µM	~50 nM ^b	28	Decreases
<i>Vibrio harveyi</i>	DPTA NONOate	50 µM	40-80 nM ^b	110	Enhances
<i>Vibrio harveyi</i>	DPTA NONOate	100 µM	100-300 nM ^b	110	Decreases
<i>Neisseria gonorrhoeae</i>	SNP	500 nM	0.5 nM ^a	86	Decreases
<i>Neisseria gonorrhoeae</i>	SNP	20 µM	20 nM ^a	87	Enhances
<i>Shewanella oneidensis</i>	DETA NONOate	200 µM	400-2,700 nM ^b	29	Enhances
<i>Shewanella oneidensis</i>	DPTA NONOate	50 µM	~100 nM ^b	c	Decreases
Gram Positive					
<i>Bacillus licheniformis</i>	SNP	100-500 nM	0.1-0.5 nM ^a	31	Decreases
<i>Staphylococcus epidermidis</i>	SNP	10 µM	10 nM ^a	31	Decreases
<i>Staphylococcus aureus</i>	gaseous NO	200 ppm	~7,000,000 nM ^b	135	Decreases
<i>Staphylococcus aureus</i>	DETA NONOate	1-1000 µM	>125,000 nM	88	Decreases
<i>Staphylococcus aureus</i>	DETA NONOate	1-1000 µM	~900-2,000 nM	88	Enhances
Multispecies Biofilm					
From Water System	SNP	100-500 nM	0.1-0.5 nM ^a	31	Decreases

^aConcentration of NO released by SNP is calculated according to the measurements reported in reference 31.
^bConcentration of NO directly measured. ^cUnpublished data from the Boon Laboratory.

Physics, Dürnten, Switzerland). The oxygen concentration in these experiments was

maintained at 2.6 mg/L. Proteomics experiments revealed 11 proteins that are differentially expressed at the two NO concentrations. Notably, the flagellar and flagellar assembly proteins were among those modulated by NO and expressed at higher levels in planktonic cells, which is consistent with the observed change in growth mode. Although the underlying biochemical pathway(s) responsible for this NO-induced phenotype remains unknown, it is an NO-specific phenomenon; ammonium ion, nitrite, O₂, temperature, and pH have no significant effect on the growth mode or proteome of *N. europaea*.

NO Regulation of a Biofilm in *Pseudomonas aeruginosa*. The most extensively studied example of NO-dependent biofilm regulation is in *P. aeruginosa*. *P. aeruginosa* is a principal pathogen in cystic fibrosis and many hospital-acquired infections, and its pathogenicity is linked to biofilm formation (27). In an early study of the effect of NO on biofilm formation, it was shown that *P. aeruginosa* remain in the biofilm state until exposed to 25 nM to 2.5 mM sodium nitroprusside (SNP), which corresponds to approximately 0.025–2500 nM NO, at which point they revert to a free-swimming, planktonic lifestyle (16). When the bacteria are exposed to NO concentrations greater than ~25 μM, however, biofilm formation was enhanced relative to biofilm formation in the absence of NO. Thus, in *P. aeruginosa*, nanomolar NO is seen to cause biofilm dispersal. In addition, it was demonstrated that *P. aeruginosa* lacking the *rhl* quorum sensing circuit die because of overproduction of NO (50); quorum sensing is a critical mechanism involved in the regulation of biofilm formation in *P. aeruginosa* (51), as well as many other bacteria (40, 42, 52-54). It should be noted that SNP is used as a NO donor in many of the studies reviewed here (Table 1.1). Effective concentrations of NO

are estimated to be 1000 times lower than the concentration of NO donor used (17). For example, 25 μM SNP is expected to produce ~ 25 nM NO in solution (16). While it certainly releases NO, SNP can also release cyanide and has been linked to nitrosative stress independent of NO production (55). Therefore, studies in which SNP has been used as an NO donor require some caution before one can conclude that NO causes the observed effect.

It has been suggested that the response of *P. aeruginosa* to NO is linked to its denitrification abilities. For example, biofilms of a *P. aeruginosa* mutant lacking nitrite reductase (ΔnirS) do not disperse; NirS generates NO from nitrite under anaerobic conditions. However, a NO reductase mutant (ΔnorCB) exhibits greatly enhanced dispersal (16). Furthermore, the *P. aeruginosa* transcription factors ANR (anaerobic regulation of arginine deaminase and nitrate reduction) and DNR (dissimilative nitrate respiration regulator) help regulate a network of genes needed to respond to low oxygen tension and the presence of nitrate, nitrite, and other N-oxides (56, 57). Interestingly, DNR, a member of the FNR (fumarate and nitrate reductase regulatory protein) family, has been demonstrated to be a heme protein capable of binding NO (58). Its affinity for NO appears to be in the micromolar range, however, and thus, DNR is likely not responsible for the observation that low nanomolar concentrations of NO can disperse *P. aeruginosa* biofilms.

Little is known about the detailed molecular mechanism of *P. aeruginosa* biofilm dispersal, but it appears that this response to NO is a metabolic response and not a toxic response. Exposure to NO is associated with the regulation of intracellular levels of c-di-GMP through the sophisticated interaction of many diguanylate cyclases and

phosphodiesterases (59). However, of the proteins known to play a role in NO-mediated biofilm dispersal in *P. aeruginosa*, it is unclear if any of them serve as a primary, sensitive NO sensor. A chemotaxis transducer BdlA (biofilm dispersal locus A) has been implicated in c-di-GMP degradation and biofilm dispersal upon detection of NO through a currently unknown mechanism (59, 60). The PASa domain of BdlA can bind heme and may detect NO (61). However, BdlA appears to also respond to many environmental cues in addition to NO, including succinate, Ag^+ , Hg^{2+} , and As^{3+} . The c-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 (62). The c-di-GMP phosphodiesterases DipA (63) and NbdA (64) have been linked to decreasing cyclic-di-GMP concentrations upon exposure to NO. However, the mechanism of action for both is NO-induced upregulation of *dipA* and *nbdA* expression, suggesting they each act downstream of initial NO sensing. Nonetheless, a bioinformatics study has suggested that conserved Met and His residues in NbdA could coordinate with copper ions and potentially sense NO (65).

Other Examples of Clinically Relevant NO-Regulated Biofilm Formation. NO-mediated regulation of biofilm formation was first observed in *N. europaea* (15) and *P. aeruginosa* (16), but NO is now generally considered to regulate biofilm formation in a wide variety of bacteria (17, 66) (Table 1.1). A few additional representative examples of NO-mediated biofilm formation are discussed below. For example, *Ne. gonorrhoeae*, the causative agent for gonorrhea, has been shown to form biofilms during cervical infection. As a human pathogen, *Ne. gonorrhoeae* has evolved the ability to resist NO-mediated killing (from host inducible nitric oxide synthase) and reduce intracellular NO

levels using NorB, a respiratory nitric oxide reductase (67). Furthermore, *Ne. gonorrhoeae* can grow under anaerobic conditions in the presence of nitrite, and NorB is also responsible for the reduction of NO produced by the respiratory nitrite reductase AniA (68). Thus, *Ne. gonorrhoeae* encodes NO binding proteins such as NsrR (69) that respond to NO for denitrification or detoxification purposes. In terms of NO-responsive biofilm phenotypes, one study found both *norB* and *aniA* insertion mutants of *Ne. gonorrhoeae* strain 1291 to be defective in biofilm formation, which led the authors to conclude that biofilm formation is linked to NO concentration (70). Interestingly, in the same study, it was demonstrated that application of a very low concentration of NO, 500 nM SNP (~500 pM NO), at the beginning of biofilm growth (under aerobic conditions where NorB is not expected to be expressed) resulted in a significant reduction in biomass. In a follow-up study, however, it was demonstrated that *Ne. gonorrhoeae* biofilms are tolerant to high concentrations of NO (up to 20 μ M SNP, ~20 nM) if grown under anaerobic conditions where *norB* is expressed, but nitrite has been removed (71). This may be due to the fact that *Ne. gonorrhoeae* can use NO as a substrate for anaerobic growth when nitrite is limited. Taken together, the authors of these studies suggest that when NO is present at sublethal concentrations, either under aerobic conditions or when *norB* is expressed in the presence of nitrite, biofilm formation is inhibited, implying that *Ne. gonorrhoeae* is capable of sensing and responding to low concentrations of NO. No pico- or nanomolar NO sensors have been described for *Ne. gonorrhoeae* to date, however.

In *Staphylococcus aureus*, a causative agent for rhinosinusitis, a recent study found that ~10-fold less biofilm biomass accumulates in the presence of relatively high NO

concentrations (~125–1000 μM estimated from the concentration of the NO donor (72)) than in the absence of NO. In this study, the NO donor used was a NONOate. NONOates are N-diazeniumdiolate compounds that are stable as solids but release NO in solution at neutral or acidic pH; as such, they are commonly used as NO-donating reagents (73). In contrast, when *S. aureus* biofilms were exposed to lower levels of NO (0.9–2 μM), biofilm biomass increased. Interestingly, in a healthy individual, a normal concentration of sinonasal NO is considered to be between 6 and 25 ppm (~200 and 830 μM) (74), while the NO levels in patients with rhinosinusitis are usually much lower, between 0.021 and 0.07 ppm (~0.7 and 2.3 μM) (75). The mechanism for this dual nature of NO-dependent biofilm regulation is not yet understood, but these results suggest that *S. aureus* can sense NO in a concentration-dependent manner and, further, that NO may be physiologically important for regulating the human microbiome.

In addition to the examples discussed above, NO regulation of biofilm formation has been documented in *Legionella pneumophila* and several species of *Shewanella* and *Vibrio*. In these organisms, however, unlike those described above, significantly more is currently understood about the mechanism of NO sensing and signal transduction. In these cases, the primary NO sensor has been demonstrated to be a member of the H-NOX family. These sensors will be described below.

■ A PRIMARY NO SENSOR IN BACTERIA: THE H-NOX FAMILY

A family of bacterial heme proteins with as much as 40% primary sequence identity to the heme domain from mammalian sGC has been identified on the basis of genomic analysis (76). We and others have confirmed this discovery through biochemical characterization of predicted bacterial family members (18-20, 77-87). They all have ligand binding properties similar to those of sGC, confirming their membership in a family of sensitive and selective NO-sensing proteins. H-NOX family members are able to discriminate between NO and molecular oxygen as ligands; the molecular basis of ligand discrimination has been debated (79, 88, 89). Nonetheless, this ability to discriminate between ligands is essential to the role of H-NOX as a NO sensor.

H-NOX domains have now been predicted in the genomes of more than 260 sequenced species from all subgroups of bacteria. Generally, H-NOX genes are encoded in putative operons with signaling proteins such as histidine kinases in two-component signaling, GGDEF diguanylate cyclases, and/or EAL and HD-GYP c-di-GMP phosphodiesterases. Histidine kinases found in NO/H-NOX signal transduction pathways are collectively termed HaHKs (H-NOX-associated histidine kinase), and c-di-GMP synthases/phosphodiesterases in NO/H-NOX signaling pathways are collectively termed HaCEs (H-NOX-associated cyclic-di-GMP processing enzymes). Although the function of H-NOX proteins is not yet fully understood, from currently available data, it appears they are bacterial NO sensors that broadly regulate biofilm formation and other bacterial community behaviors such as quorum sensing and symbiosis. H-NOX proteins have been recently reviewed (90); thus, here we only revisit NO/H-NOX signaling in the context of NO regulation of biofilm formation (Figure 1.3).

NO/H-NOX Regulation of Biofilm Formation in *L. pneumophila*. The first published observation of biofilm regulation by an NO/H-NOX signaling pathway was in *L. pneumophila* (85). *L. pneumophila* encodes two H-NOX proteins, one cocistronic with a

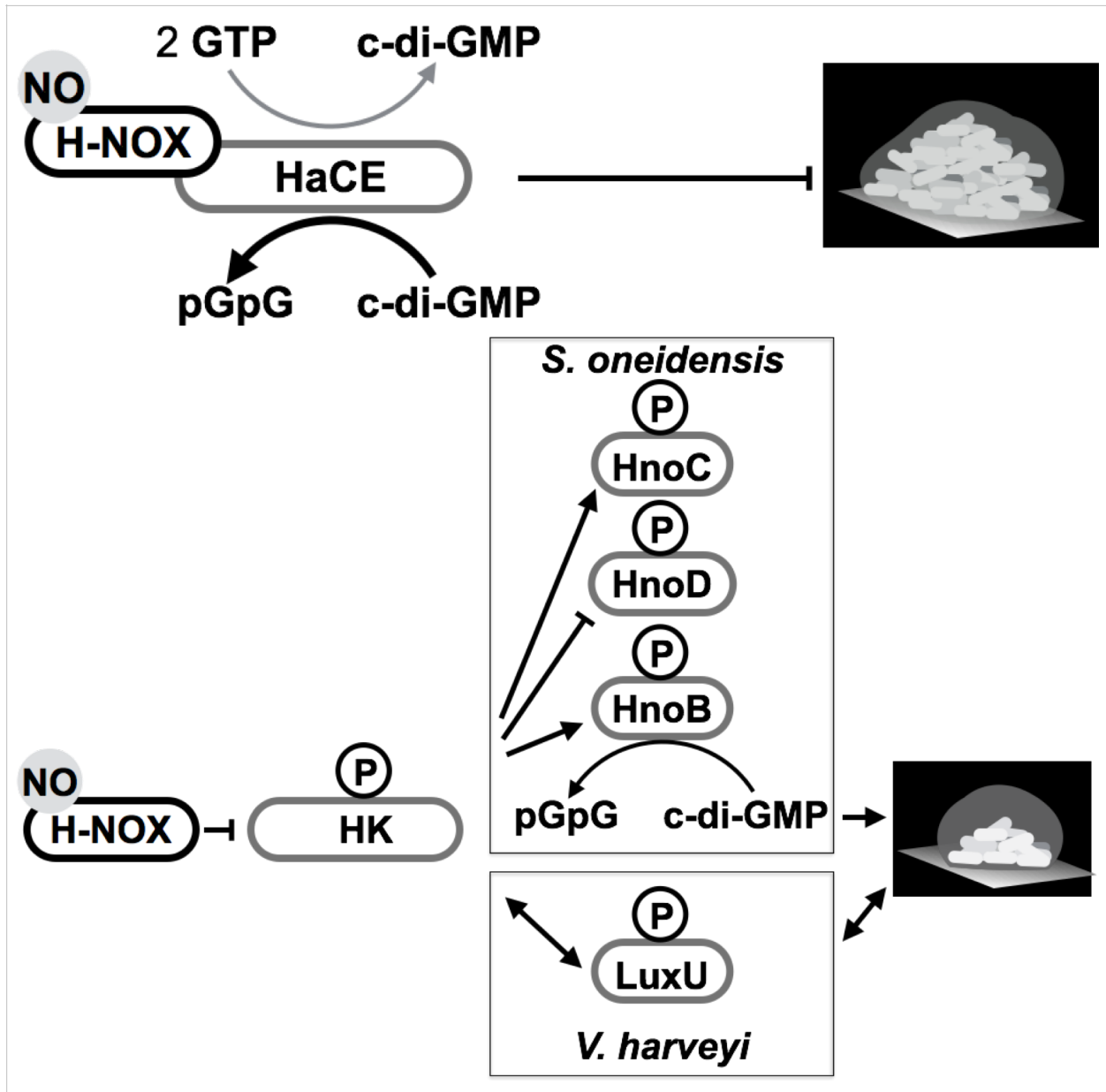


Figure 1.3: NO regulates biofilm formation through ligation to H-NOX. (A) NO-bound H-NOX directly regulates both the c-di-GMP synthesis and phosphodiesterase activities of a bifunctional HaCE in *Shewanella woodyi*. A decreased c-di-GMP concentration leads to biofilm repression in the presence of NO. (B) NO-bound H-NOX directly regulates histidine kinase autophosphorylation, which ultimately regulates downstream biofilm formation through c-di-GMP signaling in *Shewanella oneidensis* and quorum sensing in *Vibrio harveyi*.

HaHK and one cocistronic with a HaCE. The HaCE in *L. pneumophila* has both GGDEF and EAL domains but was found to have only c-di-GMP synthase and not phosphodiesterase activity. NO-bound H-NOX was shown to inhibit this cyclase activity, thereby inhibiting the formation of c-di-GMP. The authors showed that deletion of the HaCE- adjacent H-NOX gene resulted in a *L. pneumophila* strain with a hyperbiofilm phenotype. This phenotype was reversed by deletion of the associated HaCE, suggesting that this pathway, and therefore NO, plays an essential role in biofilm regulation of *L. pneumophila*.

NO/H-NOX Regulation of Biofilm Formation in *S. woodyi*. In contrast to the HaCE from *L. pneumophila*, the HaCE from *S. woodyi* has been characterized as an enzyme with dual activity (91), capable of acting as both diguanylate cyclase and phosphodiesterase. Using purified proteins, we have shown that *S. woodyi* H-NOX and HaCE are binding partners (19, 92). Bound to H-NOX in the absence of NO, HaCE behaves primarily as a c-di-GMP synthase, but bound to NO-H-NOX, HaCE acts primarily as a phosphodiesterase (19). Consistent with these results, we found that 50 nM NO, delivered from a NONOate, causes a decrease in both the level of biofilm formation and *in vivo* c-di-GMP concentration, a phenotype that is absent in the *hnoX* deletion strain, confirming that NO affects biofilm formation in *S. woodyi* via H-NOX/c-di-GMP signaling.

NO/H-NOX Regulation of Biofilm Formation in *S. oneidensis*. *S. oneidensis* encodes one H-NOX gene cocistronic with a HaHK. With purified proteins, it was shown that the autophosphorylation activity of HaHK is regulated by the ligation state of H-NOX (84). We identified and biochemically characterized the first response regulator cognate

to an H-NOX-associated histidine kinase (HaRR; H-NOX-associated response regulator) from *Pseudoalteromonas atlantica*, an organism with an H-NOX/HaHK operon (87) homologous to that in *S. oneidensis*. This response regulator was identified by use of bioinformatic tools and is annotated as an HD-GYP c-di-GMP phosphodiesterase. A multiple-sequence alignment revealed degeneracy, however, and we found that this HD- GYP HaRR lacks phosphodiesterase activity. It does, however, exhibit autophosphatase activity (87), presumably as a means of autoregulation, as seen with other response regulators (93). It is possible that this HD-GYP HaRR indirectly manipulates intracellular c-di-GMP levels via a binding event. Subsequently, the homologous HD-GYP HaRR, as well as two additional HaRRs, an EAL-type c-di-GMP phosphodiesterase, and a helix–turn–helix transcription factor were identified and characterized in *S. oneidensis* (20). Further, it was documented that c-di-GMP hydrolysis by the EAL HaRR was inhibited by NO-bound H-NOX, not only directly by inhibiting HaHK phosphorylation of this EAL response regulator but also indirectly by the unphosphorylated HD-GYP HaRR. The authors reported further that ~200 μ M DETA NONOate (the authors report production of ~400 to ~2700 nM NO under these conditions) caused an increase in *S. oneidensis* biofilm biomass, hypothesizing that biofilm formation is a defense mechanism against environmental NO (20). It should be noted, however, that this biofilm growth was evaluated anaerobically, which is not ideal for *S. oneidensis* biofilm formation; in fact, an anaerobic environment has been reported to cause rapid dispersal of *S. oneidensis* biofilms (94). When *S. oneidensis* is grown aerobically in the presence of lower NO concentrations (~100 nM from NONOate), our laboratory found that NO actually inhibits its biofilm formation (unpublished results). This

discrepancy could be due to aerobic versus anaerobic conditions or the difference in NO concentration. These findings in *S. oneidensis* highlight the complexity of multi-component signaling networks in bacteria, suggesting stringent regulation of biofilm formation.

NO/H-NOX Regulation of Quorum Sensing and Biofilm Formation in *V. harveyi*.

V. harveyi is an extremely well-studied model organism for QS because cultures become bioluminescent via luciferase expression in response to a high cell density. *V. harveyi* has at least three QS circuits that together regulate the phosphorylation state of a phosphotransfer protein called LuxU, which regulates synthesis of the QS master transcriptional regulator LuxR, ultimately regulating bioluminescence and other QS responses. Unexpectedly, several years ago, we demonstrated that 50 nM NO from NONOate caused a significant increase in *V. harveyi* bioluminescence (18) suggesting NO may affect QS. Interestingly, several species of *Vibrio* bacteria have a predicted *hnoX* gene in the same putative operon as a gene that encodes a soluble histidine kinase that is highly identical to the kinase domain of one of the *V. harveyi* quorum-sensing kinases, LuxQ. These specific H-NOX-associated histidine kinases are termed HqsK (H-NOX-associated quorum-sensing kinase). We were able to demonstrate that in *V. harveyi*, H-NOX participates in the LuxU/LuxR QS pathway by regulating the activity of HqsK (18), which like the other QS kinases is able to exchange phosphate with LuxU. We further showed that NO-bound H-NOX regulates HqsK autophosphorylation and phosphoexchange with LuxU, providing a mechanistic explanation for our observation that NO affects QS. In a subsequent study, we found that NO regulates biofilm formation and flagellar synthesis via H-NOX/HqsK in a dose-dependent manner (95).

NO/H-NOX Regulation of Symbiosis in *Vibrio fischeri*. Another organism with an H-NOX/HqsK signaling pathway is *V. fischeri*, a bioluminescent marine bacterium that forms a specific symbiotic relationship with *Euprymna scolopes*, the Hawaiian bobtail squid. Squid-generated NO is thought to play a role in establishing this symbiosis (14, 96). NO is normally detectable in the ducts, appendages, and mucus lining of the squid, but upon colonization by *V. fischeri*, NO production is weakened. Furthermore, when the squid is treated with NO scavengers during incubation with *V. fischeri*, aggregates of *V. fischeri* are significantly larger (50–200 μm in diameter) than their normal size in the presence of NO (<20 μm in diameter), suggesting a role for NO in the regulation of bacterial clumping, which is likely related to biofilm formation.

The role of H-NOX as an NO sensor involved in colonization has been investigated. Surprisingly, an *hnoX* deletion strain of *V. fischeri* is able to outcompete the wild-type strain for colonization of the squid light organ (86). It is currently hypothesized that NO/H-NOX is involved in the tight regulation of iron acquisition in *V. fischeri*. The light organ is in an iron-deficient environment, so bacteria tend to scavenge the nutrient upon colonization. High concentrations of iron can be toxic to growth, so NO/H-NOX may be involved in the tight regulation of iron uptake. This would explain why an *hnoX* deletion displays an initial growth advantage upon colonization due to the absence of this strict regulation of iron uptake. In support of this hypothesis, the *hnoX* deletion strain loses fitness after a longer time, presumably because of the absence of the iron uptake regulation.

■ SYNTHESIS OF NITRIC OXIDE IN BACTERIA

One of the major outstanding questions about the role of NO in bacteria is the source of the NO signal. The *V. fischeri*/*Eu. scolopes* symbiosis suggests that, at least in some cases, NO may serve as an interkingdom signal, generated by eukaryotic NOS enzymes. Another possible source is NO produced by nitrite reductases, which are used by facultative anaerobic bacteria in the presence of nitrate/nitrite under anaerobic conditions. Interestingly, nitrate/nitrite-reducing bacteria have been suggested to be an important mammalian symbiont with roles in the nitrogen cycle (97, 98). Another intriguing possibility is bacterial homologues of the oxygenase domain of mammalian NOS (bNOS), which are briefly reviewed here.

Bacterial homologues of mammalian NOS enzymes were recently discovered (99), and thus far, 403 species of bacteria, mostly Gram-positive, are known to encode a bNOS gene. Unlike mammalian NOSs, which are composed of an oxygenase domain and a reductase domain connected by a calmodulin binding domain, bNOS genes encode an oxygenase domain only. Although bNOS enzymes can convert L-arginine to L-citrulline and NO if an appropriate reductase domain is provided *in trans*, it is currently not clear if bNOS necessarily produces NO *in vivo*, although many studies are consistent with NO production. For example, in *Streptomyces turgidiscabris*, a plant pathogen responsible for potato scab disease, bNOS is thought to be involved in the nitration of tryptophan (100), rather than explicit NO production. In other organisms, bNOS is believed to generate NO as a final product.

The function of synthesized NO is not fully understood, but it appears to be cytoprotective. In *Bacillus subtilis* and *Bacillus anthracis*, many studies have suggested that bNOS is involved in the protection of bacteria against oxidative damage, presumably by NO-mediated suppression of Fenton chemistry and/or upregulation of catalase activity (101-103). Furthermore, deletion of the *nos* gene from the extremophile *Deinococcus radiodurans* severely compromises this organism's ability to survive exposure to radiation (104).

Interestingly, there is also evidence of a possible interkingdom signaling role for NO in studies of bNOS function. *Caenorhabditis elegans* is a nonparasitic, transparent nematode that lives in temperate soil environments. *C. elegans* subsists on *Bacillus sp.* as their primary food and is a rare eukaryote that does not encode a NOS gene. *C. elegans* that are fed wild-type *Bacillus* or *E. coli* transgenically expressing bNOS live longer than *C. elegans* fed either wild-type *E. coli* (do not encode bNOS) or bnos-deficient *Bacillus* (105).

Schreiber *et al.* demonstrated that NO from bNOS does not appear to affect biofilm development or bacterial motility in *B. subtilis* 3610 but found ~10-fold less biofilm in a Δ *bnos* mutant than in the wild type, suggesting NO may inhibit biofilm dispersal (106). In *Lactobacillus plantarum*, it was found that even nanomolar concentrations of exogenous NO from SNP were toxic and therefore reduced the level of biofilm formation. Endogenously produced NO from bNOS, however, was found to be critical for biofilm formation (107). Furthermore, when heterologously expressed in *Pseudomonas putida*, bNOS from *B. subtilis* was shown to produce NO endogenously, enhance bacterial motility, and decrease the level of biofilm formation (108).

NOS and NO/H-NOX/HaHK Signaling and Bacterial Symbiosis. Recently, the first characterization of a bacterium that encodes both a NOS and H-NOX was demonstrated, resembling the mammalian system capable of both synthesizing and sensing NO (109). *Silicibacter sp.* strain TrichCH4B is a marine alphaproteobacterium that possesses both the *hnoX* and *nos* genes in its genome. In this study, the authors demonstrate that *Silicibacter sp.* strain TrichCH4B NOS is activated by its algal symbiont *Trichodesmium erythraeum*. This activation of NOS leads to endogenous NO formation that can ligate to the H-NOX protein. NO/H-NOX signaling, through histidine kinase and a response regulator eventually leads to an increase in cyclic di-GMP levels resulting in biofilm, suggesting NO plays a key role in bacterial symbiosis. In general, the molecular details of NO sensing and signaling pathways in bacteria are poorly understood at present.

In conclusion, The H-NOX domain has been demonstrated to be a primary NO sensor in several environmental and facultative pathogenic bacteria that regulates biofilm formation in response to NO ligation. Many bacteria, including *P. aeruginosa* and *N. europaea*, do not encode H-NOX but have documented nanomolar NO-mediated biofilm phenotypes; therefore, it is likely that an additional biofilm-regulating NO sensor is yet to be characterized. Indeed, the condition and concentration dependent aspects of NO signaling in bacteria may indicate the existence of multiple NO signaling pathways that regulate biofilm formation, aside from the better established NO signaling pathways for detoxification and denitrification.

Chapter 2 describes the identification and first complete biochemical characterization of a novel NO sensing protein NosP and a NosP-associated two-component signaling

pathway in *Pseudomonas aeruginosa*. However, the histidine kinase proved challenging to work with in the laboratory. Therefore, NosP and the NosP-associated kinase were investigated in *Vibrio cholerae* in Chapter 3 and in *Legionella pneumophila* in Chapter 4. In Chapter 5, we discuss the implications and further aspects of this research.

References

1. Alderton WK, Cooper CE, & Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J* 357(Pt 3):593-615.
2. Stuehr DJ, Santolini J, Wang ZQ, Wei CC, & Adak S (2004) Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem* 279(35):36167-36170.
3. Daff S (2010) NO synthase: structures and mechanisms. *Nitric Oxide* 23(1):1-11.
4. Derbyshire ER & Marletta MA (2012) Structure and regulation of soluble guanylate cyclase. *Annu Rev Biochem* 81:533-559.
5. Ignarro LJ, Byrns RE, Buga GM, & Wood KS (1987) Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ Res* 61(6):866-879.
6. Ignarro LJ (1991) Signal transduction mechanisms involving nitric oxide. *Biochem Pharmacol* 41(4):485-490.
7. Murad F (1994) Regulation of cytosolic guanylyl cyclase by nitric oxide: the NO-cyclic GMP signal transduction system. *Adv Pharmacol* 26:19-33.
8. Denninger JW & Marletta MA (1999) Guanylate cyclase and the .NO/cGMP signaling pathway. *Biochim Biophys Acta* 1411(2-3):334-350.
9. MacMicking J, Xie QW, & Nathan C (1997) Nitric oxide and macrophage function. *Annu Rev Immunol* 15:323-350.
10. Bogdan C (2001) Nitric oxide and the immune response. *Nat Immunol* 2(10):907-916.

11. Cruz-Ramos H, Crack J, Wu G, Hughes MN, Scott C, Thomson AJ, Green J, & Poole RK (2002) NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. *EMBO J* 21(13):3235-3244.
12. D'Autreaux B, Tucker NP, Dixon R, & Spiro S (2005) A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature* 437(7059):769-772.
13. Bodenmiller DM & Spiro S (2006) The yjeB (nsrR) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. *J Bacteriol* 188(3):874-881.
14. Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L, & McFall-Ngai MJ (2004) NO means 'yes' in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell Microbiol* 6(12):1139-1151.
15. Schmidt I, Steenbakkens PJ, op den Camp HJ, Schmidt K, & Jetten MS (2004) Physiologic and proteomic evidence for a role of nitric oxide in biofilm formation by *Nitrosomonas europaea* and other ammonia oxidizers. *J Bacteriol* 186(9):2781-2788.
16. Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, & Webb JS (2006) Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol* 188(21):7344-7353.
17. Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, & Kjelleberg S (2009) Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. *Microb Biotechnol* 2(3):370-378.
18. Henares BM, Higgins KE, & Boon EM (2012) Discovery of a nitric oxide responsive quorum sensing circuit in *Vibrio harveyi*. *ACS Chem Biol* 7(8):1331-1336.

19. Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA, & Boon EM (2012) Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry* 51(10):2087-2099.
20. Plate L & Marletta MA (2012) Nitric oxide modulates bacterial biofilm formation through a multicomponent cyclic-di-GMP signaling network. *Mol Cell* 46(4):449-460.
21. Davey ME & O'Toole G A (2000) Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* 64(4):847-867.
22. O'Toole G, Kaplan HB, & Kolter R (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* 54:49-79.
23. Stoodley P, Sauer K, Davies DG, & Costerton JW (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56:187-209.
24. Webb JS, Givskov M, & Kjelleberg S (2003) Bacterial biofilms: prokaryotic adventures in multicellularity. *Curr Opin Microbiol* 6(6):578-585.
25. Hall-Stoodley L, Costerton JW, & Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2(2):95-108.
26. Flemming HC & Wingender J (2010) The biofilm matrix. *Nat Rev Microbiol* 8(9):623-633.
27. Costerton JW, Stewart PS, & Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284(5418):1318-1322.
28. Donlan RM & Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15(2):167-193.
29. Fux CA, Costerton JW, Stewart PS, & Stoodley P (2005) Survival strategies of infectious biofilms. *Trends Microbiol* 13(1):34-40.

30. Van Wey AS, Cookson AL, Roy NC, McNabb WC, Soboleva TK, & Shorten PR (2011) Bacterial biofilms associated with food particles in the human large bowel. *Mol Nutr Food Res* 55(7):969-978.
31. Swidsinski A, Loening-Baucke V, Lochs H, & Hale LP (2005) Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. *World J Gastroenterol* 11(8):1131-1140.
32. Stewart PS & Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* 358(9276):135-138.
33. Hoiby N, Bjarnsholt T, Givskov M, Molin S, & Ciofu O (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 35(4):322-332.
34. Romero D & Kolter R (2011) Will biofilm disassembly agents make it to market? *Trends Microbiol* 19(7):304-306.
35. Stock AM, Robinson VL, & Goudreau PN (2000) Two-component signal transduction. *Annu Rev Biochem* 69:183-215.
36. Laub MT & Goulian M (2007) Specificity in two-component signal transduction pathways. *Annu Rev Genet* 41:121-145.
37. Mascher T, Helmann JD, & Uden G (2006) Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* 70(4):910-938.
38. Cock PJ & Whitworth DE (2007) Evolution of prokaryotic two-component system signaling pathways: gene fusions and fissions. *Mol Biol Evol* 24(11):2355-2357.
39. Rodrigue A, Quentin Y, Lazdunski A, Mejean V, & Foglino M (2000) Two-component systems in *Pseudomonas aeruginosa*: why so many? *Trends Microbiol* 8(11):498-504.

40. Fuqua C, Parsek MR, & Greenberg EP (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* 35:439-468.
41. Miller MB & Bassler BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55:165-199.
42. Ng WL & Bassler BL (2009) Bacterial quorum-sensing network architectures. *Annu Rev Genet* 43:197-222.
43. Romling U, Gomelsky M, & Galperin MY (2005) C-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol* 57(3):629-639.
44. Jenal U & Malone J (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* 40:385-407.
45. Hengge R (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 7(4):263-273.
46. Romling U, Galperin MY, & Gomelsky M (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* 77(1):1-52.
47. Ryjenkov DA, Tarutina M, Moskvina OV, & Gomelsky M (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J Bacteriol* 187(5):1792-1798.
48. Schmidt AJ, Ryjenkov DA, & Gomelsky M (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J Bacteriol* 187(14):4774-4781.

49. Dow JM, Fouhy Y, Lucey JF, & Ryan RP (2006) The HD-GYP domain, cyclic di-GMP signaling, and bacterial virulence to plants. *Mol Plant Microbe Interact* 19(12): 1378-1384.
50. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, Allen HL, DeKievit TR, Gardner PR, Schwab U, Rowe JJ, Iglewski BH, McDermott TR, Mason RP, Wozniak DJ, Hancock RE, Parsek MR, Noah TL, Boucher RC, & Hassett DJ (2002) *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* 3(4):593-603.
51. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, & Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280(5361):295-298.
52. Whitehead NA, Barnard AM, Slater H, Simpson NJ, & Salmond GP (2001) Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* 25(4):365-404.
53. Antunes LC, Ferreira RB, Buckner MM, & Finlay BB (2010) Quorum sensing in bacterial virulence. *Microbiology* 156(Pt 8):2271-2282.
54. Galloway WR, Hodgkinson JT, Bowden SD, Welch M, & Spring DR (2011) Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chem Rev* 111(1):28-67.
55. Poole RK (2005) Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem Soc Trans* 33(Pt 1):176-180.
56. Schreiber K, Krieger R, Benkert B, Eschbach M, Arai H, Schobert M, & Jahn D (2007) The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J Bacteriol* 189(11):4310-4314.

57. Trunk K, Benkert B, Quack N, Munch R, Scheer M, Garbe J, Jansch L, Trost M, Wehland J, Buer J, Jahn M, Schobert M, & Jahn D (2010) Anaerobic adaptation in *Pseudomonas aeruginosa*: definition of the Anr and Dnr regulons. *Environ Microbiol* 12(6):1719-1733.
58. Giardina G, Rinaldo S, Johnson KA, Di Matteo A, Brunori M, & Cutruzzola F (2008) NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. *J Mol Biol* 378(5):1002-1015.
59. Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, & Kjelleberg S (2009) Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *J Bacteriol* 191(23):7333-7342.
60. Morgan R, Kohn S, Hwang SH, Hassett DJ, & Sauer K (2006) BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *J Bacteriol* 188(21):7335-7343.
61. Petrova OE & Sauer K (2012) PAS domain residues and prosthetic group involved in BdlA-dependent dispersion response by *Pseudomonas aeruginosa* biofilms. *J Bacteriol* 194(21):5817-5828.
62. Petrova OE, Cherny KE, & Sauer K (2015) The diguanylate cyclase GcbA facilitates *Pseudomonas aeruginosa* biofilm dispersion by activating BdlA. *J Bacteriol* 197(1):174-187.
63. Roy AB, Petrova OE, & Sauer K (2012) The phosphodiesterase DipA (PA5017) is essential for *Pseudomonas aeruginosa* biofilm dispersion. *J Bacteriol* 194(11):2904-2915.

64. Li Y, Heine S, Entian M, Sauer K, & Frankenberg-Dinkel N (2013) NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. *J Bacteriol* 195(16):3531-3542.
65. Galperin MY, Nikolskaya AN, & Koonin EV (2001) Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* 203(1): 11-21.
66. McDougald D, Rice SA, Barraud N, Steinberg PD, & Kjelleberg S (2011) Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol* 10(1):39-50.
67. Householder TC, Fozo EM, Cardinale JA, & Clark VL (2000) Gonococcal nitric oxide reductase is encoded by a single gene, norB, which is required for anaerobic growth and is induced by nitric oxide. *Infect Immun* 68(9):5241-5246.
68. Householder TC, Belli WA, Lissenden S, Cole JA, & Clark VL (1999) cis- and trans-acting elements involved in regulation of aniA, the gene encoding the major anaerobically induced outer membrane protein in *Neisseria gonorrhoeae*. *J Bacteriol* 181(2):541-551.
69. Isabella VM, Lapek JD, Jr., Kennedy EM, & Clark VL (2009) Functional analysis of NsrR, a nitric oxide-sensing Rrf2 repressor in *Neisseria gonorrhoeae*. *Mol Microbiol* 71(1):227-239.
70. Falsetta ML, Bair TB, Ku SC, Vanden Hoven RN, Steichen CT, McEwan AG, Jennings MP, & Apicella MA (2009) Transcriptional profiling identifies the metabolic phenotype of gonococcal biofilms. *Infect Immun* 77(9):3522-3532.

71. Falsetta ML, McEwan AG, Jennings MP, & Apicella MA (2010) Anaerobic metabolism occurs in the substratum of gonococcal biofilms and may be sustained in part by nitric oxide. *Infect Immun* 78(5):2320-2328.
72. Jardeleza C, Foreman A, Baker L, Paramasivan S, Field J, Tan LW, & Wormald PJ (2011) The effects of nitric oxide on *Staphylococcus aureus* biofilm growth and its implications in chronic rhinosinusitis. *Int Forum Allergy Rhinol* 1(6):438-444.
73. Sheffler LA, Wink DA, Melillo G, & Cox GW (1995) Characterization of nitric oxide-stimulated ADP-ribosylation of various proteins from the mouse macrophage cell line ANA-1 using sodium nitroprusside and the novel nitric oxide-donating compound diethylamine dinitric oxide. *J Leukoc Biol* 57(1):152-159.
74. Lundberg JO, Farkas-Szallasi T, Weitzberg E, Rinder J, Lidholm J, Anggaard A, Hokfelt T, Lundberg JM, & Alving K (1995) High nitric oxide production in human paranasal sinuses. *Nat Med* 1(4):370-373.
75. Deja M, Busch T, Bachmann S, Riskowski K, Campean V, Wiedmann B, Schwabe M, Hell B, Pfeilschifter J, Falke KJ, & Lewandowski K (2003) Reduced nitric oxide in sinus epithelium of patients with radiologic maxillary sinusitis and sepsis. *Am J Respir Crit Care Med* 168(3):281-286.
76. Iyer LM, Anantharaman V, & Aravind L (2003) Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins. *BMC Genomics* 4(1):5.
77. Karow DS, Pan D, Tran R, Pellicena P, Presley A, Mathies RA, & Marletta MA (2004) Spectroscopic characterization of the soluble guanylate cyclase-like heme

domains from *Vibrio cholerae* and *Thermoanaerobacter tengcongensis*. *Biochemistry* 43(31):10203-10211.

78. Pellicena P, Karow DS, Boon EM, Marletta MA, & Kuriyan J (2004) Crystal structure of an oxygen-binding heme domain related to soluble guanylate cyclases. *Proc Natl Acad Sci U S A* 101(35):12854-12859.

79. Boon EM, Huang SH, & Marletta MA (2005) A molecular basis for NO selectivity in soluble guanylate cyclase. *Nat Chem Biol* 1(1):53-59.

80. Boon EM & Marletta MA (2005) Ligand discrimination in soluble guanylate cyclase and the H-NOX family of heme sensor proteins. *Curr Opin Chem Biol* 9(5):441-446.

81. Boon EM & Marletta MA (2005) Ligand specificity of H-NOX domains: from sGC to bacterial NO sensors. *J Inorg Biochem* 99(4):892-902.

82. Boon EM, Davis JH, Tran R, Karow DS, Huang SH, Pan D, Miazgowiec MM, Mathies RA, & Marletta MA (2006) Nitric oxide binding to prokaryotic homologs of the soluble guanylate cyclase beta1 H-NOX domain. *J Biol Chem* 281(31):21892-21902.

83. Ma X, Sayed N, Beuve A, & van den Akker F (2007) NO and CO differentially activate soluble guanylyl cyclase via a heme pivot-bend mechanism. *EMBO J* 26(2):578-588.

84. Price MS, Chao LY, & Marletta MA (2007) *Shewanella oneidensis* MR-1 H-NOX regulation of a histidine kinase by nitric oxide. *Biochemistry* 46(48):13677-13683.

85. Carlson HK, Vance RE, & Marletta MA (2010) H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Mol Microbiol* 77(4):930-942.

86. Wang Y, Dufour YS, Carlson HK, Donohue TJ, Marletta MA, & Ruby EG (2010) H-NOX-mediated nitric oxide sensing modulates symbiotic colonization by *Vibrio fischeri*. *Proc Natl Acad Sci U S A* 107(18):8375-8380.
87. Arora DP & Boon EM (2012) Nitric oxide regulated two-component signaling in *Pseudoalteromonas atlantica*. *Biochem Biophys Res Commun* 421(3):521-526.
88. Tsai AL, Berka V, Martin E, & Olson JS (2012) A "sliding scale rule" for selectivity among NO, CO, and O(2) by heme protein sensors. *Biochemistry* 51(1):172-186.
89. Tsai AL, Martin E, Berka V, & Olson JS (2012) How do heme-protein sensors exclude oxygen? Lessons learned from cytochrome c', *Nostoc punctiforme* heme nitric oxide/oxygen-binding domain, and soluble guanylyl cyclase. *Antioxid Redox Signal* 17(9):1246-1263.
90. Plate L & Marletta MA (2013) Nitric oxide-sensing H-NOX proteins govern bacterial communal behavior. *Trends Biochem Sci* 38(11):566-575.
91. Liu N, Pak T, & Boon EM (2010) Characterization of a diguanylate cyclase from *Shewanella woodyi* with cyclase and phosphodiesterase activities. *Mol Biosyst* 6(9):1561-1564.
92. Lahiri T, Luan B, Raleigh DP, & Boon EM (2014) A structural basis for the regulation of an H-NOX-associated cyclic-di-GMP synthase/phosphodiesterase enzyme by nitric oxide-bound H-NOX. *Biochemistry* 53(13):2126-2135.
93. Zapf J, Madhusudan M, Grimshaw CE, Hoch JA, Varughese KI, & Whiteley JM (1998) A source of response regulator autophosphatase activity: the critical role of a residue adjacent to the Spo0F autophosphorylation active site. *Biochemistry* 37(21):7725-7732.

94. Thormann KM, Saville RM, Shukla S, & Spormann AM (2005) Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. *J Bacteriol* 187(3):1014-1021.
95. Henares BM, Xu Y, & Boon EM (2013) A nitric oxide-responsive quorum sensing circuit in *Vibrio harveyi* regulates flagella production and biofilm formation. *Int J Mol Sci* 14(8):16473-16484.
96. Visick KL & Ruby EG (2006) *Vibrio fischeri* and its host: it takes two to tango. *Curr Opin Microbiol* 9(6):632-638.
97. Lundberg JO, Weitzberg E, Cole JA, & Benjamin N (2004) Nitrate, bacteria and human health. *Nat Rev Microbiol* 2(7):593-602.
98. Hyde ER, Luk B, Cron S, Kusic L, McCue T, Bauch T, Kaplan H, Tribble G, Petrosino JF, & Bryan NS (2014) Characterization of the rat oral microbiome and the effects of dietary nitrate. *Free Radic Biol Med* 77:249-257.
99. Torreilles J (2001) Nitric oxide: one of the more conserved and widespread signaling molecules. *Front Biosci* 6:D1161-1172.
100. Kers JA, Wach MJ, Krasnoff SB, Widom J, Cameron KD, Bukhalid RA, Gibson DM, Crane BR, & Loria R (2004) Nitration of a peptide phytotoxin by bacterial nitric oxide synthase. *Nature* 429(6987):79-82.
101. Gusarov I & Nudler E (2005) NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. *Proc Natl Acad Sci U S A* 102(39):13855-13860.
102. Shatalin K, Gusarov I, Avetissova E, Shatalina Y, McQuade LE, Lippard SJ, & Nudler E (2008) *Bacillus anthracis*-derived nitric oxide is essential for pathogen virulence and survival in macrophages. *Proc Natl Acad Sci U S A* 105(3):1009-1013.

103. Gusarov I, Shatalin K, Starodubtseva M, & Nudler E (2009) Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. *Science* 325(5946): 1380-1384.
104. Patel BA, Moreau M, Widom J, Chen H, Yin L, Hua Y, & Crane BR (2009) Endogenous nitric oxide regulates the recovery of the radiation-resistant bacterium *Deinococcus radiodurans* from exposure to UV light. *Proc Natl Acad Sci U S A* 106(43): 18183-18188.
105. Gusarov I, Gautier L, Smolentseva O, Shamovsky I, Eremina S, Mironov A, & Nudler E (2013) Bacterial nitric oxide extends the lifespan of *C. elegans*. *Cell* 152(4): 818-830.
106. Schreiber F, Beutler M, Enning D, Lamprecht-Grandio M, Zafra O, Gonzalez-Pastor JE, & de Beer D (2011) The role of nitric-oxide-synthase-derived nitric oxide in multicellular traits of *Bacillus subtilis* 3610: biofilm formation, swarming, and dispersal. *BMC Microbiol* 11:111.
107. Iarullina DR, Vakatoва LV, Krivoruchko AV, Rubtsova EV, & Il'inskaia ON (2013) Exogenous and endogenous nitric oxide influence on biofilm formation by *Lactobacillus plantarum*. *Mikrobiologiya* 82(4):417-421.
108. Liu P, Huang Q, & Chen W (2012) Heterologous expression of bacterial nitric oxide synthase gene: a potential biological method to control biofilm development in the environment. *Can J Microbiol* 58(3):336-344.
109. Rao M, Smith BC, & Marletta MA (2015) Nitric Oxide Mediates Biofilm Formation and Symbiosis in *Silicibacter* sp. Strain TrichCH4B. *MBio* 6(3):e00206-00215.

Chapter 2

NosP: Discovery of a novel nitric oxide sensor and nitric oxide-responsive signaling pathway in *Pseudomonas aeruginosa*¹

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, I describe the discovery of a novel nitric oxide sensor (NosP; NO sensing protein) as well as a novel NO-responsive pathway in *P. aeruginosa*. I demonstrate that biofilms of a *P. aeruginosa* mutant lacking components of the NosP pathway loses the ability to disperse in response to NO. Upon cloning, expressing, and purifying NosP, I found that it binds heme and ligates to NO with a dissociation rate constant that is comparable to other well-established NO-sensing proteins. Moreover, I show that NO-bound NosP is able to regulate the phosphorelay activity of a hybrid histidine kinase that is involved in biofilm regulation in *Pseudomonas aeruginosa*. Here I present evidence of a novel NO-responsive pathway that regulates biofilm formation in *Pseudomonas aeruginosa*.

¹ This chapter is adapted from the following submission
Hossain S and Boon EM (2016). NosP: Discovery of a novel nitric oxide sensor and nitric oxide-responsive signaling pathway in *Pseudomonas aeruginosa*. *PNAS* (submitted).

NosP: Discovery of a novel nitric oxide sensor and nitric oxide-responsive signaling pathway in *Pseudomonas aeruginosa*

Introduction

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 research 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 system for biofilm formation, 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 causes *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⁺, Hg²⁺, and As³⁺. 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 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.

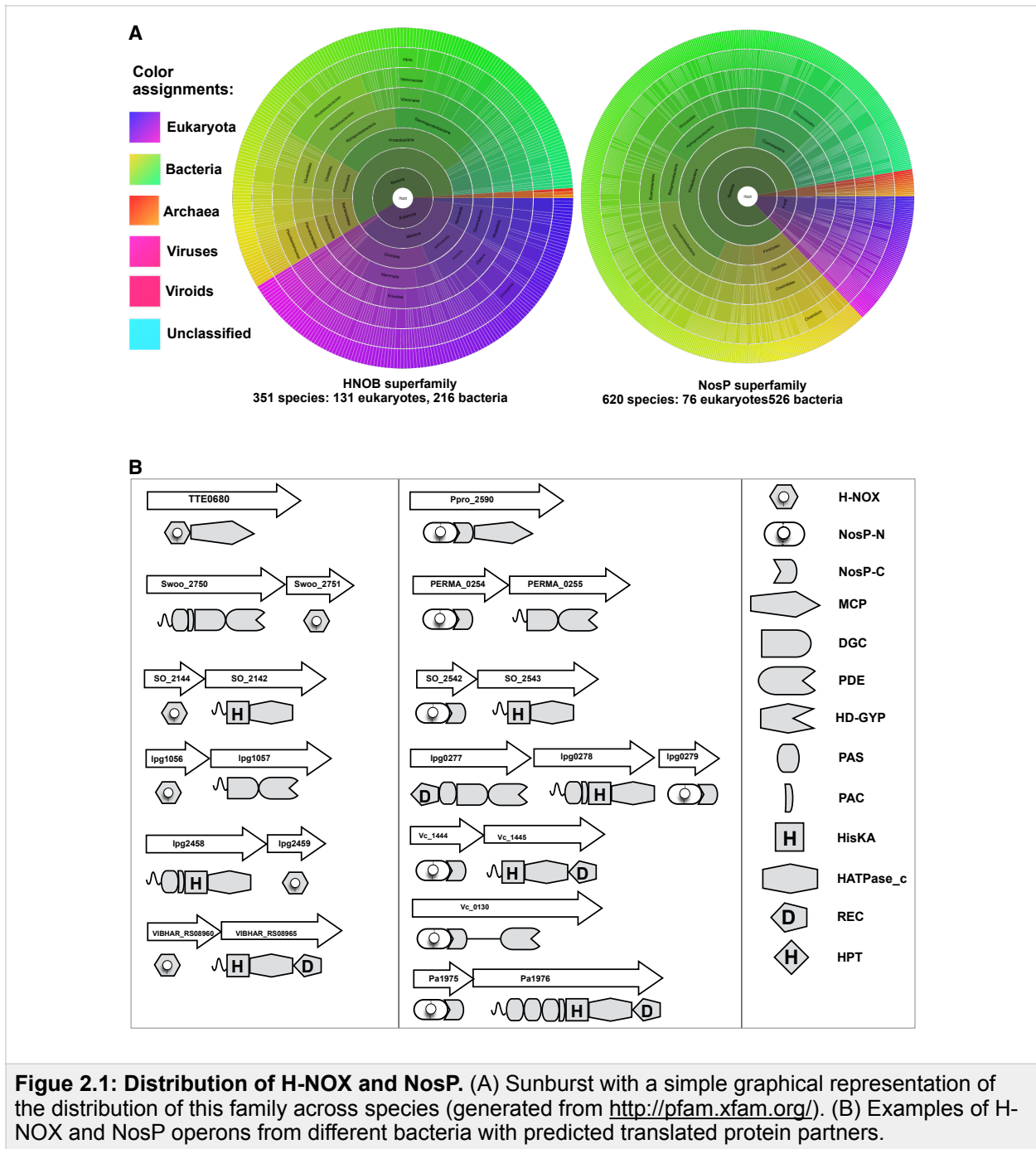
The molecular basis for NO-mediated biofilm regulation has been demonstrated in some bacteria, including *Legionella pneumophila* (25), *Shewanella oneidensis* (26), *Shewanella woodyii* (27), *Vibrio harveyi* (28, 29) and *Silicibacter sp.* strain TrichCH4B (30). In these bacteria, the NO sensor H-NOX (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 sensing proteins in bacteria called NosP (nitric oxide sensing protein). In *P. aeruginosa*, NosP binds heme, and upon ligating to NO at the heme iron, modulates the activity of a co-cistronic 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, that is widely distributed in bacteria (found in about 620 independent sequenced species, see Figure 2.1A). This domain was previously predicted to be a sensory domain by Borziak *et al.* (33), due to its appearance 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 (Figure 2.1B). Interestingly, the signaling proteins co-cistronic 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 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 hyper-biofilm phenotype (35), suggesting involvement of FIST in biofilm regulation. Most relevant to this study, in *Pseudomonas aeruginosa*, a FIST domain (Pa1975) is co-cistronic with the hybrid histidine kinase Pa1976 (Figure 2.1B). 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 co-cistronic 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*. As such, we named it NosP, for NO sensing protein.

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 *E. coli*. Upon purification, we found that it has the yellow-orange color common for hemoproteins (Figure 2.2A). In order to confirm that NosP is a heme protein, we performed a heme pulldown assay. As illustrated in Figure 2.1B, *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 electronic 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 ~350nm to ~450nm. UV-visible spectra of NosP as the Fe^{II}, Fe^{II}-CO, and Fe^{II}-NO complexes at room temperature are shown in figure 2.2 and are compared with those of H-NOX and other histidyl-ligated heme proteins in Table 2.1.

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 nm and 524 nm (Figure 2.2C). This spectrum is similar to those of hexa-coordinated hemoproteins, such as CoxA, cytochrome c, and the truncated globins, where the iron is ligated to two axial ligands, usually histidine and an additional amino acid (see Table 2.1) (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

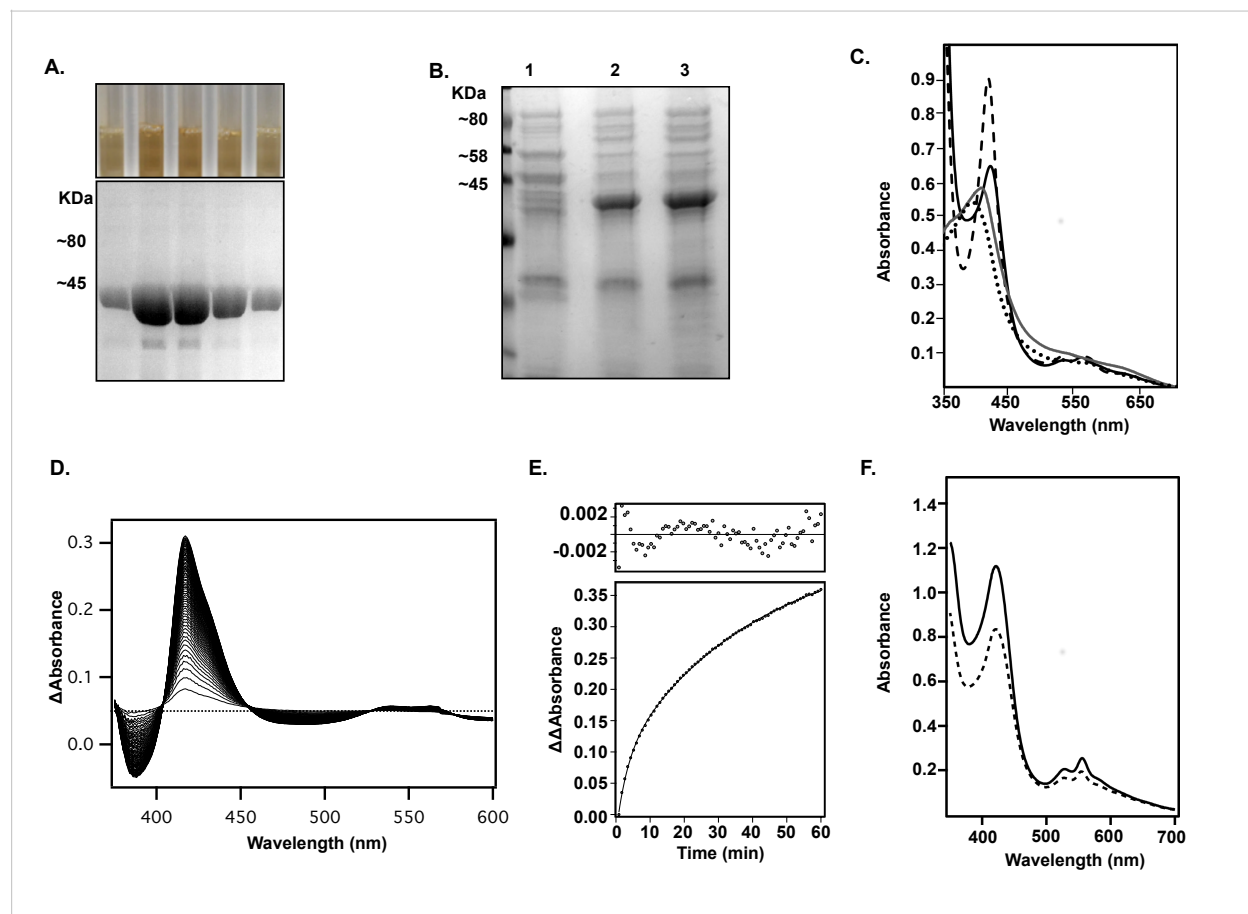


Figure 2.2: NosP is a hemoprotein that ligates NO. (A) Purified hexaHis-tagged NosP is yellow-orange in color. Top, NosP fractions as eluted from IMAC. Below, a Coomassie-stained SDS-PAGE of the same purified fractions. (B) *E. coli* lysate with overexpressed NosP (lane 2 and 3), but not lysate from 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 same protein band with more stringent wash steps. (C) NosP has the ligand binding specificity of a NO sensor. Electronic 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 electronic absorption spectroscopy with saturating CO and 30 mM dithionite as a trap for the released NO. Measured rates and amplitudes [$k_1 = k_{\text{off}} = (1.8 \pm 0.5) \times 10^{-4} \text{ s}^{-1}$, $k_2 = (13 \pm 2) \times 10^{-4} \text{ s}^{-1}$] were independent of CO and dithionite at all concentrations tested (3 – 300 mM). (D) The 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) The N-terminal domain of NosP is sufficient to bind heme. Electronic absorption spectra of ferrous NosP (black line) and ferrous NosP-NT (dashed line), with a λ_{\max} at 420 nm.

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.

The 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 constructed a truncated mutant of NosP (NosP-NT) that contains the first 235 residues (the N-terminal domain) with a hexaHis-tag at its C-terminus. When the mutant construct was purified, it retained its yellow-orange color indicating that it is bound to heme, similarly to the full-length protein. The electronic spectroscopy of this mutant is also consistent with the full-length protein, indicating that heme binding is contained within the N-terminus of NosP (Figure 2.2F and Table 2.1).

The 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 recombination of the dissociated NO. The rate of NO dissociation was followed by the formation of the Fe^{II}-CO complex at 416 nm. The measured rate was independent of CO and dithionite at all concentrations tested (3, 30, and 300 mM dithionite). Representative spectra for NosP are shown in Figure 2.2D and Table 2.1 compares these data with other Fe^{II}-NO heme proteins.

Figure 2.2E shows the data fit with two parallel exponentials ($k_1 = (1.8 \pm 0.5) \times 10^{-4} \text{ s}^{-1}$, $k_2 = (13 \pm 2) \times 10^{-4} \text{ s}^{-1}$) of the form $f(x) = A \times (1 - e^{-kx})$. 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-

Table 2.1-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
CytC	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	38, 39, 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	$3.6 \pm 0.8 \times 10^{-4} \text{s}^{-1}$			46
SwH-NOX	$15.2 \pm 3.5 \times 10^{-4} \text{s}^{-1}$			27
VhH-NOX	$4.6 \pm 0.9 \times 10^{-4} \text{s}^{-1}$			28
NosP	$1.8 \pm 0.5 \times 10^{-4} \text{s}^{-1}$			This article

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 $1.8 \times 10^{-4} \text{ s}^{-1}$ for NosP is very similar to that of sGC $3.6 \times 10^{-4} \text{ s}^{-1}$ (46) and other H-NOX domains (Table 2.1), 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 from NosP.

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 co-cistronic kinase (Pa1976; named *nahK* for NosP-associated histidine kinase), using targeted type II intron disruption (TargeTron®) (47). 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 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*.

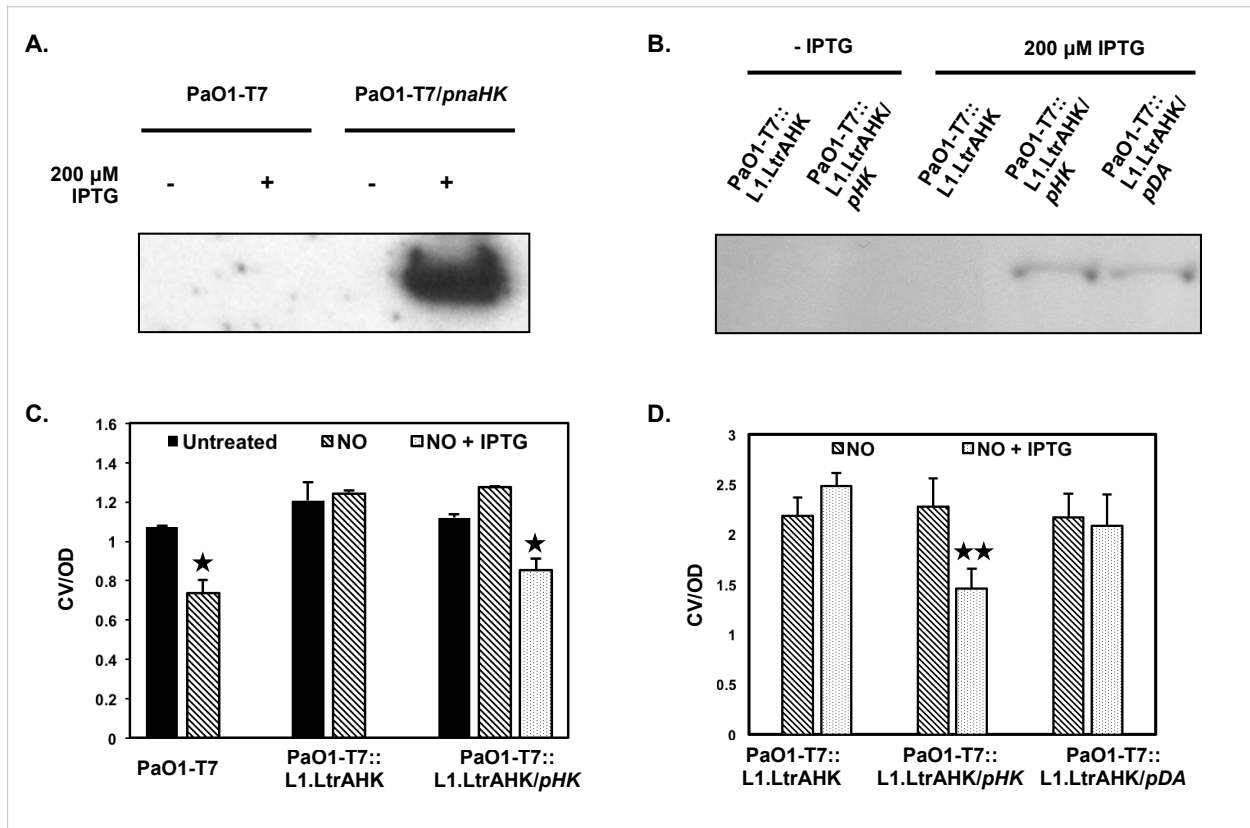


Figure 2.3: NO mediated biofilm dispersal requires NaHK. (A, B) Western blot analyses with an anti-hexaHis-tag antibody (HRP). (A) NaHK can be expressed in *P. aeruginosa* strain PaO1-T7. Anti-His western blot of NaHK in PaO1-T7 and PaO1-T7 that is transformed with an IPTG-inducible plasmid expressing 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 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 hours 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 represent one standard deviation from the mean of triplicate experiments; * = $p \leq 0.005$ in comparison to wild-type PaO1-T7; ** = $p \leq 0.005$ in comparison to PaO1-T7::L1.LtrAHK/pHK before IPTG addition.

We were able to target *nahK* (*pa1976*) with L1.LtrA to generate the strain PaO1-T7::L1.LtrAHK. Further, we complemented PaO1-T7::L1.LtrAHK with an IPTG-inducible vector that 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.LtrAHK strains. The results are shown in Figures 2.3A and 2.3B and indicate that the induction and expression of NaHK takes place only when IPTG is added to the growth media (2.3A lane 4 and 2.3B lane 4).

To characterize the biofilming characteristics of these strains, we conducted a static biofilm assay. As illustrated in Figure 2.3C, 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, 48). PaO1-T7::L1.LtrAHK, the kinase-disrupted mutant, is able to form biofilm, but it does not display an NO phenotype, as demonstrated in figure 2.3C. The NaHK kinase-complemented strain recovers the NO phenotype, but only once IPTG is added to the medium to induce *pa1976* expression (Figure 2.3C). 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 2.3D). 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 an operon with a hybrid histidine kinase (Pa1976; named NaHK for NosP-associated histidine kinase). Frequently co-cistronic proteins function together, thus we decided to study the kinase

activity of NaHK and determine if it is regulated by NosP. In order to study the kinase activity of Pa1976, we first 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 2.4A). 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 NaHK Δ N84. This truncated construct did not express or purify well, but sufficient quantities were obtained to continue with the study.

Figure 2.4B shows the autokinase activity of NaHK Δ N84 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 (49, 50). Due to phosphatase activity (51), phosphotransferase 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 2.4C, however, demonstrates stable phosphorylation of NaHK Δ N84 over 30 minutes.

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 (50). *P. aeruginosa* has three annotated HPTs, of which Pa3345 (HptB) has been shown to accept phosphate 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 2.4C lane 1. We made a mutant of NaHK Δ N84, NaHK Δ N84DA, in which the conserved aspartate (D809) in the receiver domain is

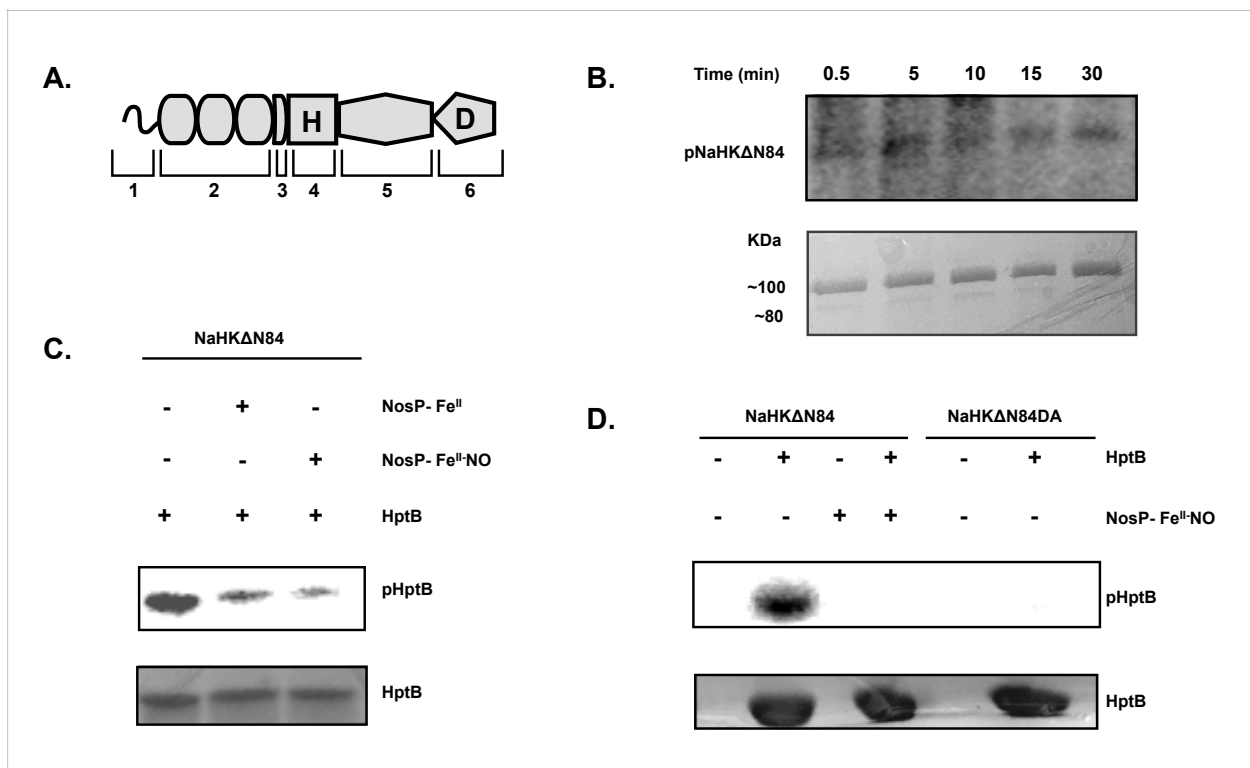


Figure 2.4: NO/NosP regulates NaHK. (A) Schematic of predicted domain organization of NaHK (pa1976). The N-terminus is predicted to be coiled-coil followed by three PAS domains, a PAC domain, a HisKA domain [His Kinase A (phosphoacceptor) domain], a HATPase_c domain (Histidine kinase-like ATPases), and a REC domain. (B) *In vitro* autophosphorylation of NaHK Δ N84 over time. Radiolabeled phosphoproteins were detected by SDS-PAGE (bottom) and autoradiography (top). (C, D) NaHK Δ N84 transfers phosphate to HptB. (C) Phosphotransfer from NaHK Δ N84 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 ³²P-labeled ATP and monitored over time: lane 1, NaHK Δ N84 + HptB; lane 2, NaHK Δ N84 + HptB + ferrous NosP; lane 3, NaHK Δ N84 + HptB + NO-bound NosP. (D) NaHK Δ N84DA cannot transfer phosphate to HptB, indicating Asp809 is necessary for phosphotransfer from NaHK Δ N84 to HptB. Top, protein loading detected by Coomassie staining. Bottom, detection of radiolabeled HptB by autoradiography. Lanes 1-5, the following proteins were incubated with ³²P-labeled ATP and monitored over time: lane 1, NaHK Δ N84; lane 2, NaHK Δ N84 + HptB; lane 3, NaHK Δ N84 + NO-bound NosP; lane 4, NaHK Δ N84 + HptB + NO-bound NosP; lane 5, NaHK Δ N84DA; lane 6, NaHK Δ N84DA + HptB.

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 NaHK Δ N84DA was used instead of NaHK Δ N84 in the phosphorelay assay (Figure 2.4D).

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 2.4C). Inhibition of NaHK Δ N84 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 NaHK Δ N84 was observed in the presence of NO-bound NosP (Figure 2.4C, lane 3). Indeed, excess (~30-fold) NO-bound NosP is able to completely inhibit phosphotransfer to HptB (Figure 2.4D, lane 3). Interestingly, this pattern of kinase inhibition is analogous to the inhibition of histidine kinase activity observed in H-NOX/NaHK (H-NOX and H-NOX-associated histidine kinase) signaling in *Vibrio harveyi* (28), *Pseudalteromonas atlantica* (52), *Shewanella oneidensis* (26), and *Silicibacter sp.* strain TrichCH4B (30). In all these systems, NO-ligated H-NOX inhibits NaHK kinase activity.

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.* (53) 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 $\Delta hapZ$ mutant was shown to be deficient in biofilm formation, due to an early attachment defect, as demonstrated by a

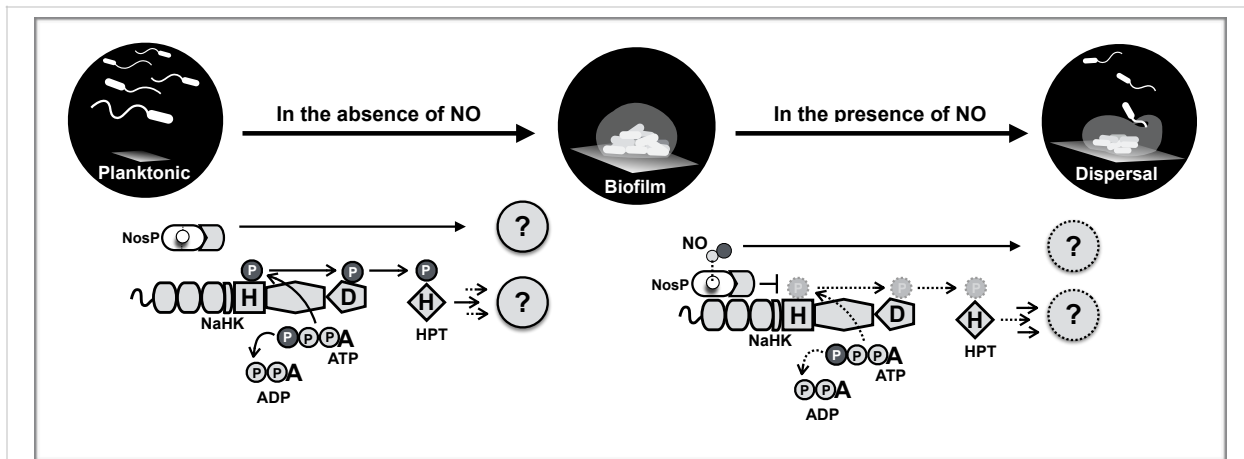


Figure 2.5: Schematic representation of NO-mediated biofilm dispersal through HptB. In the absence of NO, NosP has no effect on NaHK activity. As a result, NaHK can autophosphorylate its conserved histidine residue, transfer phosphate intramolecularly to its receiver domain and then engage in phosphotransfer 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, however, NO/NosP inhibits NaHK activity which ultimately yields a decrease in phosphate flow through the signaling pathway, and results in biofilm dispersal.

flow cell biofilm assay (53). 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 both HptB-mediated regulation of receiver domain-containing diguanylate cyclases and/or cyclic-di-GMP phosphodiesterases, as well as possible NosP interactions with these types of proteins and/or orphan kinases in *P. aeruginosa*.

Conclusion

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 2.5). In conclusion, we have identified a novel family of bacterial primary NO sensors 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 co-cistronic 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 in these and other bacterial systems.

Materials and Methods

Cloning genes for heterogenous expression and protein purification from *E. coli*.

DH5 α *E. coli* strain was used to clone *nosP*, *nosP-NT*, *nahK*, *nahK Δ N84*, *hpt*. All the constructs were introduced into pET20 (b) vector (Novagen) by the use of NdeI and XhoI restriction enzymes. The DA (D809A) mutants were generated by site directed

mutagenesis. All the primers used are listed in Table 2.2. All the generated constructs were verified via DNA sequencing.

Generation of PaO1-T7::L1LtrAHK. The NaHK gene disruption was constructed according to Yao *et al.* (54). The NaHK gene disruption was constructed according to Yao *et al.* (54). In short, by use of the clostron algorithm (<http://clostron.com/>), primer sets were generated to retarget L1.LtrA intron to the *nahK* gene. By the use of two step PCR, the desired insert was generated and with appropriate restriction enzymes (HindIII and BsrGI) was introduced into the pBL1 vector (generously provided by Dr. Alan M. Lambowitz). The pBL1 vector contains an *m*-toluic acid-inducible promoter and RK2 minireplicon. After ligation, the *pBL1nahK*, was transformed into DH5 α and clones were confirmed by DNA sequencing. This construct, *pBL1nahK*, was then transformed into the PaO1-T7 strain (generously provided by Dr. Peter Greenberg), according to the Benchmarks BioTechnique protocol (55). Following transformation, an isolated colony was inoculated into 5 ml of LB supplemented with 80 μ g/ml tetracycline. The culture was allowed to grow until it reached an OD₅₉₅ of 0.3-0.4 at which point it was induced with 2 mM *m*-toluic acid overnight at 30 °C. Serial dilutions of induced overnight cultures were plated on LB agar plates with tetracycline and incubated overnight at 37 °C. Isolated colonies were used in colony PCR with primers flanking the insertion site (Table 2.2) to isolate clones with disrupted *nahK*; these clones exhibit a +900 basepair shift due to the insertion of the intron.

Generation of PaO1-T7::L1LtrAHK/pHK. To induce expression of NaHK, we used the pLJQhis vector containing a broad-host-range *ori* compatible with *P. aeruginosa* (56). We subcloned *nahK* in this vector using NdeI and XhoI. Once the clones were

Table 2.2-Strains, Plasmids, and Primers Used

<i>E. coli</i> strains used in this work		
DH5α		For cloning genes into <i>E. coli</i>
BL21 (DE3) pLysS		For cloning genes into <i>E. coli</i>
<i>Pseudomonas aeruginosa</i> strains used for biofilm assay		
PaO1-T7		
PaO1-T7::L1.LtrAHK		This study
PaO1-T7::L1.LtrAHK/ <i>pHK</i>		This study
PaO1-T7::L1.LtrAHK/ <i>pDA</i>		This study
Plasmids Used		
pET20 (b)	To clone, express and purify proteins from <i>E. coli</i> .	This study
pBI1	To generate retargeted intron	53
pLJQ-His	To complement <i>nahK</i> disrupted strain PaO1-T7::L1.LtrAHK	55
Primers used (N represents nonspecific nucleotides)		
<i>pnosP</i>	5'-NNNNNNNCATATGTACGAAGGGCAGGGGAAGGGTCTGC-3' 5'-NNNNNNNCTCGAGGCGACGGACACCTCCGCGGGTTCGGCC-3'	
<i>pnosPNT</i>	5'-NNNNNNNCATATGTACGAAGGGCAGGGGAAGGGTCTGC-3' 5'-NNNNNNNCTCGAGCGGCTCGGCGTTGAGTTC-3'	
<i>pnahK</i>	5'-NNNNNNNCATATGGCATGCATATCAACCAGACCTTC-3' 5'-NNNNNNNCTCGAGGACCGAGGCTTCGCGCTCCAGCAAGTG-3'	
<i>pnahKΔN84</i>	5'-NNNNNNNCATATGCTCGAACTGAAGGCCAGCAAC-5' 5'-NNNNNNNCTCGAGGACCGAGGCTTCGCGCTCCAGCAAGTG-3'	
<i>pnahKΔN84DA</i>	5'-CCTGCTGATCGTCGCCTATCACCTCGACG-3' 5'-CGTCGAGGTGATAGGCGACGATCAGCAGG-3'	
Retargeting L1.LtrAHK	5'-AAAAAAGCTTATAATTATCCTTAGCCGGCGGGCAGGTGCGCCCAGATAGGGTG-3' 5'-CAGATTGTACAAATGTGGTGATAACAGATAAGTCGGGCAGCGTAACTTACCTTTCTTTGT-3' 5'-TGAACGCAAGTTTCTAATTTTCGATTCCGGCTCGATAGAGGAAAGTGTCT-3' 5'-CGAAATTAGAACTTTCGCTTCAGTAAAC-3'	
Primers used for colony PCR	5'-TCGAGGAGCAGCGTGG-3' 5'-CTCGCTGGCAGCAAC-3'	

confirmed via DNA sequencing, the plasmids were introduced into PaO1-T7::L1LtrA with the Benchmarks BioTechnique protocol (55).

Protein Expression and purification. NosP expression and purification procedure was adopted from Boon *et al.* (44), NaHK purification was adopted from Hsu *et al.* (36), and HPT expression and purification was adopted according to Ni-NTA manufacturer's (Qiagen) specifications. BL21 (DE3) pLysS cells (Novagen) were transformed with the *pnosP* plasmid. A single colony from the transformants was inoculated in 5 ml Lennox Broth (LB) for an overnight starter culture supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). This 5 ml overnight culture was diluted into 1 L yeast extract media (4.5% Yeast extract with 17 mM NaH₂PO₄ and 72 mM Na₂HPO₄, pH 7.5) with antibiotics and shaken at 37 °C until the optical density (OD₅₉₅) reached ~0.5. At the desired OD, 0.1 mM ALA (β -aminolevulinic acid) was added to the culture as it cooled to 16 °C, was induced with 25 µM IPTG (isopropyl β -D-1-thiogalactopyranoside), and then was shaken at 250 rpm at 16 °C for 12 to 16 hours. The induced cells were harvested by centrifugation and stored at -80 °C. For purification, the frozen pellet was thawed on ice and resuspended in lysis buffer (20 mM Tris-HCl, 250 mM KCl, 5 mM 2-mercaptoethanol, 10% glycerol, 50 µM EDTA, 1mM PMSF, 1% Triton X-100, 20µM hemin, pH 8.0). The resuspended pellets were then sonicated to lyse the cells and centrifuged for 1 h at 4 °C at 40,000 *xg* to clear the lysate. The cleared lysate was then passed through 1 ml of pre-equilibrated Ni-NTA beads and washed with 100 ml of wash buffer (20 mM Tris-HCl, 250 mM KCl, 5 mM 2-mercaptoethanol, 10% glycerol, 1 mM PMSF, pH 8.0) containing 10 mM imidazole. For a second wash step, 50 ml of 20 mM imidazole wash was used followed by 5 ml of a 50 mM imidazole wash for a third wash

step prior to eluting the proteins with 250 mM imidazole-containing buffer. Fractions (1 ml) were collected during elution and Bradford reagent was used in a 1:10 (protein to Bradford reagent) ratio to determine the fractions containing protein. Subsequently, the fractions containing protein were pooled together and desalted on a PD10 column (GE) in desalting buffer (50 mM Tris, 250 mM KCl, 5 μ M 2-mercaptoethanol, pH 8.0) to get rid of excess imidazole. The desalted protein was then incubated with 100 μ M hemin overnight at 4 °C. Later, protein containing heme was concentrated by passing through a membrane with a 10 KDa exclusion limit and was purified by size exclusion chromatography on a Superdex 200 column. The colored fractions were collected and glycerol was added to a final concentration of 10% to be stored at -80 °C for future use.

Expression and Purification of other proteins. All the constructs used for protein purification were transformed into BL21 (DE3) pLysS cells. TB media (1.2% Tryptone, 2.4% Yeast extract, 0.04% glycerol with 17 mM KH_2PO_4 and 72 mM K_2HPO_4 , pH 7.5) was used for growth, and no ALA was added. NaHK Δ N84, and NaHK Δ N84DA were induced at OD_{595} of 0.5 with 5 μ M IPTG. HptB (Pa3345) was induced with 25 μ M IPTG at OD_{595} of 0.5. Bacterial cultures were cooled to 16 °C prior to induction. NaHK Δ N84, and NaHK Δ N84DA purification buffer contained 500 mM NaCl instead of 250 mM KCl, with no hemin added, and the lysis buffer contained cComplete, EDTA free, protease inhibitor cocktail (Roche). Typically, following binding to 1 ml of Ni-NTA beads, three wash steps were performed: 100 ml with 10 mM imidazole, 50 ml with 20 mM imidazole and 10 ml with 30 mM imidazole. Followed by the washes, the proteins were eluted in buffer with 250 mM imidazole and dialyzed in dialysis buffer (50 mM Tris, 200 mM KCl, 10 mM MgCl_2 , 0.1 mM EDTA and 50% glycerol, 1 mM dithiothreitol, pH 8.0) overnight at

4 °C. The next day proteins were aliquoted and stored at -80 °C for later use. Purification of HptB was carried out in the same buffers used for NosP purifications excluding any addition of hemin. Purified HptB, after elution, was desalted on a PD-10 column (GE) with the same desalting buffer used for NosP. Glycerol was added to a final concentration of 10% and proteins were stored at -80 °C for future use.

Western Blot. Western blot analysis was conducted by the use of the Anti-6X His tag® antibody (HRP) from Abcam (ab1187) according to manufacturer's specifications. In short, a nitrocellulose blotting membrane was used for transfer. Following transfer, the membrane was blocked in 5% nonfat milk in TBST (Tris buffered saline with 0.05% Tween20) for an hour with gentle rocking at room temperature. Incubation with the antibody was carried out in fresh blocking buffer (1:10,000 dilution) for another hour at room temperature with rocking. After the discarding of the antibody solution, the membrane was washed four times for 10 minutes each with excess TBST. Following the wash, ECL western blotting substrate (Millipore) was added onto the membrane for one minute. The membrane was then exposed to x-ray films (Denville Scientific) and developed manually.

Kinase Assay and Autoradiography . Kinase assay conditions were adapted from Hsu *et al.* (36). Autoradiographs were analyzed by the use of ImageJ software. Kinase assays were performed in an assay buffer containing 100 mM Tris, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, and pH 8.0. Typically, each reaction was started by the addition of an ATP solution so that the final ATP concentration was 2.5 μM with 10 μCi [γ-³²P]-ATP per reaction. Reactions were incubated for 30 minutes at room temperature before quenching with 5x SDS loading dye, unless HptB was added. Following separation on a

12.5% Tris-glycine SDS-PAGE, gels were fixed and stained with Coomassie blue and destained prior to exposure to a phosphoscreen overnight and scanned with a Typhoon phosphoimager (Amersham Biosciences) and images were analyzed with ImageJ software. For the phosphotransfer assay, HptB was added to the reaction and incubated for an additional 15 minutes after the kinase had already been incubated with ATP for 30 minutes at room temperature.

Electronic Spectroscopy. All electronic spectra were recorded on a Cary 100 spectrophotometer equipped with a constant temperature bath. NosP complexes were prepared in an oxygen-free glove bag and NO dissociation kinetics were measured as previously described (44).

Heme agarose pulldown assay. Hemin-agarose was purchased from Sigma. Hemin-agarose (40 μ l) was equilibrated with 500 μ l of assay buffer (20 mM Tris•HCl, 300mM NaCl, 1 mM PMSF, 1% triton X-100, pH 8.0). Cleared lysate (1 ml) from an induced, non-specific 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 hour at 4 °C. The beads were collected by centrifugation. The lysate supernatant fraction was discarded and the beads were washed three times with 1ml of the assay buffer containing either 10 mM imidazole or 100 mM imidazole. Following the wash steps, 50 μ l of SDS loading dye was added directly to the beads and they were boiled for three minutes at 95 °C. Samples were centrifuged again to settle the beads and 10 μ l of the supernatant fraction was analyzed via SDS PAGE.

Biofilm Assay. Overnight cultures of PaO1-T7 wild-type and mutants were subjected to microtiter dish assay described previously (57). To analyze biofilm, overnight cultures of

PaO1-T7 wild-type and mutants were diluted 100-fold in M63 media according to the microtiter dish assay described previously (57). Briefly, 100 μ l of the diluted cultures were transferred onto Coster 96-well plates. DETA NONOate ((Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino]diazene-1,1,2-diolate, or Diethylenetriamine NONOate) from Cayman was used as a source of NO. NO-treated wells were supplied with either 500 nM, or 200 μ M DETA NONOate. Plates were covered and incubated at 37 °C for 12 to 14 hours. Each condition tested was plated in 8 or 16 wells, half of which were analyzed for growth and the other half were analyzed for crystal violet staining. To analyze growth, the wells were scraped to remove cells in a biofilm before cultures were transferred to a Falcon 96-well plate and the OD₅₉₅ was measured. For crystal violet analysis, cultures were decanted by inverting the plates and dabbing the plates on paper towels. Wells were then washed three times with PBS (phosphate-buffered saline) buffer. Once dried, 125 μ l of 0.1% crystal violet solution was applied to the wells and incubated for 15 minutes. After incubation, the crystal violet solution was discarded and wells were washed three times in PBS buffer. Plates were then allowed to dry. To measure the crystal violet staining, the stain was solubilized in 125 μ l of 30% acetic acid and incubated with shaking for an additional 15 minutes. A 100 μ l portion of this solution was then transferred to a Falcon 96-well plate to measure the absorbance at λ_{540} nm in a Victor X5 plate reader.

References

1. Costerton JW, Stewart PS, & Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284(5418):1318-1322.
2. Pierce GE (2005) *Pseudomonas aeruginosa*, *Candida albicans*, and device-related nosocomial infections: implications, trends, and potential approaches for control. *J Ind Microbiol Biotechnol* 32(7):309-318.
3. Shi X & Zhu X (2009) Biofilm formation and food safety in food industries. *Trends in Food Science & Technology* 20(9):407-413.
4. Burmolle M, Thomsen TR, Fazli M, Dige I, Christensen L, Homoe P, Tvede M, Nyvad B, Tolker-Nielsen T, Givskov M, Moser C, Kirketerp-Moller K, Johansen HK, Hoiby N, Jensen PO, Sorensen SJ, & Bjarnsholt T (2010) Biofilms in chronic infections - a matter of opportunity - monospecies biofilms in multispecies infections. *FEMS Immunol Med Microbiol* 59(3):324-336.
5. Salta M, Wharton JA, Blache Y, Stokes KR, & Briand JF (2013) Marine biofilms on artificial surfaces: structure and dynamics. *Environ Microbiol* 15(11):2879-2893.
6. Blaustein RA, Shelton DR, Van Kessel JA, Karns JS, Stocker MD, & Pachepsky YA (2016) Irrigation waters and pipe-based biofilms as sources for antibiotic-resistant bacteria. *Environ Monit Assess* 188(1):56.
7. Stewart PS & Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* 358(9276):135-138.
8. Jesaitis AJ, Franklin MJ, Berglund D, Sasaki M, Lord CI, Bleazard JB, Duffy JE, Beyenal H, & Lewandowski Z (2003) Compromised host defense on *Pseudomonas*

aeruginosa biofilms: characterization of neutrophil and biofilm interactions. *J Immunol* 171(8):4329-4339.

9. Tolker-Nielsen T (2014) *Pseudomonas aeruginosa* biofilm infections: from molecular biofilm biology to new treatment possibilities. *APMIS Suppl* (138):1-51.

10. Roilides E, Simitsopoulou M, Katragkou A, & Walsh TJ (2015) How Biofilms Evade Host Defenses. *Microbiol Spectr* 3(3).

11. Cross A, Allen JR, Burke J, Duce G, Harris A, John J, Johnson D, Lew M, MacMillan B, Meers P, & et al. (1983) Nosocomial infections due to *Pseudomonas aeruginosa*: review of recent trends. *Rev Infect Dis* 5 Suppl 5:S837-845.

12. Boisvert AA, Cheng MP, Sheppard DC, & Nguyen D (2016) Microbial Biofilms in Pulmonary and Critical Care Diseases. *Ann Am Thorac Soc*.

13. Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, & Webb JS (2006) Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol* 188(21):7344-7353.

14. Romling U, Gomelsky M, & Galperin MY (2005) C-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol* 57(3):629-639.

15. Valentini M & Filloux A (2016) Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from *Pseudomonas aeruginosa* and Other Bacteria. *J Biol Chem* 291(24):12547-12555.

16. Jenal U & Malone J (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* 40:385-407.

17. Hengge R (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 7(4):263-273.

18. Romling U, Galperin MY, & Gomelsky M (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* 77(1):1-52.
19. Morgan R, Kohn S, Hwang SH, Hassett DJ, & Sauer K (2006) BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *J Bacteriol* 188(21):7335-7343.
20. Petrova OE & Sauer K (2012) PAS domain residues and prosthetic group involved in BdlA-dependent dispersion response by *Pseudomonas aeruginosa* biofilms. *J Bacteriol* 194(21):5817-5828.
21. Roy AB, Petrova OE, & Sauer K (2012) The phosphodiesterase DipA (PA5017) is essential for *Pseudomonas aeruginosa* biofilm dispersion. *J Bacteriol* 194(11):2904-2915.
22. Li Y, Heine S, Entian M, Sauer K, & Frankenberg-Dinkel N (2013) NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. *J Bacteriol* 195(16):3531-3542.
23. Galperin MY, Gaidenko TA, Mulkidjanian AY, Nakano M, & Price CW (2001) MHYT, a new integral membrane sensor domain. *FEMS Microbiol Lett* 205(1):17-23.
24. Giardina G, Rinaldo S, Johnson KA, Di Matteo A, Brunori M, & Cutruzzola F (2008) NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. *J Mol Biol* 378(5):1002-1015.
25. Carlson HK, Vance RE, & Marletta MA (2010) H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Mol Microbiol* 77(4):930-942.

26. Plate L & Marletta MA (2012) Nitric oxide modulates bacterial biofilm formation through a multicomponent cyclic-di-GMP signaling network. *Mol Cell* 46(4):449-460.
27. Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA, & Boon EM (2012) Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry* 51(10):2087-2099.
28. Henares BM, Higgins KE, & Boon EM (2012) Discovery of a nitric oxide responsive quorum sensing circuit in *Vibrio harveyi*. *ACS Chem Biol* 7(8):1331-1336.
29. Henares BM, Xu Y, & Boon EM (2013) A nitric oxide-responsive quorum sensing circuit in *Vibrio harveyi* regulates flagella production and biofilm formation. *Int J Mol Sci* 14(8):16473-16484.
30. Rao M, Smith BC, & Marletta MA (2015) Nitric Oxide Mediates Biofilm Formation and Symbiosis in *Silicibacter* sp. Strain TrichCH4B. *MBio* 6(3):e00206-00215.
31. Plate L & Marletta MA (2013) Nitric oxide-sensing H-NOX proteins govern bacterial communal behavior. *Trends Biochem Sci* 38(11):566-575.
32. Arora DP, Hossain S, Xu Y, & Boon EM (2015) Nitric Oxide Regulation of Bacterial Biofilms. *Biochemistry* 54(24):3717-3728.
33. Borziak K & Zhulin IB (2007) FIST: a sensory domain for diverse signal transduction pathways in prokaryotes and ubiquitin signaling in eukaryotes. *Bioinformatics* 23(19):2518-2521.
34. Tamayo R, Schild S, Pratt JT, & Camilli A (2008) Role of cyclic Di-GMP during el tor biotype *Vibrio cholerae* infection: characterization of the *in vivo*-induced cyclic Di-GMP phosphodiesterase CdpA. *Infect Immun* 76(4):1617-1627.

35. Pécastaings S, Allombert J, Lajoie B, Doublet P, Roques C, & Vianney A (2016) New insights into *Legionella pneumophila* biofilm regulation by c-di-GMP signaling. *Biofouling* 32(8):935-948.
36. Hsu JL, Chen HC, Peng HL, & Chang HY (2008) Characterization of the histidine-containing phosphotransfer protein B-mediated multistep phosphorelay system in *Pseudomonas aeruginosa* PAO1. *J Biol Chem* 283(15):9933-9944.
37. Soret J (1883) Analyse spectrale: Sur le spectre d'absorption du sang dans la partie violette et ultra-violette. *Compt Rend* 97:1269-1273.
38. Aono S, Nakajima H, Saito K, & Okada M (1996) A novel heme protein that acts as a carbon monoxide-dependent transcriptional activator in *Rhodospirillum rubrum*. *Biochem Biophys Res Commun* 228(3):752-756.
39. Shelver D, Kerby RL, He Y, & Roberts GP (1997) CooA, a CO-sensing transcription factor from *Rhodospirillum rubrum*, is a CO-binding heme protein. *Proc Natl Acad Sci U S A* 94(21):11216-11220.
40. Michel H, Behr J, Harrenga A, & Kannt A (1998) Cytochrome c oxidase: structure and spectroscopy. *Annu Rev Biophys Biomol Struct* 27:329-356.
41. Nakajima H, Nakagawa E, Kobayashi K, Tagawa S, & Aono S (2001) Ligand-switching intermediates for the CO-sensing transcriptional activator CooA measured by pulse radiolysis. *J Biol Chem* 276(41):37895-37899.
42. Allen JW, Leach N, & Ferguson SJ (2005) The histidine of the c-type cytochrome CXXCH haem-binding motif is essential for haem attachment by the *Escherichia coli* cytochrome c maturation (Ccm) apparatus. *Biochem J* 389(Pt 2):587-592.

43. Boon EM, Huang SH, & Marletta MA (2005) A molecular basis for NO selectivity in soluble guanylate cyclase. *Nat Chem Biol* 1(1):53-59.
44. Boon EM & Marletta MA (2005) Ligand specificity of H-NOX domains: from sGC to bacterial NO sensors. *J Inorg Biochem* 99(4):892-902.
45. Boon EM, Davis JH, Tran R, Karow DS, Huang SH, Pan D, Miazgowicz MM, Mathies RA, & Marletta MA (2006) Nitric oxide binding to prokaryotic homologs of the soluble guanylate cyclase beta1 H-NOX domain. *J Biol Chem* 281(31):21892-21902.
46. Stone JR & Marletta MA (1994) Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* 33(18):5636-5640.
47. Yao J & Lambowitz AM (2007) Gene targeting in Gram-negative bacteria by use of a mobile group II intron ("Targetron") expressed from a broad-host-range vector. *Appl Environ Microbiol* 73(8):2735-2743.
48. Barraud N, Kelso MJ, Rice SA, & Kjelleberg S (2015) Nitric oxide: a key mediator of biofilm dispersal with applications in infectious diseases. *Curr Pharm Des* 21(1): 31-42.
49. Egger LA, Park H, & Inouye M (1997) Signal transduction via the histidyl-aspartyl phosphorelay. *Genes Cells* 2(3):167-184.
50. Stock AM, Robinson VL, & Goudreau PN (2000) Two-component signal transduction. *Annu Rev Biochem* 69:183-215.
51. Zhu Y, Qin L, Yoshida T, & Inouye M (2000) Phosphatase activity of histidine kinase EnvZ without kinase catalytic domain. *Proc Natl Acad Sci U S A* 97(14): 7808-7813.

52. Arora DP & Boon EM (2012) Nitric oxide regulated two-component signaling in *Pseudoalteromonas atlantica*. *Biochem Biophys Res Commun* 421(3):521-526.
53. Xu L, Venkataramani P, Ding Y, Liu Y, Deng Y, Yong GL, Xin L, Ye R, Zhang L, Yang L, & Liang ZX (2016) A Cyclic di-GMP-binding Adaptor Protein Interacts with Histidine Kinase to Regulate Two-component Signaling. *J Biol Chem* 291(31):16112-16123.
54. Yao J & Lambowitz AM (2007) Gene targeting in Gram-negative bacteria by use of a mobile group II intron ("Targetron") expressed from a broad-host-range vector. *Applied and environmental microbiology* 73(8):2735-2743.
55. Chuanchuen R, Narasaki CT, & Schweizer HP (2002) Benchtop and microcentrifuge preparation of *Pseudomonas aeruginosa* competent cells. *BioTechniques* 33(4):760-762-763.
56. Lee JH, Lequette Y, & Greenberg EP (2006) Activity of purified QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. *Molecular microbiology* 59(2):602-609.
57. O'Toole GA (2011) Microtiter dish biofilm formation assay. *J Vis Exp* (47).

Chapter 3

Discovery of a Nitric oxide Sensitive Quorum Sensing circuit in *Vibrio cholerae*

Abstract

In this chapter, we discover the involvement of NosP in *Vibrio cholerae* quorum sensing (QS). Here, I show that a previously characterized hybrid histidine kinase VpsS, which was shown to be involved in *V. cholerae* QS, is indeed a NosP associated histidine kinase. Thus, we rename this kinase as NqsK for NosP associated quorum sensing kinase. I also clone, purify and characterize a NosP containing phosphodiesterase from *V. cholerae*, a role for which is still not understood.

Discovery of a Nitric oxide Sensitive Quorum Sensing circuit in *Vibrio cholerae*

Introduction

Vibrio cholerae is a Gram-negative bacteria that functions as a human pathogen that causes cholera, an intestinal diarrheal disease that is still currently a global health problem. The natural habitat of this bacterium is fresh and saltwater environments and the persistence of *V. cholerae* in these aquatic ecosystems has been demonstrated to be linked to seasonal outbreaks in areas of endemicity and worldwide pandemics.

V. cholerae, like many other bacteria, can exist in either a biofilm or in a free swimming state, and in particular, has also been shown to encode for an *hnoX* gene that is co-cistronic to a histidine kinase gene. Further, this H-NOX signaling network was recently biochemically characterized (1) and in this study the authors demonstrate that the *V. cholerae* H-NOX associated histidine kinase (VcHaHK) transfers phosphate to two response regulator proteins, a PDE and a degenerate HD-GYP protein. Deletion of the homologous PDE gene in *Shewanella oneidensis* results in a hyperbiofilm phenotype. This homologous PDE from *S. oneidensis* is activated ~50 fold when phosphorylated. However, when NO is ligated to the SoH-NOX protein, NO/ SoH-NOX can inhibit SoHaHK activity, which would result in a non-phosphorylated PDE response regulator, and an increase in cyclic di-GMP levels and biofilm formation in this bacterium. Given the existence of an architecturally similar pathway in *V. cholerae*, one would expect an increase in *V. cholerae* biofilms in the presence of NO. However, in 2009, Barraud *et al.* (2) demonstrated that *V. cholerae* biofilm disperses at low, nanomolar concentration of NO. Therefore, the molecular mechanism by which *V.*

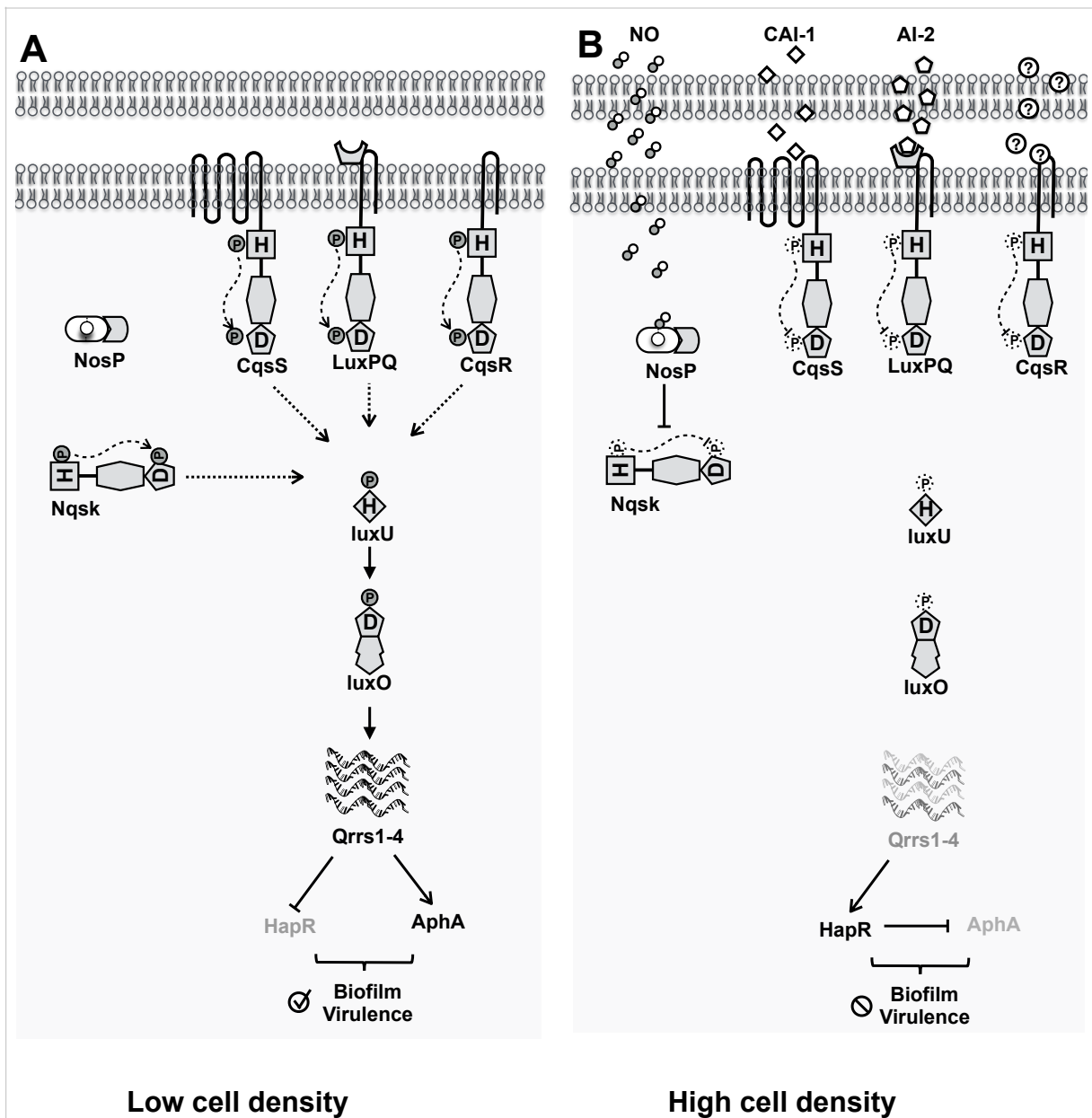


Figure 3.1: Schematic of QS circuit in *V. cholerae*. A) QS signal transduction at low cell density (LCD) and without NO. When autoinducer levels are low and no NO is present, kinase activities of NsqK, CqsS, LuxPQ, and CqsR (VC1831) predominate. Through LuxU, these four histidine kinase receptors activate LuxO via phosphorylation. Activated LuxO promotes transcription of the Qrr1-4 small RNAs (sRNAs), which in turn activate AphA expression and repress HapR production. The LCD QS regulon includes genes required for virulence factor production and biofilm formation. (B) QS signal transduction at high cell density (HCD) and/or NO is present. Autoinducer levels are high and dephosphorylation activities of the four receptors predominate. At this condition, LuxO is dephosphorylated, Qrr1-4 sRNAs are not transcribed, and AphA expression is repressed while HapR protein is produced.

cholerae senses and responds to NO is different from that observed with *S. oneidensis* and is therefore unclear.

Interestingly, quorum sensing (QS) is a process that is known to play a major role in regulating biofilm formation in *Vibrio cholerae*. Quorum sensing involves the production, release and group-wide detection of extracellular signaling molecules, called autoinducers (AIs) once a threshold concentration level has been attained. (3, 4). As the bacterial population increases, autoinducers accumulate in the environment. This allows bacteria to monitor changes in their environment and respond via collective differential expression of genes. Such coordinated group behavior is an important process in biofilming bacteria as it allows bacteria to count themselves. Studies have shown that *V. cholerae* relies on QS to switch between motile and biofilm lifestyles.

Biofilm formation is dependent on the production of an exopolysaccharide matrix (EPS) (5) and in *V. cholerae*, EPS synthesis is encoded for by the *Vibrio* polysaccharide (*vps*) genes (6-8). *Vps gene* expression in *V. cholerae* is typically repressed through the quorum-sensing signaling cascade via HapR (haemagglutinin called HA/protease) activation (Figure 3.1) (9). In *V. cholerae*, HapR translation is regulated via the Qrr sRNAs 1-4 (quorum-regulated small RNAs) (10, 11) in conjugation with Hfq, an RNA binding protein (12). The expressions of the small RNAs is under the regulation of LuxO, as only phosphorylated LuxO facilitates the expression of the Qrr sRNAs 1-4. LuxO receives its phosphate from phosphorylated LuxU, which is only phosphorylated under low cell density (LCD) conditions by hybrid histidine kinases (HK) (see Figure 3.1).

Furthermore, in *V. cholerae*, the quorum-sensing signal transduction system negatively regulates biofilm formation (13). Quorum sensing is activated when the concentration of AIs reach a threshold level that allows binding to their cognate receptors. *V. cholerae* produces two AIs, known as cholerae autoinducer-1 (CAI-1) and autoinducer-2 (AI-2), that are detected via the hybrid histidine kinases CqsS and LuxQ, respectively (13, 14). At a low cell density (when the AI concentration is low), CqsS and LuxPQ act as kinases and are autophosphorylated and further engage in phosphorelay with LuxU (an HPT protein) and then phosphotransfer to the σ^{54} dependent RR LuxO (14). When the cell density is high (High AI concentration), LuxQ and CqsS bind to their respective AIs and act as phosphatases which causes a reversal of phosphate flow, causing LuxU and LuxO to become dephosphorylated. HapR subsequently becomes derepressed resulting in the inhibition of *vps* gene expression, and consequently less biofilm. Although CqsS and LuxPQ were thought to be the only contributors to quorum sensing, mutants missing both receptors still display identical phenotypes to the wild-type bacteria (14). This observation finally led to the discovery of two more hybrid histidine kinase proteins that also participate in *Vibrio cholerae* quorum sensing (15-17). One of these is a membrane bound histidine kinase (Vc1831), and the other is predicted to be a cytosolic protein (Vc1445). In 2009, purified receiver domains of these histidine kinases were shown to accept phosphate from phosphorylated LuxU *in vitro* (15). Moreover, overexpression of the soluble hybrid histidine kinase, Vc1445 (later names VpsS), results in a LuxO dependent up regulation of biofilm (15). More recently, Jung *et al.* (16) demonstrated that *V. cholerae* mutants lacking either Vc1445 (VpsS) or, Vc1831 (named CsqR) exhibit identical phenotypes to the LuxU mutants thus underscoring the

role of these two hybrid HKs as additional receptors in the *V. cholerae* quorum sensing system (16). The stimulus for either kinases, however, is yet to be discovered.

Interestingly, Vc1445 (VpsS) is predicted to be in an operon with Vc1444, which we have annotated as a NosP protein. Also of interest, the *hnoX* gene in *V. cholera* (unlike that observed in *Vibrio harveyi*), that is co-cistronic with a *haHK* gene which does not encode for a hybrid histidine kinase. In *Vibrio harveyi*, however, the NO/VhHNOX signaling pathway was demonstrated to participate in quorum sensing by inhibiting the activity of the H-NOX associated hybrid histidine kinase, which we named VhHqsK (H-NOX associated quorum sensing kinase), and thereby decreasing the phosphate flux to VhLuxU. Since in *V. cholerae*, the Vc1445 (VpsS) protein is a hybrid histidine kinase that can receive phosphate from phosphorylated LuxU *in vitro*, and is predicted to be co-cistronic with a predicted NosP protein, we hypothesize that NO/VcNosP can participate in the *V. cholerae* QS pathway, through interaction with and regulation of Vc1445 (VpsS) activity. In this chapter, we demonstrate that Vc1445 (VpsS) is an active hybrid histidine kinase that can transfer phosphate to VcLuxU *in vitro*. We also demonstrate that Vc1444 is indeed a NosP that can bind NO and when bound, VcNosP can suppress VcHqsK autophosphorylation activity. Therefore, we propose that Vc1445 is a NosP associated quorum sensing kinase (NqsK) that can regulate quorum sensing and possibly also regulate biofilm formation in an NO-dependent manner through its interaction with VcNosP.

Results and Discussion:

Purified VcNosP exhibits ligand-binding properties that are consistent with NO sensing. We cloned and expressed *V. cholerae* NosP (VC1444; 42 KDa) in *E. coli*.

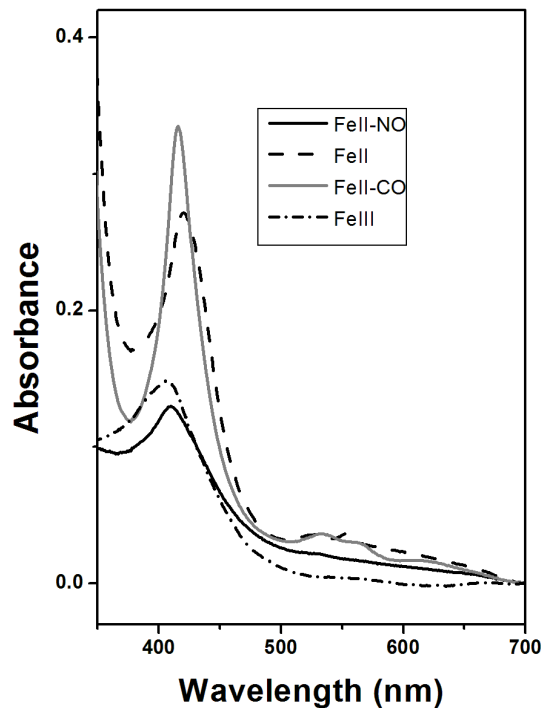


Figure 3.2: UV-Visible spectra of VcNosP in unligated and ligated states. Black dot and dash line is of FeIII, black Dash line is of FeII, grey solid line is of FeII-CO and black solid is the spectrum of FeII-NO.

Upon purification, we found that it has the yellow-orange color common for hemoproteins. A representative UV-visible spectra of VcNosP as the Fe^{II}, Fe^{II}-CO, and Fe^{II}-NO complexes at room temperature are shown in Figure 3.2 and are compared with those of H-NOX and other histidyl-ligated heme proteins along with PaNosP (NosP from *Pseudomonas aeruginosa*) in Table 3.1.

Purified VcNosP has a Soret maximum of ~411 nm, which is indicative of a mixture of ferrous and ferric complexes. Ferricyanide treatment of purified VcNosP results in the formation of the ferric state, a complex that is indicative of a histidine-ligated, high-spin, five-coordinate complex with a Soret maximum at 409 nm as it is with PaNosP (NosP from *Pseudomonas aeruginosa*). Sodium dithionite treatment of ferric NosP anaerobically reduces the protein and shifts the Soret maximum to 420 nm with split α/β

Table 3.1- UV/Visible Peak Positions and NO disassociation kinetics

Protein	Soret (nm)	β (nm)	α (nm)	ref
Fe^{II}				
sGC	431		555	
VcH-NOX	429		568	
CooA	425.5	529.5	559.5	
CytC	420	526	556	
PaNosP	420	524	554	Chapter 2
VcNosP	420	524	554	This Chapter
VcNosPECL	422	523	557	This Chapter
VhNosP	420	538	568	This Chapter
Fe^{II}-CO				
sGC	423	541	567	
VcH-NOX	429	541	566	
CooA	422	539.5	569	
PaNosP	416	538	565	Chapter 2
VcNosP	416	538	565	This Chapter
VcNosPECL	417	539	568	This Chapter
Fe^{II}-NO				
sGC	398	537	571	
VcH-NOX	398	540	573	
PaNosP	396	534	573	Chapter 2
VcNosP	396	534	574	This Chapter
VcNosPECL	399	534	574	This Chapter
VhNosP	397	NR	NR	
NR= Not Recorded				

bands at 554 nm and 524 nm (Figure 3.2). This spectrum is similar to those of hexa-coordinated hemoproteins, such as CooA, cytochrome c, the truncated globins, as well as PaNosP, where the iron is ligated to two axial ligands, usually histidine and an additional amino acid (see Table 3.1) (18-22). As with PaNosP, 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 (23-25). 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 (as was the case with PaNosP), that ferrous VcNosP ligates to two axial ligands, probably water with a amino acid ligand from protein, or two amino acid ligands from the protein. In any case, the sixth ligand is displaced upon binding CO or NO.

Vc1445 transfers phosphate to VcLuxU. Figure 3.3 A shows the autokinase activity of NqsK over time. NqsK is a hybrid histidine kinase with a receiver domain at its C-terminus. Despite the intrinsic instability of phosphorylated hybrid histidine kinases due to phosphatase activity (26), phosphotranferase activity, and/or the intrinsic chemical instability of phosphorylated aspartate, the autophosphorylation assay in Figure 3.3A (lanes 1-4) demonstrates stable phosphorylation of NqsK over 30 minutes. Compared with the PaNahK, this kinase purifies well and is stable.

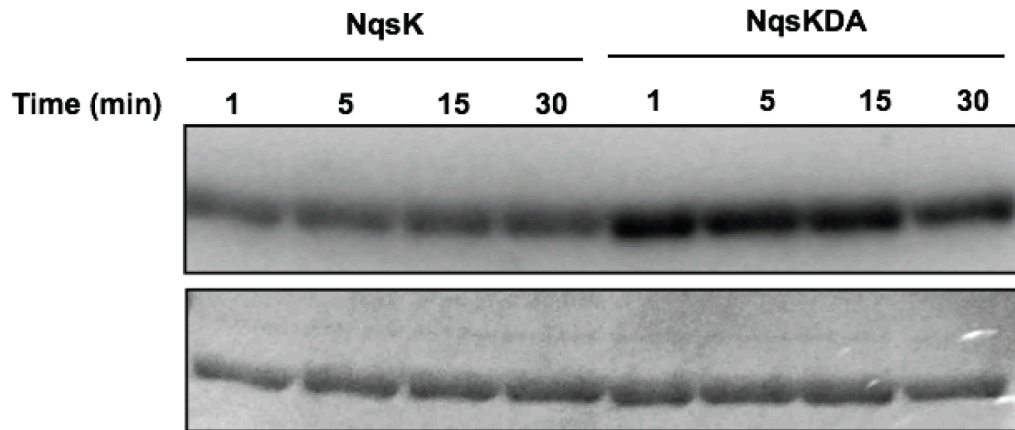
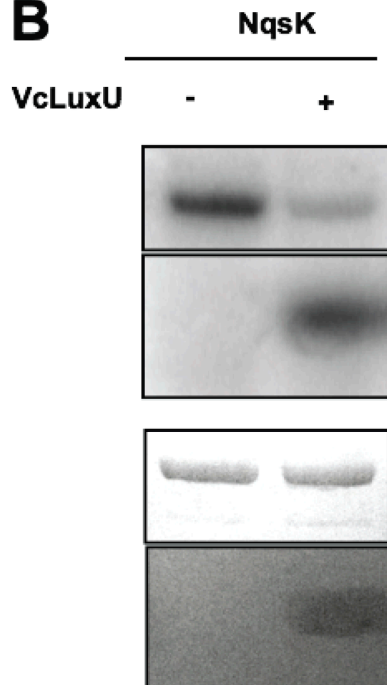
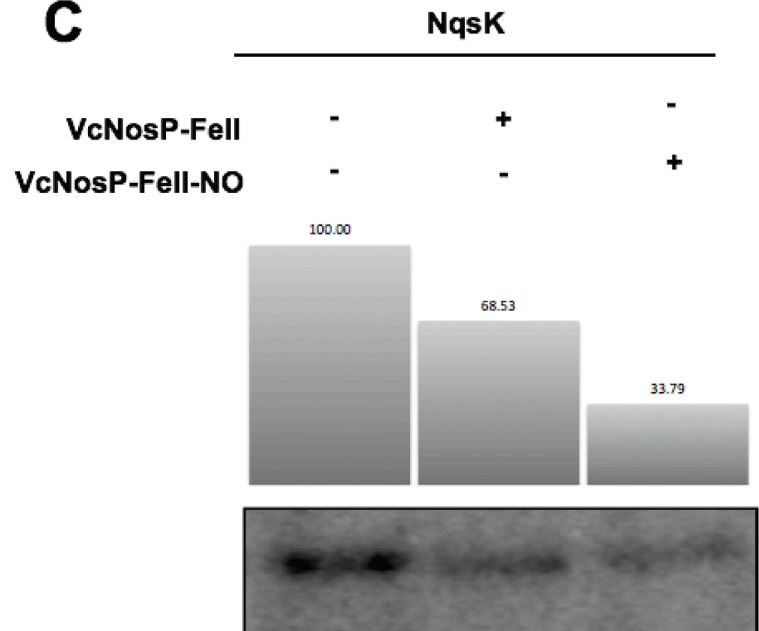
A**B****C**

Figure 3.3: NO/VcNosP inhibits autophosphorylation of NqsK. (A) *In vitro* autophosphorylation of VcNqsK and VcNqsKDA over time. Radiolabeled phosphoproteins were detected by SDS-PAGE (bottom) and autoradiography (top). (B) Phosphotransfer from VcNqsK to LuxU. Radiolabeled phosphoproteins were detected by SDS-PAGE (bottom) and autoradiography (top). Following proteins were incubated with ^{32}P -labeled ATP and monitored over time: lane 1, VcNaHK; and lane 2, VcNaHK + LuxU. (C) NaHK autophosphorylation regulation of NO/NosP. Following proteins were incubated with ^{32}P -labeled ATP and monitored over time: lane 1, VcNaHK; lane 2, VcNaHK and VcNosP-Fell; lane 3, VcNaHK and VcNosP-Fell-NO.

Hybrid histidine kinases typically transfer phosphate from the aspartate in the receiver domain to a histidine-containing phosphotransfer protein (HPT) to transduce the signal downstream (27), as we have noted with *P. aeruginosa* Pa3345 (HptB), which accepts phosphate from NaHK (28). In *V. harveyi*, HqsK transfers its phosphate to VhLuxU (29), which is essentially an HPT protein. Therefore, we cloned, expressed, and purified VcLuxU (Vc1022) in order to study phosphotransfer from NqsK to LuxU. Upon incubation of purified LuxU with phosphorylated NqsK, phosphotransfer is evident, as illustrated in Figure 3.3B lane 2. We also made a mutant of NqsK, NqsKDA, in which the conserved aspartate, D505 in the receiver domain is mutated to alanine to prevent phosphotransfer from the histidine residue. This mutant expresses and purifies well from *E. coli* and as expected showed enhanced phosphorylation over time (Figure 3.3A, lanes 5-8). We want to perform further studies on phosphotransfer with this mutant in the future.

NosP/NqsK signaling is NO sensitive. We hypothesized that NO/VcNosP might regulate the kinase and signal transduction activities of NqsK. To evaluate this hypothesis, ferrous and NO-bound VcNosP were added to the phosphorelay assay (Figure 3.3C). Inhibition of NqsK autophosphorylation was observed when excess VcNosP was present in either the Fe^{II} or the Fe^{II}-NO bound form. The greatest degree of inhibition, however, was observed in the presence of NO-bound VcNosP (Figure 3.3C lane 3). Interestingly, this pattern of kinase inhibition is analogous to the inhibition of histidine kinase activity observed in H-NOX/HaHK (H-NOX and H-NOX-associated histidine kinase) signaling in *Vibrio harveyi* (30), *Pseudalteromonas atlantica* (31), *Shewanella oneidensis* (1), *Silicibacter* sp. strain TrichCH4B (32), and *Pseudomonas*

aeruginosa. In all these systems, the NO-ligated sensor protein is the most potent inhibitor of the co-cistronic kinase activity. Furthermore, the inhibition of kinase activity in the presence of NO suggests that *V. cholerae* can detect NO as a signaling molecule that ultimately acts to modulate gene expression further downstream through regulating

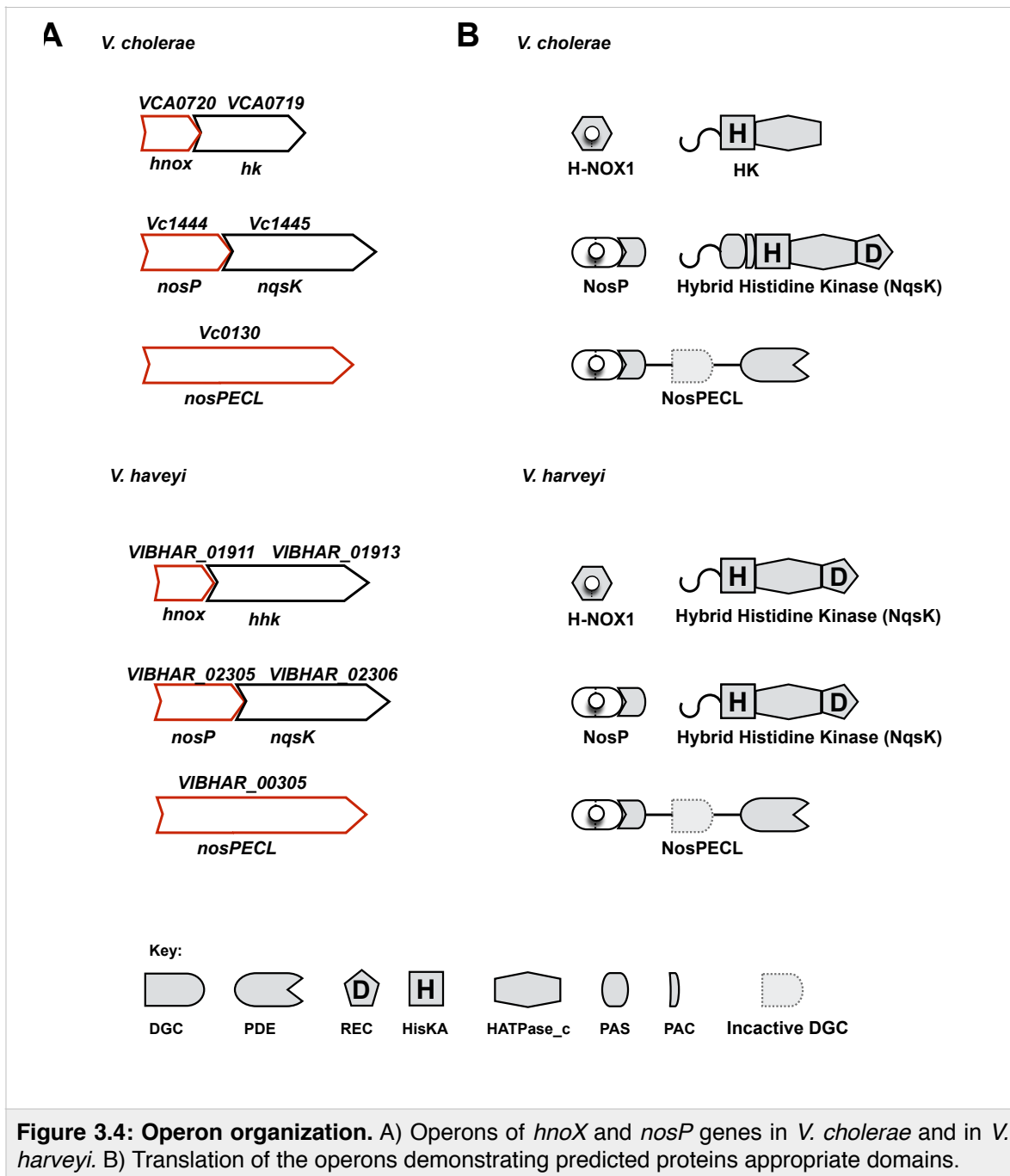


Figure 3.4: Operon organization. A) Operons of *hnox* and *nosP* genes in *V. cholerae* and in *V. haveyi*. B) Translation of the operons demonstrating predicted proteins appropriate domains.

LuxO. The presence of NO would mimic a high cell density state (Figure 3.1B). Although, NqsK was already linked to the quorum sensing in *V. cholerae*, we are the first to show direct phosphotransfer from NqsK to LuxU, thus substantiating its involvement in *V. cholerae* quorum sensing.

quorum sensing and cyclic di-GMP in *V. cholerae*

Cyclic di-GMP has been shown to play a role in *V. cholerae* biofilm formation and in quorum sensing (33). Aside from containing the stand alone NosP, *V. cholerae* also contains another annotated NosP in its genome (Figure 3.4). This annotated NosP is coded for in the same polypeptide with a DGC and a PDE di-domain containing protein. A quick alignment of the annotated DGC domain of Vc0130 with a known active DGC from *Shewanella woodyi* HaCE (34) reveals that this domain is significantly smaller and lacks crucial elements that a DGC domain should contain including the GGDEF active site (highlighted in red in Figure 3.5). The PDE domain of Vc0130, on the other hand contains all the elements of an active PDE with ECL in its active site. Hence, we decided to name this protein NosPECL.

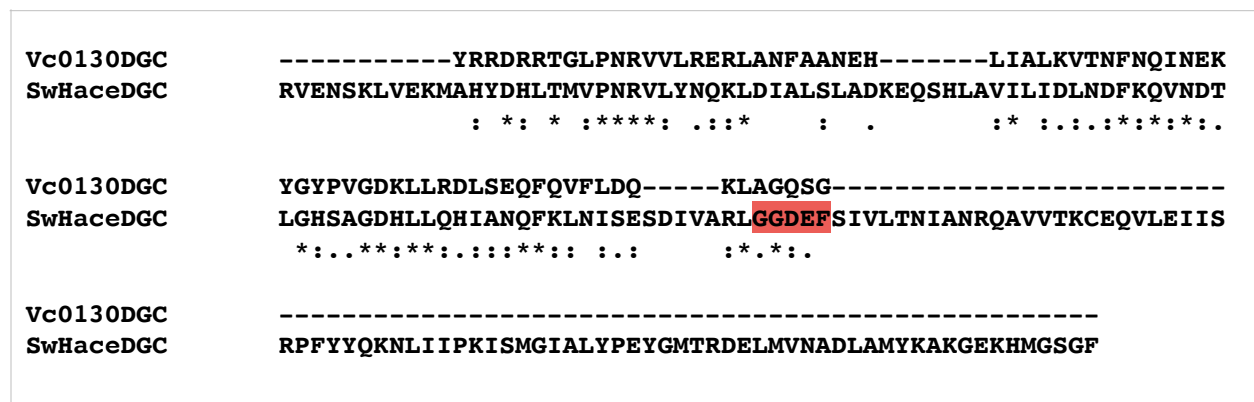


Figure 3.5: Alignment of DGC domain. Alignment of Vc0130 degenerate GGDEF with a well characterized GGDEF protein from *S. woodyi*.

Vc0130, has been previously studied *in vivo* by the Camilli group without reference to its NO binding ability (33). By the use of quantitative real-time PCR (qPCR), the authors demonstrated that the transcription of VC0130 is up-regulated in biofilm in a mouse model of infection. In addition, overexpression of this protein in a strain of *V. cholerae* resulted in reduced intracellular cyclic di-GMP levels and biofilm formation. It is worth mentioning that they did not use purified Vc0130 in their assay. Due to its involvement in *V. cholerae* biofilm, we decided to evaluate the role for Vc0130 protein biochemically.

Vc0130 is a hemoprotein. We cloned Vc0130 with a hexa-His₆ tag and purified it from *E. coli*. Vc0130 purifies with color indicating the presence of heme. We then decided to characterize this protein spectroscopically as we did with VcNosP. The soret maxima for unligated and different ligated states of this protein are listed in Table 3.1.

Vc0130 is an active PDE *in vitro*. We assayed purified Vc0130 for phosphodiesterase activity (Figure 3.6). The ferric state of Vc0130 is active and is able to hydrolyze cyclic di-GMP (Figure 3.6A). Measuring the area under the curve, we determined that only 28% of the total cyclic di-GMP was hydrolyzed in its ferric state. 34% of cyclic di-GMP was hydrolyzed when Vc0130 was treated with sodium dithionite to get to ferrous state (Figure 3.6B). NO ligated Vc0130 showed a slight increase in hydrolysis (45%) as seen from Figure 3.6C. To our surprise, when we assayed Vc0130 prior to adding excess heme, 100% cyclic di-GMP was hydrolyzed. The total hydrolysis of its substrate prior to incubation with excess heme was an unexpected finding for us. In our hypothesis that Vc0130 may be an NO sensor, we expected Vc0130 to have the greatest effect on its substrate when it is ligated to NO. It is important to note that when we assayed this protein for PDE activity before heme treatment, the pooled purified protein did contain a

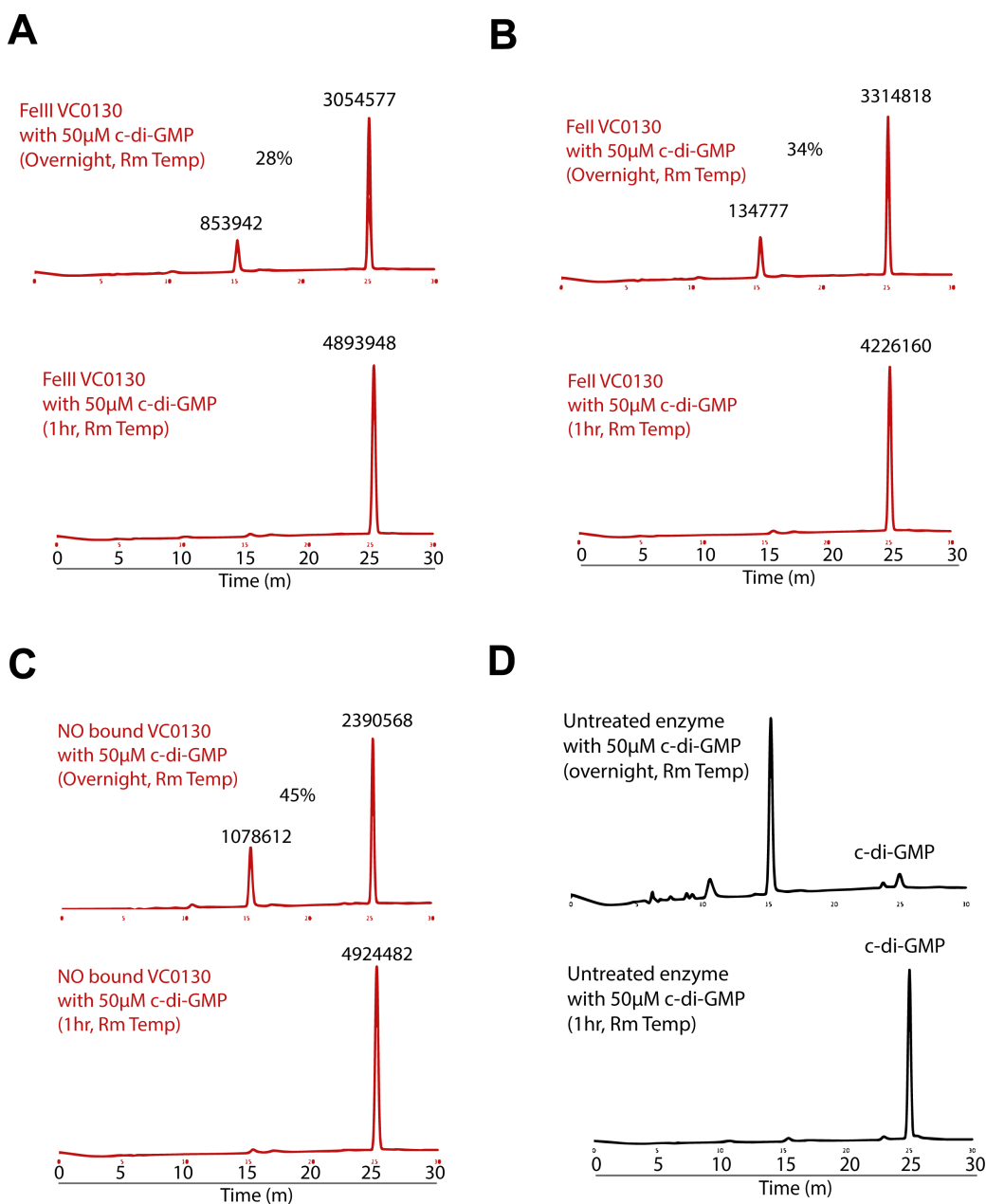


Figure 3.6: Cyclic di-GMP hydrolysis assay. A) Overnight hydrolysis of cyclic di-GMP with ferric NosPECL. 28 percent cyclic di-GMP was hydrolyzed with ferric NosPECL. B) Overnight hydrolysis of cyclic di-GMP with ferrous NosPECL. 34 percent cyclic di-GMP was hydrolyzed with ferrous NosPECL. C) Overnight hydrolysis of cyclic di-GMP with NO/NosPECL. 45 percent cyclic di-GMP was hydrolyzed with NO/NosPECL. D) Overnight hydrolysis of cyclic di-GMP with untreated NosPECL. ~95% percent cyclic di-GMP was hydrolyzed with untreated NosPECL.

small population that was heme bound as was evident by the slight color. Thus, this finding poses the following questions that need to be addressed in future studies: Is Vc0130 heme bound in *V. cholerae*? Could Vc0130 be acting as a heme sensor and not an NO sensor? If so, what does sensing heme have to do with *V. cholerae* biology?

Conclusion

In conclusion, we propose that VcNosP (Vc1444) may function as a primary NO sensor in *V. cholerae* that participates in *V. cholerae* quorum sensing via NqsK as *V. cholerae* H-NOX is co-cistronic to a histidine kinase that cannot directly transfer phosphate to VcLuxU. Based on previous studies, however, we know that intracellular levels of cyclic-di-GMP may be regulated by the VcH-NOX operon (1). Consequently, it would be interesting to see if VcHaHK can also regulate quorum sensing through regulation of total cyclic di-GMP levels or through phosphorylation of LuxO. LuxO is a receiver domain containing DNA binding protein. No one has ever investigated whether LuxO can be phosphorylated by any other proteins other than LuxU. Also, in *V. harveyi*, the NosP is co-cistronic to a hybrid histidine kinase (Figure 3.4), which begs the question whether this kinase is also able to transfer phosphate to VhLuxU and therefore participate in *V. harveyi* quorum sensing. More experiments have to be conducted to evaluate possible cross-talks between the H-NOX and the NosP signaling. Moreover, the role for NosPECLs that are encoded for in both *V. cholerae* and in *V. harveyi* should be elucidated. In order to approach these questions, we have already cloned the *nosP*, *nosPhhk* and *nospECL* from *V. harveyi*, and we are currently working to demystify the biochemical and therefore biological relevance of these multiple sensors and the NosPECLs.

Materials and Method:

Cloning

Genes were PCR amplified from *Vibrio cholerae* genomic DNA and cloned into pET20b vector appending His₆ at the C terminus by the use of NdeI and XhoI restrictions enzymes. Proteins were expressed in E. The appropriate primers are listed in Table 3.2.

Protein Expression and Purification

Expression and purification of NosPs. BL21 (DE3) pLysS cells (Novagen) were transformed with the *pnosPs* and *pnosPECL* plasmid. VcNosP, VhNosP and VcNosPECL were purified the same way as PaNosP from *Pseudomonas aeruginosa*. Yeast extract media was used to grow the protein and was induced with 25 μ M IPTG at 16°C overnight. ALA (0.1mM) was added at the time of induction. All the steps for purification was exactly the same as it was for PaNosP purification.

Expression and Purification of other proteins. All the other proteins were purified as follows. BL21 (DE3) pLysS cells were transformed and grown in TB media (1.2% Tryptone, 2.4% Yeast extract, 0.04% glycerol with 17 mM KH₂PO₄ and 72 mM K₂HPO₄, pH 7.5). Induction was carried out at OD₅₉₅ of 0.5 with 25 μ M IPTG. Bacterial cultures were cooled to 16 °C prior to induction. Purification buffer was the same except no hemin was added. Typically, following binding to 1 ml of Ni-NTA beads, three wash steps were performed: 100 ml with 10 mM imidazole, 50 ml with 20 mM imidazole and 10 ml with 30 mM imidazole. Followed by the washes, the proteins were eluted in buffer with 250 mM imidazole and dealt on a PD-10 column (GE) in buffer containing 50 mM

Table 3.2- Strains, Plasmids, and Primers Used

<i>E. coli</i> strains used in this work	
DH5α	For cloning genes into <i>E. coli</i>
BL21 (DE3) pLysS	For protein Expression
Plasmids Used	
pET20 (b)	To clone, express and purify proteins from <i>E. coli</i> .
Primers used (N represents nonspecific nucleotides)	
<i>pvcnosP</i>	5'-NNNNNNNCATATGGTGTTCATCTCACGTATACAAAACCTCGTTC-3' 5'-NNNNNNNCTCGAGTGCCTCATAAAATACAACAGAAG-3'
<i>pvcnosPECL</i>	5'-NNNNNNNCATATGGTGTTCATCTCACGTATACAAAACCTCGTTC-3' 5'-NNNNNNNCTCGAGACCCAAGCGTGAAGGTGACCCAAG-3'
<i>pvcnsqK</i>	5'-NNNNNNNCATATGTTTACGGTCTCGCGTCTCATCCCAG-3' 5'-NNNNNNNCTCGAGTGGATTAAGGAAACGTTTCACAACCTCCATGATG-3'
<i>pvcluxU</i>	5'-NNNNNNNCATATGAGAGAATGGATCAACCAAAG-3' 5'-NNNNNNNCTCGAGATGAACTAAATCGGAGTAGC-3'
<i>pvcnsqKDA</i>	5'-CTGGTGCTAATGGATATTTCCATGCCG-3' 5'-CGGCATGGAAATATCCATTAGCACCAG-3'
<i>pvhnosP</i>	5'-NNNNNNNCATATGCAGTGCCAATCATTATTTACTCATATTACCGAC-3' 5'-NNNNNNNCTCGAGTCTTTCATAAGGTTTATGGAAAATTGCCGATG-3'
<i>pvhnsqK</i>	5'-NNNNNNNCATATGAAAGATAAATATTTAGAGATTTACCAAC-3' 5'-NNNNNNNCTCGAGCGCGGTTTCAACTAACGATTGAATTG-3'
<i>pvhnosPECL</i>	5'-NNNNNNNCATATGCGAGCATTCTCTCAACTGGTTCAAATG-3' 5'-NNNNNNNCTCGAGAAACAAGCGTGTGCGTTCACCCAAATAATAAC-3'

Tris, 200 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA and 10% glycerol, 1 mM dithiothreitol, pH 8.0. Purified proteins were then aliquoted and stored at -80 °C for later use.

UV-Visible Spectroscopy. All spectra were recorded on a Cary 100 spectrophotometer equipped with a constant temperature bath. NosP complexes were prepared in an oxygen-free glove bag and NO dissociation kinetics were measured as previously described (24).

Kinase Assay. Kinase assays were performed in an assay buffer containing 100 mM Tris pH 8.0, 50 mM KCl, 5 mM MgCl₂ and 1 mM DTT. Typically, each reaction was started by the addition of an ATP solution so that the final ATP concentration was 1 mM with 2 μCi [γ -³²P]-ATP per reaction. Reactions were incubated for an appropriate time at room temperature before quenching with 5x SDS loading dye. Following separation on a 12.5% Tris-glycine SDS-PAGE, gels were fixed and stained with Coomassie blue and destained prior to exposure to a phosphoscreen overnight, were scanned with a Typhoon phosphoimager (Amersham Biosciences) and images were analyzed with ImageJ software. For the phosphotransfer assay, HptB was added to the reaction and incubated for an additional 15 minutes after the kinase had already been incubated with ATP for 30 minutes at room temperature.

Phosphodiesterase assays. PDE assays were performed at room temperature in an assay buffer containing 50 mM Tris pH 8.0, 5 mM MgCl₂ and 50 μM c-di-GMP. Reactions were initiated by the addition of 200 nM of purified protein. Reactions were

quenched by addition of 10 mM CaCl₂ and subsequently heated to 95 °C for 5 min to precipitate protein. Precipitated protein was removed by centrifugation and the resulting supernate was filtered through a 0.22 μm membrane and analyzed by HPLC on a reverse phase C18 column (Shimazu) with an ion pairing buffer system. Buffer A (10 mM TBAOH (tetrabutylammonium hydroxide), 10 mM KH₂PO₄, 1% Methanol) Buffer B (2.8 mM TBAOH, 100 mM KH₂PO₄, 30% Methanol) Nucleotides were eluted with the following gradient: 40% Solvent B over 5 min, 40–55% Solvent B over 3 min, 55–100% Solvent B over 9 min, maintained at 100% Solvent B for 4 min, 100–40% Solvent B over 4 min, and maintained at 40% Solvent B for 5 min.

References

1. Plate L & Marletta MA (2012) Nitric oxide modulates bacterial biofilm formation through a multicomponent cyclic-di-GMP signaling network. *Mol Cell* 46(4):449-460.
2. Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, & Kjelleberg S (2009) Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *Journal of bacteriology* 191(23):7333-7342.
3. Miller MB & Bassler BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55:165-199.
4. Pappenfort K & Bassler BL (2016) Quorum sensing signal-response systems in Gram-negative bacteria. *Nat Rev Microbiol* 14(9):576-588.
5. Flemming HC, Neu TR, & Wozniak DJ (2007) The EPS matrix: the "house of biofilm cells". *J Bacteriol* 189(22):7945-7947.
6. Yildiz FH & Schoolnik GK (1999) *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc Natl Acad Sci U S A* 96(7):4028-4033.
7. Yildiz FH, Dolganov NA, & Schoolnik GK (2001) VpsR, a Member of the Response Regulators of the Two-Component Regulatory Systems, Is Required for Expression of vps Biosynthesis Genes and EPS(ETr)-Associated Phenotypes in *Vibrio cholerae* O1 El Tor. *J Bacteriol* 183(5):1716-1726.

8. Teschler JK, Zamorano-Sanchez D, Utada AS, Warner CJ, Wong GC, Linington RG, & Yildiz FH (2015) Living in the matrix: assembly and control of *Vibrio cholerae* biofilms. *Nat Rev Microbiol* 13(5):255-268.
9. Tsou AM, Cai T, Liu Z, Zhu J, & Kulkarni RV (2009) Regulatory targets of quorum sensing in *Vibrio cholerae*: evidence for two distinct HapR-binding motifs. *Nucleic Acids Res* 37(8):2747-2756.
10. Lenz DH, Mok KC, Lilley BN, Kulkarni RV, Wingreen NS, & Bassler BL (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* 118(1):69-82.
11. Tu KC & Bassler BL (2007) Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev* 21(2): 221-233.
12. Vogel J & Luisi BF (2011) Hfq and its constellation of RNA. *Nat Rev Microbiol* 9(8):578-589.
13. Hammer BK & Bassler BL (2003) Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol* 50(1):101-104.
14. Miller MB, Skorupski K, Lenz DH, Taylor RK, & Bassler BL (2002) Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* 110(3): 303-314.
15. Shikuma NJ, Fong JC, Odell LS, Perchuk BS, Laub MT, & Yildiz FH (2009) Overexpression of VpsS, a hybrid sensor kinase, enhances biofilm formation in *Vibrio cholerae*. *J Bacteriol* 191(16):5147-5158.

16. Jung SA, Chapman CA, & Ng WL (2015) Quadruple quorum-sensing inputs control *Vibrio cholerae* virulence and maintain system robustness. *PLoS Pathog* 11(4):e1004837.
17. Jung SA, Hawver LA, & Ng WL (2016) Parallel quorum sensing signaling pathways in *Vibrio cholerae*. *Curr Genet* 62(2):255-260.
18. Aono S, Nakajima H, Saito K, & Okada M (1996) A novel heme protein that acts as a carbon monoxide-dependent transcriptional activator in *Rhodospirillum rubrum*. *Biochem Biophys Res Commun* 228(3):752-756.
19. Shelver D, Kerby RL, He Y, & Roberts GP (1997) CooA, a CO-sensing transcription factor from *Rhodospirillum rubrum*, is a CO-binding heme protein. *Proc Natl Acad Sci U S A* 94(21):11216-11220.
20. Michel H, Behr J, Harrenga A, & Kannt A (1998) Cytochrome c oxidase: structure and spectroscopy. *Annu Rev Biophys Biomol Struct* 27:329-356.
21. Nakajima H, Nakagawa E, Kobayashi K, Tagawa S, & Aono S (2001) Ligand-switching intermediates for the CO-sensing transcriptional activator CooA measured by pulse radiolysis. *J Biol Chem* 276(41):37895-37899.
22. Allen JW, Leach N, & Ferguson SJ (2005) The histidine of the c-type cytochrome CXXCH haem-binding motif is essential for haem attachment by the *Escherichia coli* cytochrome c maturation (Ccm) apparatus. *Biochem J* 389(Pt 2):587-592.
23. Boon EM, Huang SH, & Marletta MA (2005) A molecular basis for NO selectivity in soluble guanylate cyclase. *Nat Chem Biol* 1(1):53-59.
24. Boon EM & Marletta MA (2005) Ligand specificity of H-NOX domains: from sGC to bacterial NO sensors. *J Inorg Biochem* 99(4):892-902.

25. Boon EM, Davis JH, Tran R, Karow DS, Huang SH, Pan D, Miazgowicz MM, Mathies RA, & Marletta MA (2006) Nitric oxide binding to prokaryotic homologs of the soluble guanylate cyclase beta1 H-NOX domain. *J Biol Chem* 281(31):21892-21902.
26. Zhu Y, Qin L, Yoshida T, & Inouye M (2000) Phosphatase activity of histidine kinase EnvZ without kinase catalytic domain. *Proc Natl Acad Sci U S A* 97(14):7808-7813.
27. Stock AM, Robinson VL, & Goudreau PN (2000) Two-component signal transduction. *Annu Rev Biochem* 69:183-215.
28. Hsu JL, Chen HC, Peng HL, & Chang HY (2008) Characterization of the histidine-containing phosphotransfer protein B-mediated multistep phosphorelay system in *Pseudomonas aeruginosa* PAO1. *J Biol Chem* 283(15):9933-9944.
29. Henares BM, Xu Y, & Boon EM (2013) A nitric oxide-responsive quorum sensing circuit in *Vibrio harveyi* regulates flagella production and biofilm formation. *Int J Mol Sci* 14(8):16473-16484.
30. Henares BM, Higgins KE, & Boon EM (2012) Discovery of a nitric oxide responsive quorum sensing circuit in *Vibrio harveyi*. *ACS Chem Biol* 7(8):1331-1336.
31. Arora DP & Boon EM (2012) Nitric oxide regulated two-component signaling in *Pseudoalteromonas atlantica*. *Biochem Biophys Res Commun* 421(3):521-526.
32. Rao M, Smith BC, & Marletta MA (2015) Nitric Oxide Mediates Biofilm Formation and Symbiosis in *Silicibacter sp.* Strain TrichCH4B. *MBio* 6(3):e00206-00215.
33. Tamayo R, Schild S, Pratt JT, & Camilli A (2008) Role of cyclic Di-GMP during eltor biotype *Vibrio cholerae* infection: characterization of the *in vivo*-induced cyclic Di-GMP phosphodiesterase CdpA. *Infect Immun* 76(4):1617-1627.

34. Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA, & Boon EM (2012) Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry* 51(10):2087-2099.

Chapter 4

NosP in *Legionella pneumophila* is involved in Cyclic di-GMP metabolism

Abstract

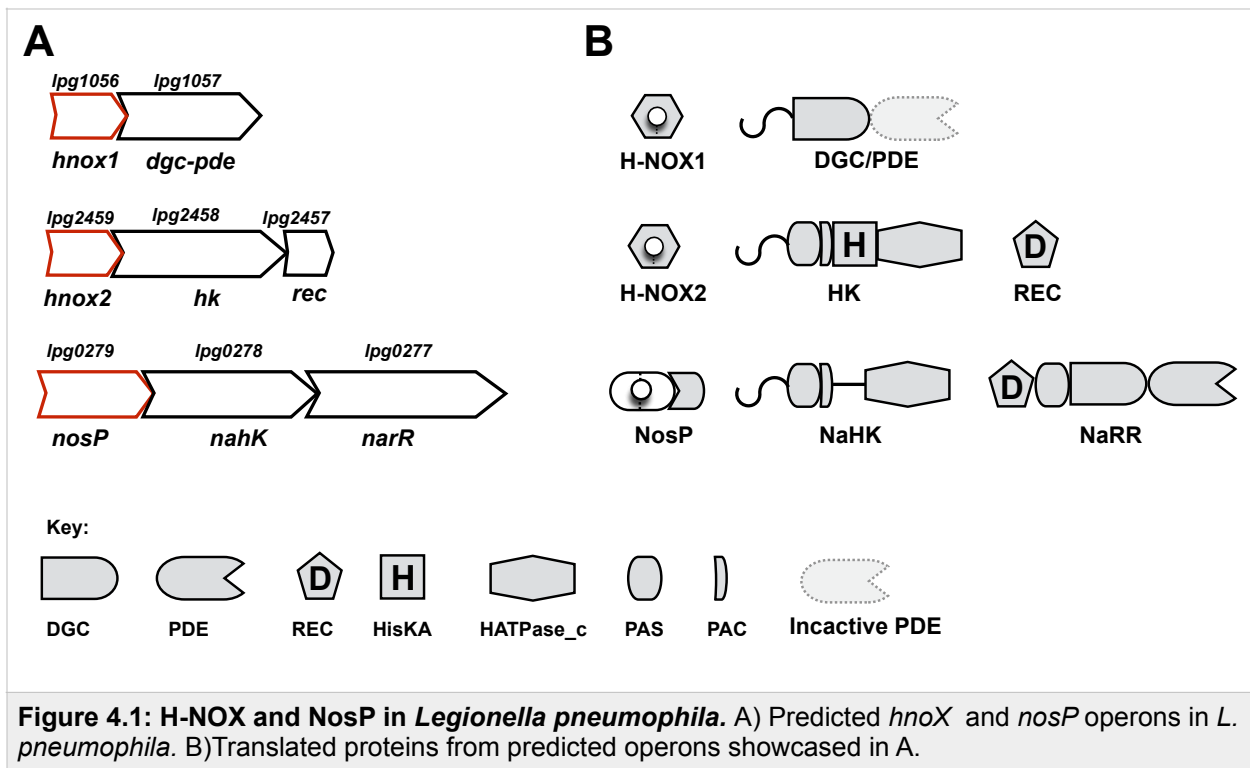
In this chapter we explore the role of NosP in cyclic di-GMP metabolism. In *Legionella pneumophila*, NosP is in the same operon with a histidine kinase (NaHK) and a response regulator protein (NaRR) with dual activities. The NaRR protein contains both a cyclase and a phosphodiesterase domain. In this chapter, we demonstrate that both domains of the NaRR protein are active and NaRR protein can receive phosphate from NaHK, and that NaHK autophosphorylation is inhibited by nitric oxide ligated NosP.

NosP in *Legionella pneumophila* is involved in Cyclic di-GMP metabolism

Introduction

Legionella pneumophila is a Gram-negative, aerobic bacteria that resides in aquatic environments all over the globe. As a pathogen, *L. pneumophila* causes Legionnaires disease, a severe form of pneumonia (1). In its natural aquatic environments, *L. pneumophila* survives and replicates inside free-living amoebae. In addition, occurrences of multi-species *L. pneumophila* biofilms in fresh water, as well as monospecies biofilms in the laboratory environments, have been reported (2-4). Due to a high tolerance to harsh environments and constant release of bacteria in circulating water, it is of particular interest to understand the mechanisms controlling biofilm formation by *L. pneumophila* and, in particular, the molecular mechanism by which nitric oxide regulates *L. pneumophila* biofilm formation.

Biofilm formation has been linked to elevated levels of cyclic di-GMP in many bacteria (5). Involvement of nitric oxide in cyclic di-GMP regulation that ultimately regulates biofilm has been demonstrated in *Legionella pneumophila* (3) and in *Shewanella woodyi* (6). *Legionella pneumophila* contains two *hnoX* genes: *hnoX1* and *hnoX2* (Figure 4.1). The role for H-NOX2 is yet to be explored. However, in their 2010 publication, Carlson *et al.* 2010, showed that H-NOX1 shares an operon with an active GGDEF protein (3). The authors demonstrate that the $\Delta hnoX1$ strain displays a hyper-biofilm phenotype as does the over-expression of the co-cistronic GGDEF protein Lpg1057. This hyperbiofilm phenotype is reversed when Lpg1057 is deleted in the $\Delta hnoX$ strain. The authors also demonstrated that the NO bound form of H-NOX1 inhibits the cyclase activity of



Lpg1057, thus linking nitric oxide to cyclic di-GMP regulation and biofilm formation in this bacterium (3).

In a recent study, five cyclic di-GMP processing enzymes were found to be involved in mediating biofilm formation in *L. pneumophila*. Of the five enzymes involved, Lpl0329 (homologue of Lpg 0277, or NaRR) from the LENS strain of *L. pneumophila* was shown to be important in preventing biofilm formation (7). Lpl0329, (Lpg0277 in the Philadelphia strain) is in the same operon with an unorthodox histidine kinase Lpl0330 (Lpg0278 in the Philadelphia strain) and Lpl0331 (Lpg0279 in the Philadelphia strain), which is a NosP protein (see Figure 4.1A and 4.1B). Levet-Paulo *et al.* (8), demonstrated that Lpl0329 is regulated by Lpl0330. Lpl0330 can transfer a phosphoryl group to Lpl0329, and phosphotransfer to Lpl0330 results in a decrease of diguanylate cyclase activity without affecting the phosphodiesterase activity (8). This observation clearly demonstrates the differential regulation of a dual enzyme under phosphorylation

conditions. Despite the understanding of the signaling downstream of the kinase, nothing upstream has been established. There has been no report of any stimulus that regulates the kinase itself. Due to the fact that this kinase and the bifunctional enzyme is in the same putative operon with a NosP protein, we hypothesize that the kinase is under the regulation of NosP and NO is the stimulus for this pathway. Thus in this chapter, we demonstrate that LpgNosP can bind NO, and that NO/LpgNosP is able to regulate the autophosphorylation activity of Lpg0278, which we named NaHK (NosP associated histidine kinase). We reconfirm the phosphorelay to Lpg0277 that we named NaRR (NosP associated response regulator) via NaHK, presenting for the first time an alternate NO signaling pathway in *L. pneumophila*.

Results and Discussion:

LpgNosP ligates to NO. We cloned and purified LpgNosP from *E. coli*. Purified LpgNosP, as with the other NosPs discussed before, purifies as a colored protein. The spectroscopic analysis of LpgNosP reveals that LpgNosP purifies as a mixture of the ferric and ferrous states as revealed by Soret max of 410 nm. Treatment of LpgNosP with potassium ferricyanide results in a Soret max of 413 nm, as was observed with other NosPs (Figure 4.2) discussed in previous chapters. Anaerobic treatment of LpgNosP with excess sodium dithionite results in a shift in Soret maximum to 420 nm with split α/β as was seen with other NosPs (Figure 4.2, Fell and the insert, solid black line). Treatment of ferrous LpgNosP with DEA-NONOate results in a Soret max of 396 nm (Figure 4.2, dot and dash line). Table 4.1 summarizes and compares the ferrous and NO bound states of NosPs characterized by us from various systems.

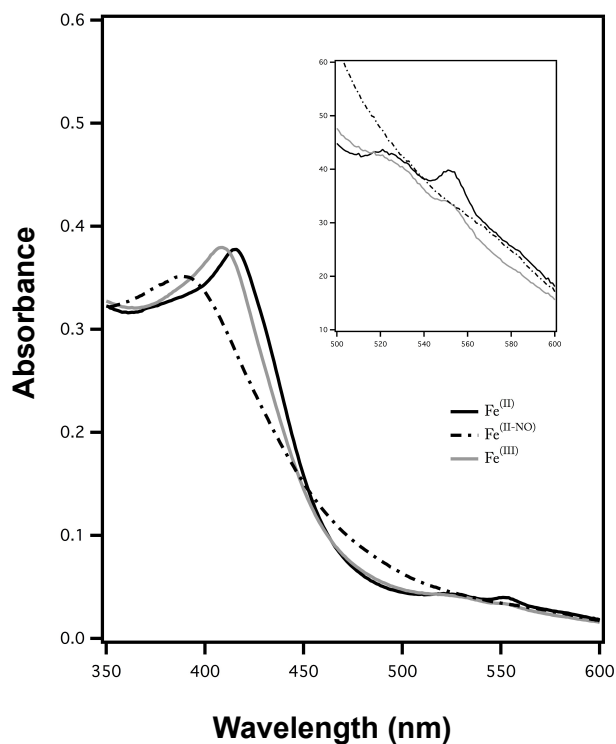


Figure 4.2: Spectroscopic characterization of LpgNosP. The inset is a close up of the spectra between 500 nm and 600 nm, highlighting the α/β region.

Lpg0278 is an active kinase. We cloned and purified Lpg0278 with hexa-His₆ tag at the C-terminus from *E. coli*. Upon purification, we tested the autokinase activity of Lpg0278 by incubating Lpg0278 with radioactive [γ -³²P]-ATP. Figure 4.3A shows the activity of the kinase over 30 minutes. Unlike the PaNaHK and VcNaHK (from chapter 2 and chapter 3), detecting the phosphorylated kinase was not a problem, as this kinase is not a hybrid kinase and lacks an internal receiver domain.

Lpg0278 transfers phosphate to Lpg0277. We cloned and purified Lpg0277 with a C-terminal hexa-His tag from *E. coli* to evaluate whether Lpg0277 can receive phosphate from Lpg0278, as was seen for Lpl0330 and Lpl0329 (8). Figure 4.3B (lane 2) shows the phosphorylation of Lpg0277 when incubated with phosphorylated Lpg0278. In fact, a disappearance of phosphorylation of Lpg2778 is observed when Lpg0277 is present

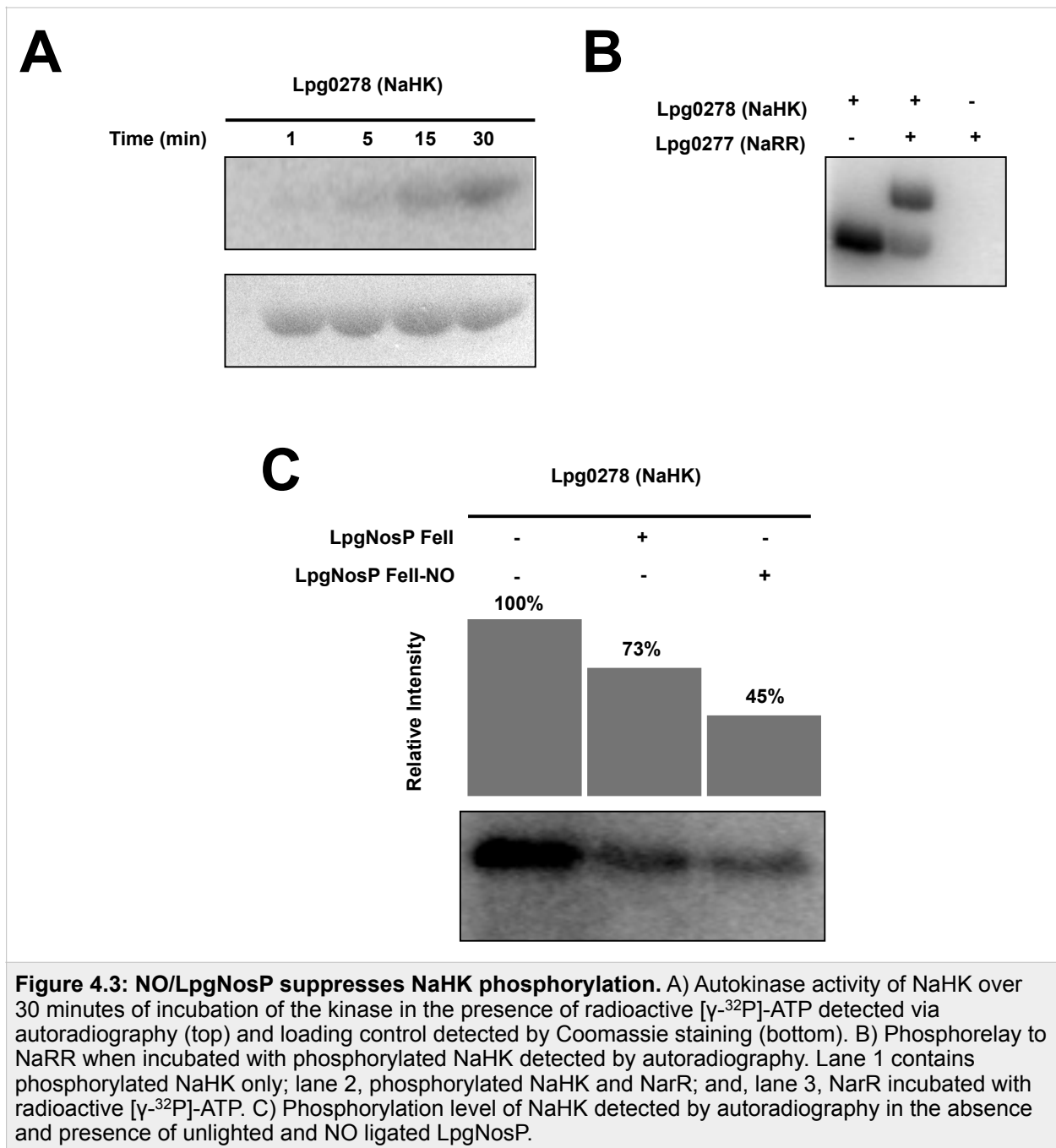
Table 4.1- UV-Visible of NosPs

NosP	Fe^{II}	α	β	Fe^{II}-NO	Source
PaNosP	420	554	524	396	Chapter 2
VcNosP	420	556	529	396	Chapter 3
VcNosPECL	422	554	523	399	Chapter 3
VhNosP	420	568	538	397	Chapter 3
LpgNosP	420	543	522	396	This chapter
SwNosP-A	420	554	528	406	This chapter

(compare Figure 4.3B lane 1 to 4.3B lane 2). As expected, Lpg0277 is unable to autophosphorylate when incubated with [γ -³²P]-ATP, as seen in Figure 4.3B lane 3.

NO ligated LpgNosP (Lpg0279) inhibits autophosphorylation of Lpg0278. In order to assess whether NO ligated LpgNosP has an effect on the kinase activity, we assayed autokinase activity of Lpg0278 in the presence of ferrous LpgNosP and in the presence of NO ligated LpgNosP. Figure 4.3C lane 1 shows autophosphorylation activity of the kinase in the absence of LpgNosP. The autokinase activity decreases when the kinase is incubated with [γ -³²P]-ATP in the presence of ferrous LpgNosP. Even greater suppression of autophosphorylation is observed when Lpg0278 is incubated with NO ligated LpgNosP. This type of suppression of kinase activity is also observed in other NosP/kinase systems as we have shown in chapter 2 for *P. aeruginosa* and in chapter 3 for *V. cholerae*. This pattern of inhibition of autophosphorylation by NO ligated NosP is similar to H-NOX/kinase systems.

Conclusion: Given our observation that Lpg0278 phosphorylation is affected by NO ligated NosP, we propose that NosP may function as an accessory protein that acts as a



sensory domain for NaHK. We also observe that NaHK is able to relay phosphate to NaRR, the bifunctional cyclic-di-GMP processing enzyme. It is crucial that we evaluate the activities of the antagonistic cyclic-di-GMP processing domains, and compare the activities of both domains dependent on the phosphorylation state of the receiver

domain. We have tested NaRR for diguanylate cyclase and phosphodiesterase activities, and as expected, when we assay this protein at two different temperatures using the malachite green assay, we find that the protein is active as both a diguanylate cyclase and a phosphodiesterase (Figure 4.4). Similar results were observed by Levet-Paulo *et al.* (2011) when they assayed Lpl0329. A protein sequence comparison

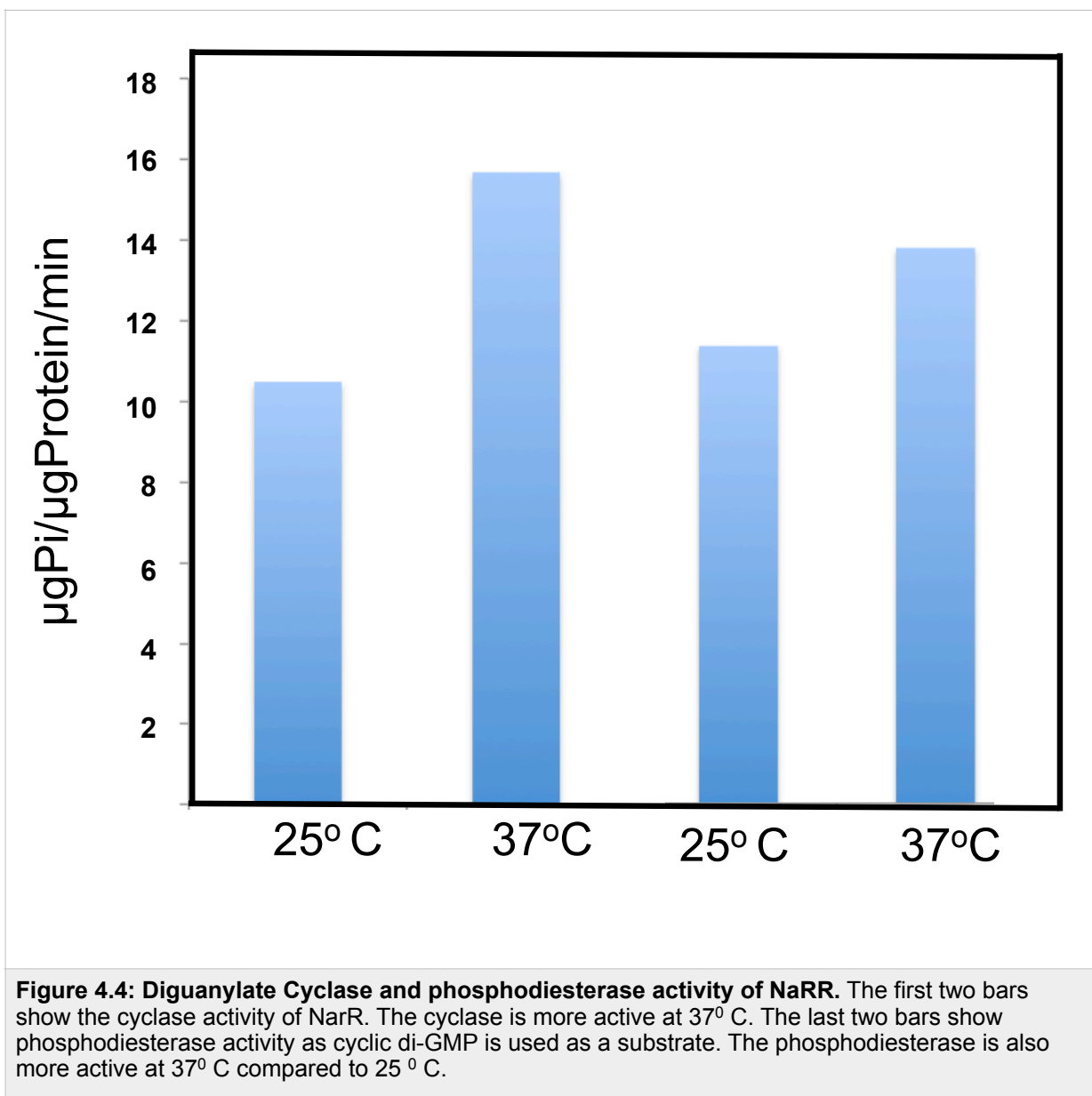


Figure 4.4: Diguanylate Cyclase and phosphodiesterase activity of NaRR. The first two bars show the cyclase activity of NarR. The cyclase is more active at 37°C. The last two bars show phosphodiesterase activity as cyclic di-GMP is used as a substrate. The phosphodiesterase is also more active at 37°C compared to 25°C.

between Lpl0329 and NaRR reveals that they are not identical (Figure 4.5). Therefore, despite the data presented by Levet-Paulo *et al.* (2011), where they observe a decrease in diguanylate cyclase activity upon phosphorylation, we need to assay NaRR for diguanylate cyclase and phosphodiesterase activities in its phosphorylated and non-phosphorylated states. To determine if phosphorylation will have the same effect on NaRR as it did on Lpl0329, we have made several mutants of NaRR (listed in Table 4.2). These mutations include a phosphomimic (conserved aspartate is mutated to glutamate) mutant as well as a constitutively active receiver domain mutant (deletion of a conserved aspartate residue that is not the aspartate that gets phosphorylated) and a receiver domain mutant that can't be phosphorylated, where the conserved aspartate has been mutated to asparagine. We are currently evaluating activities of NaRR utilizing these mutants to have a better understanding of our system along with utilizing the GGAAF (cyclase inactive) and AAL (phosphodiesterase inactive) variants to better. *L. pneumophila* contains two H-NOXs in its genome (Figure 4.1) where H-NOX1 is encoded adjacent to a DGC/PDE and H-NOX2 with a histidine kinase. Interestingly, a reversal in the ratio of NosP to H-NOX proteins, however, is observed in the bacterium *Shewanella woodyi*, which contains two NosP genes. One of the NosPs, NosP-A (Swoo_2247) is in an operon with a histidine kinase and the other, NosP-B (Swoo_3184), is found in an operon with a GGDEF protein. This is of interest to us because *Shewanella woodyi* contains an H-NOX domain that when bound to NO, can act on the co-cistronic DGC/PDE which in turn regulates the total intracellular cyclic di-GMP levels which ultimately affects biofilm formation in this bacterium (6). To better understand the role of the NosP protein, however, we need to biochemically assess the

```

1p10329      MSTESFHLIVIDDNPAIHQDFMKILNVSKNSALLNKFDAELFGDETEQLGISLPEFKIDV
lpg0277      MSTESFRILVIDDNPAIHQDFMKILNVSKNSVLLNKFDAELFDDETEQLGISLPEFEIDV
*****:*****.*****.*****.*****:***

1p10329      ATQGIEGIEKVKRSLEEGRPYPLAFVDIRMPGIDGIETIKRIWAIDPQIQIVICSAYS
lpg0277      ATQGIEGIEKVKRSLEEGRPYPLAFVDIRMPGIDGIETIKRIWAIDPQIQIVICSAYS
*****

1p10329      YSWESTVNTLGLSDNLLVLKPPFDVIAVRQLASALTQKWVLARETQKHTEFLNQLVEERT
lpg0277      YSWESTVNTLGLSDNLLVLKPPFDVIAVRQLASALTQKWVLARETQKHTEFLNQLVEERT
*****

1p10329      ESLQQSFSLLRATIESSSDGILVVDLQGKIVDYNQFVKLWNIPESIMKKTSESQLIQYM
lpg0277      ESLQQSFSLLRATIESSSDGILVVDLQGKIVDYNQFVKLWNIPESIMKKTSESQLIQYM
*****

1p10329      STQLLNSEQHAQHVKESIHIDSSIQIYHFRNGKVVVECCSKPHRVGPQTVGRVWSFRDI
lpg0277      STQLLNSEQHAQHVKESIHIDSSIQIYHFRNGKVVVECCSKPHRVGPQTVGRVWSFRDI
*****

1p10329      TEQTRLKEKLEYQATHDALTNLNRLLLDRIEHAISYARHKMKFAILFFDLDRFKMIN
lpg0277      TEQTRLKEKLEYQATHDALTNLNRLLLDRIEHAISYARHKMKFAILFFDLDRFKMIN
*****

1p10329      DLSLSHEAGDQLLCAVAKRLRSLVRKEDTLARLGGDEFVMLFQSFNSEEQIAGVAQKILKS
lpg0277      DLSLSHEAGDQLLCAVAKRLRSLVRKEDTLARLGGDEFVMLFQSFNSEEQIAGVAQKILKS
*****

1p10329      FQKPFHIAERDINIHVSIGISVYPTDGKTVNTLLSNADMAMYQAKFRGGNQFSFYTEKLN
lpg0277      FQKPFHIAERDINIHVSIGISVYPTDGKTVNTLLSNADMAMYQAKFRGGNQFSFYTEKLN
*****

1p10329      KKTNQFQLELDLQKAIHHEFFLLYQPQFTVNTHEIRSMEALIRWNHPQKGIILPLDFI
lpg0277      KKTNQFQLELDLQKAIHHEFFLLYQPQFTVNTHEIRSMEALIRWNHPQKGIILPLDFI
*****

1p10329      PTAESSGLIVPIGEWVLRETCKQIGTWRMTGLPEIRVACNVASKQLRQDFPAMVKDLLK
lpg0277      PTAESSGLIVPIGEWVLRETCKQIGTWRMTGLPEIRVACNVASKQLRQDFPAMVEDLLK
*****:*****.*****

1p10329      EYSVEPHCLELEITENVIDKEIQRTIKKLSLGVLIILDDFGTGNSSLNLLKQVAVDSL
lpg0277      EYSVPHCLELEITENVIDKEIQRTIKKLSLGVLIILDDFGTGNSSLNLLKQVAVDSL
*****:*****.*****

1p10329      KIDQSFIQNISKSPGDEAIDAIIAIAQSMFNIIAEGVETQNQLKFLKRRNCNDIQGFL
lpg0277      KIDQSFIQNISKSPGDEAIDAIIAIAQSMFNIIAEGVETQNQLKFLKRRNCNDIQGFL
*****

1p10329      MSKPIPPEEIEKLLQGNRTK
lpg0277      MSKPIPSEEIEKLLQGNRTK
*****.*****

```

Figure 4.5: Protein alignment. Lpl0329 is aligned with NaRR (Lpg 0278) and the changes are highlighted.

function of the two NosP domains in *Shewanella woodyi*. We have already cloned the NosP-A and NosP-B from *S. woodyi* and characterized NosP-A spectroscopically (Table 4.1), which exhibits similar characteristics to other purified and characterized NosP domains.

In conclusion, we propose that the sensory module for NaHK is NosP in *L. pneumophila*. We also propose that NosP is an alternate NO sensor in addition to the H-NOX proteins in *L. pneumophila*. Further studies need to be conducted to clearly elucidate the roles of these proteins in this bacterium.

Materials and Method:

Cloning

Genes were PCR amplified from *Legionella pneumophila* genomic DNA and cloned into pET20b vector appending His₆ at the C terminus by the use of NdeI and XhoI restrictions enzymes. Proteins were expressed in *E. coli* BL21 (DE3) pLysS. The appropriate primers are listed in Table 4.2.

Table 4.2- Strains, Plasmids, and Primers Used

<i>E. coli</i> strains used in this work		
DH5α		For cloning genes into <i>E. coli</i>
BL21 (DE3) pLysS		For Protein purification
Plasmids Used		
pET20 (b)	To clone, express and purify proteins from <i>E. coli</i> .	This study
Primers used (N represents nonspecific nucleotides)		
<i>plpgnosP</i>	5'-NNNNNNNCATATGATGAAAATTGAATCATTTC AATAC-3' 5'-NNNNNNNCTCGAGTGGACTCTCTAACAGGGTCGTAATG-3'	
<i>pswnosP-A</i>	5'-NNNNNNNCATATGACTAGATAAAAACAAAATATGCAGTCAG-3' 5'-NNNNNNNCTCGAGGTTTATATCTCTGGAGAGCTTAATGAG-3'	
<i>plpgNaHK</i>	5'-NNNNNNNCATATGACAGAAATGCATCGGTTGTTGCAGCGAC-3' 5'-NNNNNNNCTCGAGCCTCGTACTCAAGGTTTTGGGATTG-3'	
<i>plpgnarR</i>	5'-NNNNNNNCATATGAGTACTGAATCTTTTCGCATTCTGGTCATTG-5' 5'-NNNNNNNCTCGAGTTTTGTCTATTTCCTTGCAGAAAGTTTTTC-3'	

Protein Expression and Purification

Expression and purification of NosP. BL21 (DE3) pLysS cells (Novagen) were transformed with the *pnosP* plasmids. LpgNosP and SwNosP-A were purified the same way as PaNosP from *Pseudomonas aeruginosa*. Yeast extract media was used to grow the protein and was induced with 25 μ M IPTG at 16°C overnight. ALA (0.1mM) was added at the time of induction. All the steps for purification were exactly the same as they were for PaNosP purification.

Expression and Purification of other proteins. All the other proteins were purified as follows. BL21 (DE3) pLysS cells were transformed and grown in TB media (1.2% w/v Tryptone, 2.4% w/v Yeast extract, 0.04% v/v glycerol with 17 mM KH_2PO_4 and 72 mM K_2HPO_4 , pH 7.5). Induction was carried out at OD_{595} of 0.5 with 25 μ M IPTG. Bacterial cultures were cooled to 16 °C prior to induction. Purification buffer was the same as previously described in chapter 2, except no hemin was added. Typically, following binding to 1 ml of Ni-NTA agarose beads, three wash steps were performed: 100 ml with 10 mM imidazole, 50 ml with 20 mM imidazole and 10 ml with 30 mM imidazole (with Tris, KCl, glycerol etc). Followed by the washes, the proteins were eluted in buffer with 250 mM imidazole and desalted on a PD-10 column (GE) in buffer containing 50 mM Tris pH 8.0, 200 mM KCl, 10 mM MgCl_2 , 0.1 mM EDTA and 10% glycerol, 1 mM dithiothreitol. Proteins were aliquoted and stored at -80 °C.

UV-Visible Spectroscopy. All spectra were recorded on a Carey 100 spectrophotometer equipped with a temperature controlled cuvette chamber. NosP complexes were prepared in an oxygen-free glove bag and NO dissociation kinetics were measured as previously described (9).

Kinase Assay. Kinase assays were performed in an assay buffer containing 100 mM Tris pH 8.0, 50 mM KCl, 5 mM MgCl₂ and 1 mM DTT. Typically, each reaction was started by the addition of an ATP solution so that the final ATP concentration was 1 mM with 2 μCi [γ -³²P]-ATP per reaction. Reactions were incubated for an appropriate time at room temperature before quenching with 5x SDS loading dye. Following separation on a 12.5% Tris-glycine SDS-PAGE, gels were fixed, stained with Coomassie blue, and destained prior to exposure to a phosphorscreen overnight. The phosphor screen was scanned by a Typhoon phosphorimager (Amersham Biosciences) and images were analyzed with ImageJ software. For the phosphotransfer assay, NaRR was added to the reaction and incubated for an additional 15 minutes after the kinase had already been incubated with ATP for 30 minutes at room temperature.

Cyclase/Phosphodiesterase assay. To evaluate the cyclase and phosphodiesterase activity, we used Malachite Green to detect the formation of inorganic phosphate via the following reaction scheme (Table 4.3). When inorganic phosphate is present a shift in absorbance maxima from 446 nm (yellow) to 640 nm (green) takes place. The

Table 4.3 - Reactions involved in Malachite Green Assay

Reactions in cyclase activity
GTP + GTP → cyclic di-GMP + 2 PPI (inorganic pyrophosphate)
2 PPI + IPP (Inorganic pyrophosphatase) → 4 Pi (orthophosphate)
Reactions in phosphodiesterase activity
cyclic di-GMP → pGpG (inorganic pyrophosphate)
pGpG + CIP (Calf intestinal phosphatase) → Pi (inorganic phosphate)
Reactions involved in detection
Pi + (NH ₄)MoO ₄ + H ⁺ → H ₃ PMo ₁₂ O ₄₀
H ₃ PMo ₁₂ O ₄₀ (yellow) + HMG ²⁺ + H ⁺ → (MG ⁺)(H ₂ PMo ₁₂ O ₄₀) (green)

SensoLyte® MG phosphate assay kit was purchased from AnaSpec. Synthetic GTP was purchased from Promega, synthetic c-di-GMP and pGpG were purchased from Biolog. For the enzyme assays, 200 nM NaRR was added to a prepared solution containing 0.2 mM GTP as the substrate for the cyclase assay, or 0.1 mM cyclic di-GMP as the substrate for the phosphodiesterase assay the reaction buffer (50 mM Tris pH 7.5, 10 mM MgCl₂,) containing 5 mM MgCl₂, in a final volume of 100 µL. The reaction was incubated for 10 min, either at 25°C or 37°C, followed by boiling at 100 °C to quench the reaction. After cooling, the mixture was centrifuged at 14000 rpm (16,873 g) for 5 min to remove any precipitate. 70 µL of the supernate was mixed with either 10 µL of 3U/ml IPP or 1U/ml CIP in a 96-well plate and incubated at 25 °C for 10 min. Following this, 20 µL of Malachite Green reagent was added, mixed, and the plate was read at 600 nm using a Perkin Elmer Viktor X5 microplate reader. The absorbance readings were corrected for protein only and GTP only backgrounds.

References

1. Fields BS, Benson RF, & Besser RE (2002) *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev* 15(3):506-526.
2. Pagnier I, Merchat M, & La Scola B (2009) Potentially pathogenic amoeba-associated microorganisms in cooling towers and their control. *Future Microbiol* 4(5): 615-629.
3. Carlson HK, Vance RE, & Marletta MA (2010) H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Molecular microbiology* 77(4):930-942.
4. Thomas JM & Ashbolt NJ (2011) Do free-living amoebae in treated drinking water systems present an emerging health risk? *Environ Sci Technol* 45(3):860-869.
5. Arora DP, HOSSAIN S, Xu Y, & Boon EM (2015) Nitric Oxide Regulation of Bacterial Biofilms. *Biochemistry* 54(24):3717-3728.
6. Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA, & Boon EM (2012) Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry* 51(10): 2087-2099.
7. Pecastaings S, Allombert J, Lajoie B, Doublet P, Roques C, & Vianney A (2016) New insights into *Legionella pneumophila* biofilm regulation by c-di-GMP signaling. *Biofouling* 32(8):935-948.
8. Levet-Paulo M, Lazzaroni JC, Gilbert C, Atlan D, Doublet P, & Vianney A (2011) The atypical two-component sensor kinase Lpl0330 from *Legionella pneumophila*

controls the bifunctional diguanylate cyclase-phosphodiesterase Lpl0329 to modulate bis-(3'-5')-cyclic dimeric GMP synthesis. *J Biol Chem* 286(36):31136-31144.

9. Boon EM & Marletta MA (2005) Ligand specificity of H-NOX domains: from sGC to bacterial NO sensors. *J Inorg Biochem* 99(4):892-902.

Chapter 5

Final Thoughts

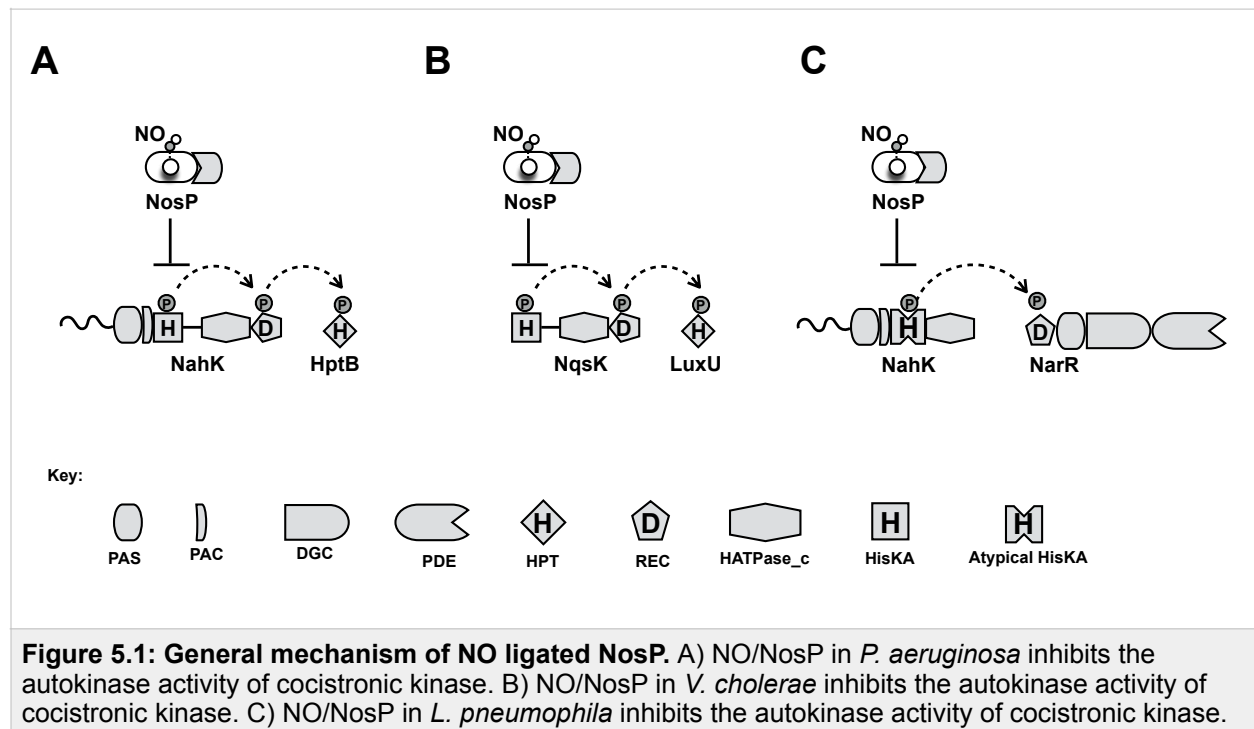
Abstract

In science you must not talk before you know. In art you must not talk before you do. In literature you must not talk before you think. [John Ruskin, "The Eagle's Nest," 1872]

In this final chapter, a summary of our understanding of NosP is discussed as well as the future direction of our research.

■ A Novel Nitric Oxide Sensor in Bacteria: NosP

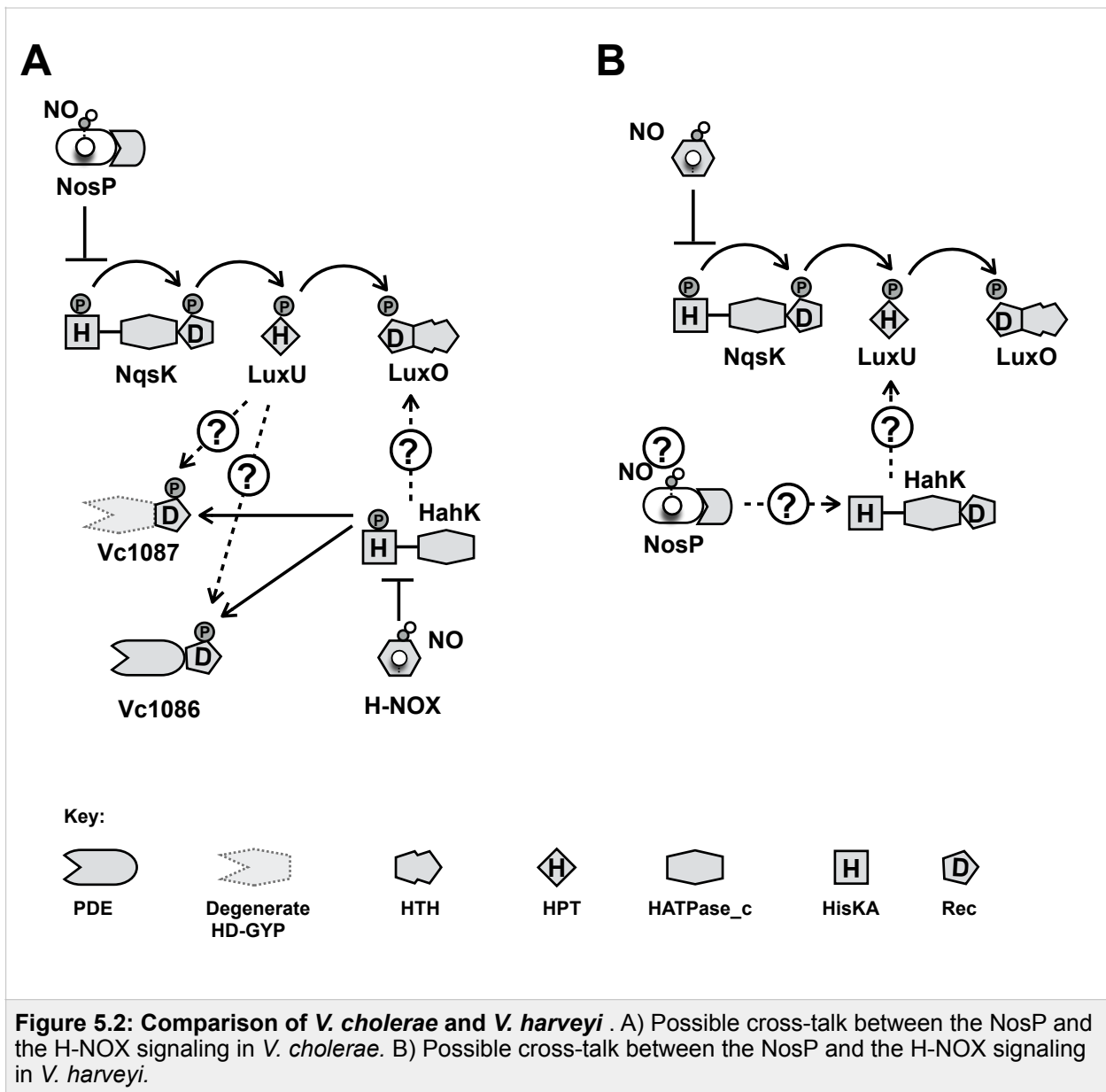
We are the first and only group to study the NosP protein and its function in bacteria. NosP is a hemoprotein that is able to ligate to diatomic gas molecules which includes nitric oxide. We have demonstrated that ligating to nitric oxide allows NosP to modulate the activity of co-cistronic kinases (chapters 2, 3, 4, Figure 5.1). A number of questions need to be answered, however. For example, what is the dissociation constant for NO? In order for a protein to be a sensor of nitric oxide, the protein must be able to bind NO at a very low concentration (nano to femtomolar range). One of the reasons why that is so is that NO is a diatomic radical gas with a very short half life due to its reactivity. Also, NO is not as available in the environment as oxygen and carbon monoxide (CO). Thus, a sensor for NO must be able to discriminate against oxygen due to the abundance of oxygen in the environment. From all the NosPs we have spectroscopically characterized so far, we have not seen any that bind oxygen. The second aspect to



consider would be the intrinsic affinity of heme for NO. Unless discriminated against, any hemoprotein will bind NO over oxygen or CO because of the iron core of heme. NO has the highest affinity for iron in general. Thus, without more biological experiments, it will be uncertain that NosP is a dedicated NO sensor.

■ Do all NosP Domains Sense NO?

When we look at the NosP fused to phosphodiesterase in *V. cholerae*, our data are not clear that NosP domain of Vc0130 is there to sense NO. We need to conduct the NO dissociation experiments for this domain and compare its behavior with that of other NosP domains we have characterized to better understand the purpose of the NosP domain of Vc0130. We also need to understand why in *V. harveyi*, the H-NOX protein is sensing NO and relaying the signal through the quorum sensing network instead of the way it is in *V. cholerae*. Is there an interplay between the H-NOX signaling and the NosP signaling in both of these species (Figure 5.2)? In order to evaluate this question, we can test to see whether there is any phosphotransfer between VcLuxU and the H-NOX associated receiver domains. Though it is unlikely, it has never been tested before. Also unidentified in *V. cholerae* is one of the H-NOX associated receiver domain that can bind DNA to modulate expressions of the operons that contain the receiver domains associated with H-NOX. Could this be the LuxO protein in *V. cholerae*? LuxO is an HTH-containing receiver domain that binds DNA and can regulate gene expressions. We can easily test this by setting up an experiment to observe phosphotransfer between the HahK and LuxO.



■ Is There a Need for Multiple NO Sensors?

We know that many bacteria contain both NosP and H-NOX proteins. We also know that in *Legionella pneumophila*, both the H-NOX and the NosP are involved in cyclic di-GMP regulation. What we need to understand is why does an organism have two modules to sense the same signal. Is it dependent on the NO concentration in the

environment? Is it because these modules are differentially expressed under different growth conditions? Or, is it simply to be redundant?

■ What of Other NO Sensors?

Aside from the Gram-negative bacteria, we know that many Gram-positive bacteria, e.g. *Bacillus licheniformis*, *Staphylococcus epidermidis* and *Staphylococcus aureus*, can form biofilms and respond to nitric oxide (chapter 1). None of these bacteria contain either the H-NOX protein or the NosP proteins in their genome. What is the nitric oxide sensing protein in those bacteria? To fully understand the nitric oxide signaling pathway in bacteria, we must obtain a better understanding of NO signaling in Gram-positive bacteria.

■ Science and I

It is thought that not “one” person invented the “scientific method.” In fact, it wasn’t invented but realized over time as the only method of obtaining reliable knowledge. According to the history, Aristotle was noted as an original thinker, first for devising methods for trying to arrive at reliable knowledge based on observation. Later, Roger Bacon, drawing on the writings of muslim scientists, described a repeating cycle of observation, hypothesis, experimentation, and verification. Eventually over time, what was initially known as the “experimental method” was renamed as the scientific method after the coining of the term “science” in the 1900s. In the end, the purpose of science and the scientific method is to bridge the gap between knowledge and reality.

Fundamental research is the only way to obtain that understanding. Therefore, let's not forget the value of scientific curiosity and the importance of fundamental research.