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Toward Understanding the Biological Role of the Conserved H-NOX Domain in Bacteria

A Thesis Presented

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Abstract of the Thesis

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Soluble guanylate cyclase (sGC) is a well-studied mammalian nitric oxide sensor. When the heme domain, located in the β1 subunit of the enzyme, binds NO, the cyclase domain is activated to form the second messenger molecule cGMP. Iyer et al. recently discovered through a homology search against the β1 subunit of sGC that some bacteria species also contain this heme-binding domain. These conserved heme domains, termed the *H*eme *N*itric oxide/*OX*ygen binding domain (H-NOX), are predicted to be nitric oxide sensors in bacteria. One particular species, *Shewanella woodyi*, has the gene encoding the H-NOX protein in the same predicted operon as a diguanylate cyclase. This diguanylate cyclase (DGC) lacks an annotated sensory domain. We predict that the H-NOX is serving a regulatory role for DGC activity in response to NO. To test this hypothesis we carried out several experiments designed to determine whether the activity of DGC is altered by addition of NO to the culture media. The product of DGC activity is cyclic-di-GMP (c-di-GMP). C-di-GMP concentration is correlated with cell motility; as c-di-GMP concentrations rise, bacteria become biofilm-dwelling, sessile cells, and as c-di-GMP concentrations decrease, bacteria become motile. Thus to test the effect of H-NOX/NO on DGC activity in *S. woodyi*, cell motility and static biofilms assays were employed. Upon addition of NO, an increase in biofilm formation and a corresponding decrease in swim motility was observed; this indicates that an increase in c-di-GMP production occurred when NO was present. This work provides the first evidence that H-NOX might be regulating the activity of DGC in *Shewanella woodyi*.

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List of Abbreviations

Nitric oxide	NO
Heme-Nitric oxide/OXygen binding domain	H-NOX
Shewanella woodyi	*S. woodyi, Sw*
Soluble guanylate cyclase	sGC
Diguanylate cyclase	DGC
Phosphodiesterase	PDE
bis-(3',5')-cyclic dimeric guanosine monophosphate	c-di-GMP
Crystal violet	CV
Dimethyl sulfoxide	DMSO
(Inducible) nitric oxide synthase	(i)NOS
Cyclic guanosine monophosphate	cGMP
Glutamic Acid	E
Alanine	A
Leucine	L
Aspartic Acid	D
Glycine	G
Phenylalanine	F
Threonine	T
2216 Marine Broth	MB
Dipropylenetriamine NONOate	DPTA/NO
Diethylenetriamine NONOate	DETA/NO
Polyvinyl chloride	PVC
Polystyrene	PS
Linear di-GMP	pGpG
Two dimensional	2D
Polyacrylamide electrophoresis	PAGE
Wild-type	WT
Pseudoalteromonas atlantica	*P. atlantica, Pa*
Histidine Kinase	HK
Response Regulator	RR

Crenated cell morphology	C
Mucoid cell morphology	M
Minimal growth media	3M
Yeast Extract	YE
Rifampicin-resistant *P. atlantica* strain	DB27
Deionized water	diH_2O
Isopropyl β-D-1-thiogalactopyranoside	IPTG
Thermoanaerobacter tengcongensis	*Tt*
Optical density	OD
Dithiothreitol	DTT
Luria-Bertani Media (Lennox L Broth)	LB
Polymerase chain reaction	PCR
Stony Brook Molecular Cloning Service	SBMCS
Milli-Q water	mqH_2O

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Chapter 1: Background Information

1.1 Nitric Oxide

Nitric oxide (NO) is an important signaling molecule in all forms of life[1]. In eukaryotes, NO is produced by the enzyme nitric oxide synthase (NOS), and various isoforms have been linked to changes in physiological states, including vasodilation, platelet aggregation, and the immune response[2-6]. Due to (a) the small size of the diatomic gas and (b) a sensor with high affinity and selectivity for NO, nitric oxide readily diffuses through the plasma membrane and is able to elicit the abovementioned responses at low concentrations.

One isoform of NOS is critical to the innate immune response. Inducible NOS (iNOS) forms NO in macrophages after bacterial infection[7]; therefore, iNOS contributes to nonselective killing of bacteria by the immune system through the production of high NO concentrations. However, nitric oxide has been proposed as a signaling molecule in prokaryotes, suggesting that bacteria have developed a method of detecting and responding to NO in the immediate environment at lower concentrations than observed during cell death[1, 8].

1.2 Conserved H-NOX Domain

Nitric oxide signaling in eukaryotes has been well characterized, with the chief sensor of NO established as the large heterodimer soluble guanylate cyclase (sGC)[9]. sGC contains two functional domains: a heme-binding domain and a catalytic cyclase domain. NO binds to the iron center of the heme-binding domain and through an unknown mechanism, activates the catalytic domain to produce cyclic guanylate monophosphate (cGMP). This second-messenger signaling molecule is in turn responsible for eliciting the physiological responses to NO.

Recently, it has been determined through a homology search of the heme-binding domain of sGC that this particular protein domain is also present in a number of bacterial species, particularly in strict anaerobes and facultative aerobes[10]. The entire family of homologous proteins was termed the H-NOX domain (*H*eme *N*itric Oxide and/or *Ox*ygen binding domain) after additional evidence that molecular oxygen may also serve as the

iron ligand in strict anaerobes (Figure 1-1). The H-NOX domain in the strict anaerobes has a distal pocket tyrosine responsible for stabilizing a hydrogen bond network with the O_2 ligand[11]; the H-NOX domains in mammalian sGCs and facultative aerobes lack this tyrosine residue, which is purported to allow the heme protein to selectively bind nanomolar concentrations of NO in an oxic environment[12].

1.3 Shewanella woodyi

Shewanella woodyi is a marine γ-proteobacterium which contains the conserved H-NOX domain (gi: 170727096) and a gene annotated as a diguanylate cyclase (DGC; gi: 170727095) directly downstream in the same predicted operon (Figure 1-2). DGCs catalyze the formation of bis-(3'-5')-cyclic dimeric guanine monophosphate (c-di-GMP). As described in more detail below, c-di-GMP is known to regulate bacterial cell motility. Based on present annotation and bioinformatics, the DGC in *S. woodyi* does not appear to have a regulatory or sensing domain. Moreover, it has been shown that in the bacterial species *Shewanella oneidensis***,** the H-NOX domain is unconditionally associated with a histidine kinase, with a change in enzymatic activity observed upon NO binding[13].

Therefore, we predict that the H-NOX domain in *Shewanella woodyi* is serving as a regulatory domain for the DGC through some type of protein-protein interaction. Since *Sw* H-NOX cannot bind molecular oxygen (Figure 1-3), it is purported that the ferrous heme domain is serving as a NO sensor[14]. Upon NO binding, we predict that DGC activity will be altered so as to produce a noticeable change in cellular motility. Lastly, *S. woodyi* is a robust facultative aerobe which will utilize either nitrate or nitrite as an alternative terminal electron acceptor for cellular respiration in the absence of oxygen[15]. When the bacterium utilizes the denitrification pathway during anaerobic metabolism, nitric oxide will inevitably be produced after nitrite reduction, so the source of nitric oxide may be endogenous to *S. woodyi* (Figure 1-4).

1.4 Cyclic-di-GMP

Diguanylate cyclases catalyze the formation of c-di-GMP from two guanosine triphosphate (GTP) molecules (Figure 1-5)[16]. The c-di-GMP from the active DGC acts as a second messenger in signaling cascades within the cell[17]. Although all the roles of c-di-

GMP have not been elucidated thus far, a large majority of pathways implicate c-di-GMP as the trigger for the sessile-motile transition in bacteria[18, 19]. The effective intracellular targets of c-di-GMP characterized thus far include cellulose synthases, the PilZ domain, and c-di-GMP binding riboswitches[18, 20-22]. It had been known for some time that high levels of c-di-GMP in *Gluconacetobacter xylinus* stimulate cellulose synthases to produce copious amounts of cellulose fibers; recently, it was discovered that increasing concentrations of c-di-GMP indicate a switch within the bacterial cell from the freely swimming planktonic cell to form the sessile biofilm[23-25]. The PilZ domain was identified as a possible protein responsible for this transition due to its ability to bind c-di-GMP with great affinity and its involvement in pili formation in *Pseudomonas aeruginosa*[26].

Methods of DGC activation are also an active area of research; however, the active DGC must form a dimer in order for the conserved GGDEF sequence to form the active site for GTP cyclization[27, 28]. Site-specific mutations removing the acidic aspartate (D) and glutamate (E) residues from the active site render the diguanylate cyclase incapable of forming c-di-GMP[29]. Bacterial cells, in an effort to control the levels of the signaling molecule, possess another enzyme responsible for the degeneration of c-di-GMP; phosphodiesterases (PDEs) cleave one of the phosphodiester bonds in c-di-GMP, yielding the linear, phosphorylated guanylate dimer pGpG. The PDEs contain a conserved nucleotide sequence EAL, with the glutamic acid residue suggested to play a catalytic role in the turnover of c-di-GMP[30]; however, further analysis of these phosphodiesterases appear to demonstrate that other conserved sequences, such as the loop sequence DDFGTG, might play a pivotal role in the stabilization of the magnesium ion required for the general-base reaction associated with c-di-GMP degradation[31]. Many DGCs have dual functions: they may also contain a PDE domain within the peptide sequence, which may indicate that a precise switch between c-di-GMP production and degradation is present within these bacteria.

1.5 Bacterial motility and biofilm formation

The understanding of cellular movement in bacteria is no longer confined to the belief that bacteria exist purely as freely swimming planktonic cells; instead, in response to various extracellular and intracellular signals, bacteria are constantly switching

between different levels of attachment to solid surfaces (Figure 1-6)[32]. These large, attached, structured bacterial communities are referred to as biofilms[33, 34]. The evolutionary advantages of forming biofilms are abundant, including surviving nutrient depletion, mutual cooperativity in synthesizing highly energetic compounds, and defense mechanisms to environmental stresses.

The initial attachment to a biotic or abiotic solid surface is mediated largely through environmental cues, such as molecules involved in quorum sensing[33]. The attachment is reversible and the bacteria are commonly referred to as microcolonies at this point of development; however, a genetic change is observed within the attached cells in which copious amounts of exopolysaccharides are produced, which indicates a shift toward the mature biofilm. The multicellular communities develop from the initial attached cells, and another set of signals are suggested to initiate biofilm dispersion to reform planktonic cells; these cells are now capable of forming a new, separate biofilm under the appropriate environmental conditions.

Numerous proteins within the bacteria are responsible for this dynamic process, many of which are still under investigation or yet to be discovered. However, c-di-GMP concentrations are now well-accepted to be important in many stages in this process, and as such, regulation of DGCs and PDEs is under intense investigation. Certain histidine kinases have been shown to relay a particular phosphate signal to DGC[30], altering c-di-GMP formation and therefore modulating flagellar movement and exopolysaccharide secretion[35]. Small gas molecules, such as O_2 in EcDOS and FixL, can alter PDE activity and therefore change the effective concentration of c-di-GMP within the cell[36-38]. The tight control over c-di-GMP is of extreme importance to the bacterium; if the cell encounters a particular extracellular environment, it must be able to change its internal physiology to increase the rate of survival.

1.6 Hypothesis

The H-NOX in *S. woodyi* can bind NO with great affinity[14], and its location upstream of an annotated DGC implies that the H-NOX is acting as the regulatory domain; upon binding of nitric oxide, the H-NOX is predicted to upregulate DGC activity, thereby inducing biofilm formation. The bacteria could encounter nitric oxide

through endogenous denitrification pathways; therefore, if NO is being reduced from nitrite, we predict that the H-NOX would upregulate c-di-GMP to produce a biofilm community in response to the nutrient-starved environment. If the NO source is exogenous to the bacteria, the upregulation of DGC activity would provide a protective biofilm environment for *S. woodyi* through the excretion of exopolysaccharide. The DGC found in *S. woodyi* is therefore hypothesized to be involved in the formation of a diverse biofilm community in response to an environmental stress (NO) encountered by the cells (Figure 1-7). In this thesis, my efforts to experimentally test this hypothesis are described.

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1.8 Tables and Figures

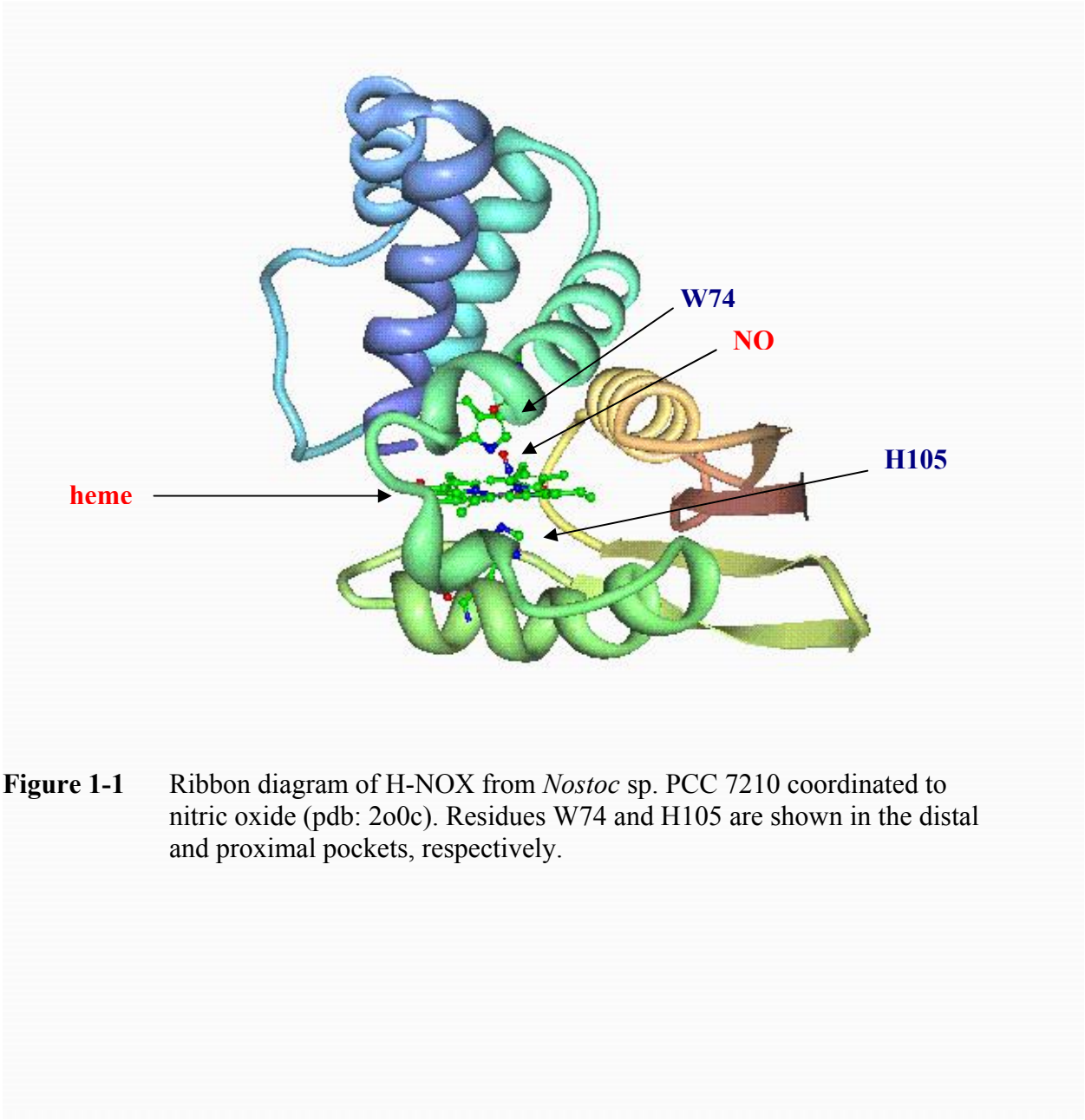

Figure 1-1 Ribbon diagram of H-NOX from *Nostoc* sp. PCC 7210 coordinated to nitric oxide (pdb: 2o0c). Residues W74 and H105 are shown in the distal and proximal pockets, respectively.

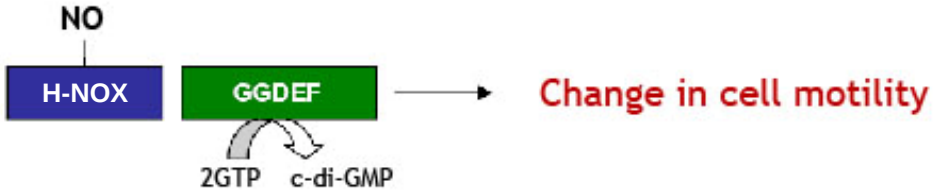

Figure 1-2 The complementary strand shows that the two genes in question are separated by only two nucleotides in *Sw* chromosome. This indicates that both genes are regulated and transcribed under the same, unknown promoter. Since the DGC lacks a sensing domain, we hypothesize that the H-NOX protein is serving as the sensory protein to regulate c-di-GMP production in response to bound NO.

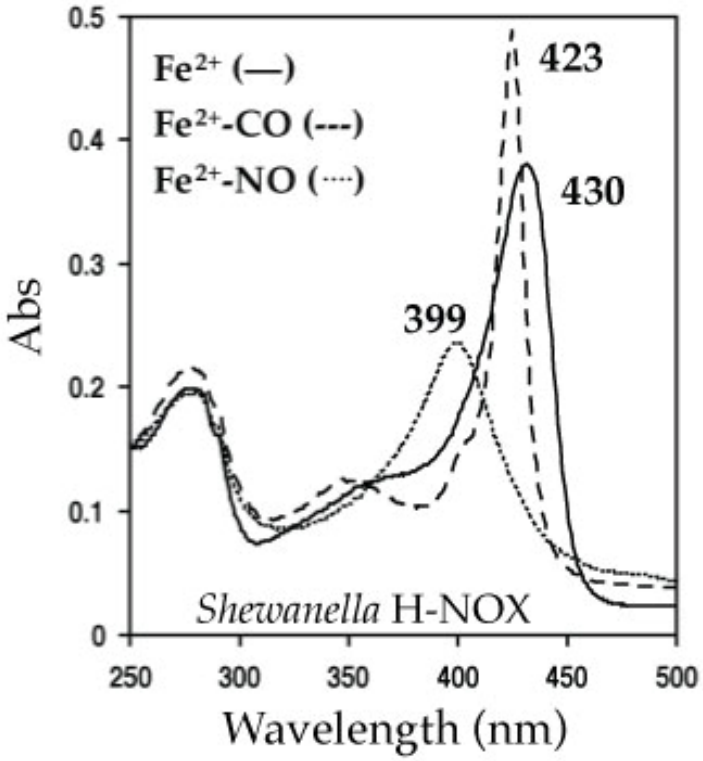

Figure 1-3 Electronic spectra of *Sw* H-NOX[14]. Soret peak of Fe(II)-NO is identical to sGC, a known NO sensor. Furthermore, the protein cannot form an oxygen complex: no shift in the Soret peak was observed between anoxic and oxic Fe(II) samples, indicating that the protein remains unbound upon exposure to O_2.

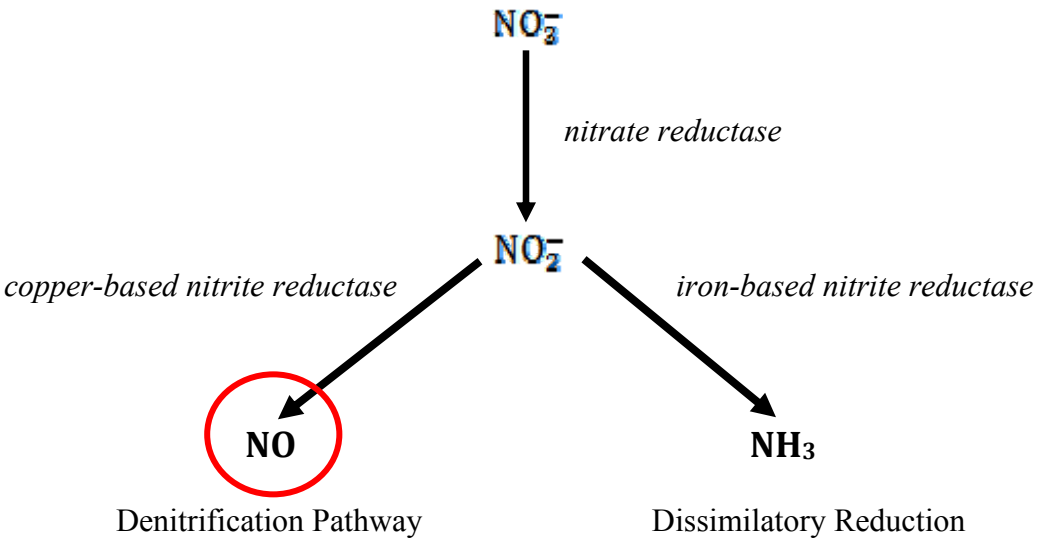

Figure 1-4 Anaerobic respiration on nitrate/nitrite sources can proceed either through denitrification or dissimilatory reduction[15]. NO can theoretically be produced in *S. woodyi* through the denitrification pathway under anoxic conditions by utilizing the multicopper nitrite reductase (gi: 170725778).

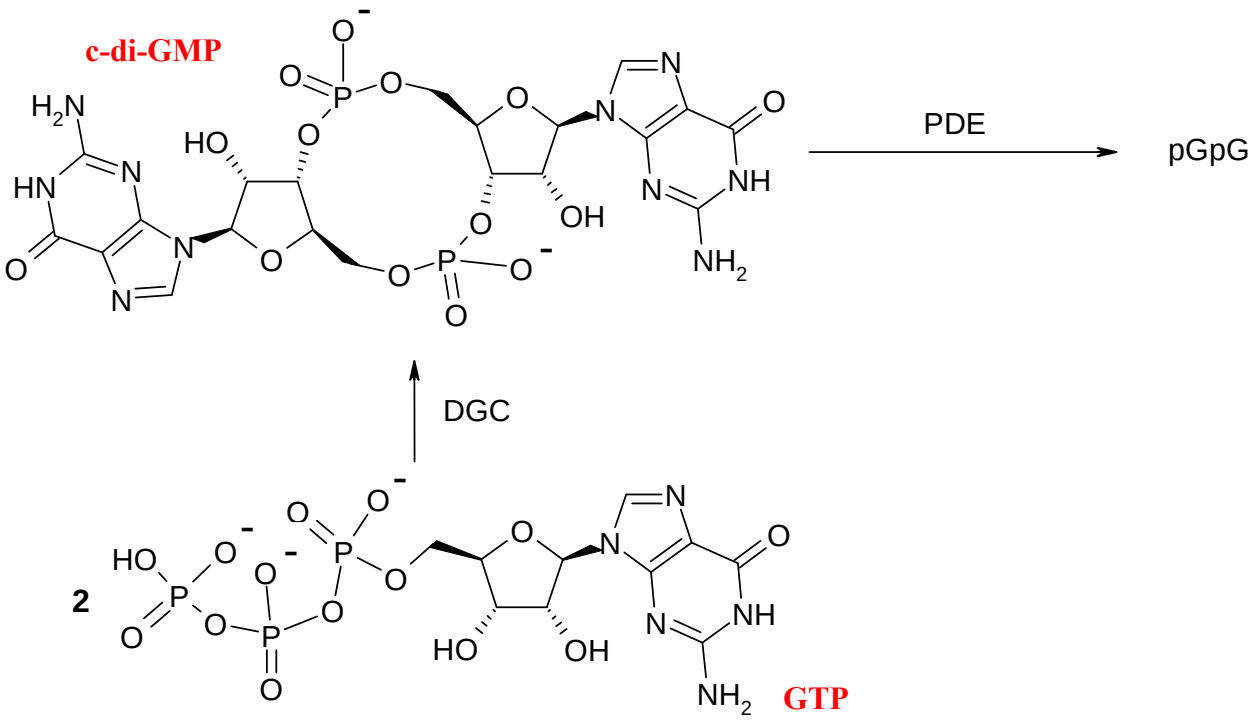

Figure 1-5 The cyclization of two GTP molecules is catalyzed by the conserved GGDEF domain in DGC. The product of the reaction, c-di-GMP, is under tight control of PDE, either present in the DGC itself or freestanding.

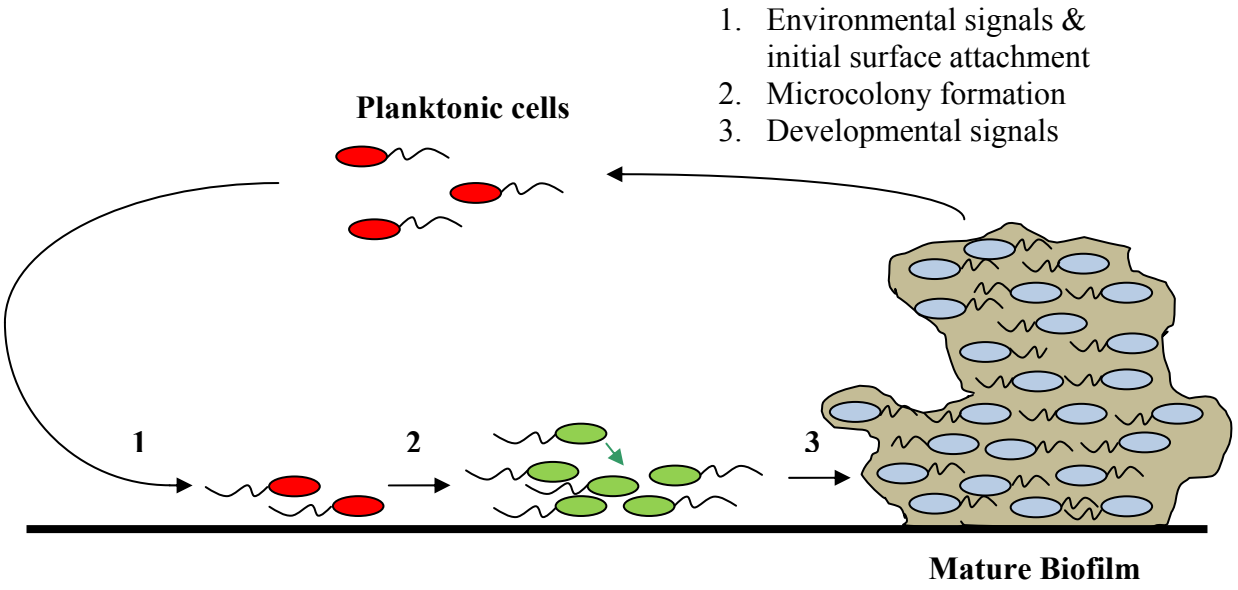

Figure 1-6 The transition between planktonic cells and a mature biofilm is a multistep process under the control of a myriad of different cues, including environmental and intracellular signals. Detachment from the biofilm reinitiates the dynamic process, which is controlled by another set of signaling molecules. Adapted from Kraigsley et al.[30]

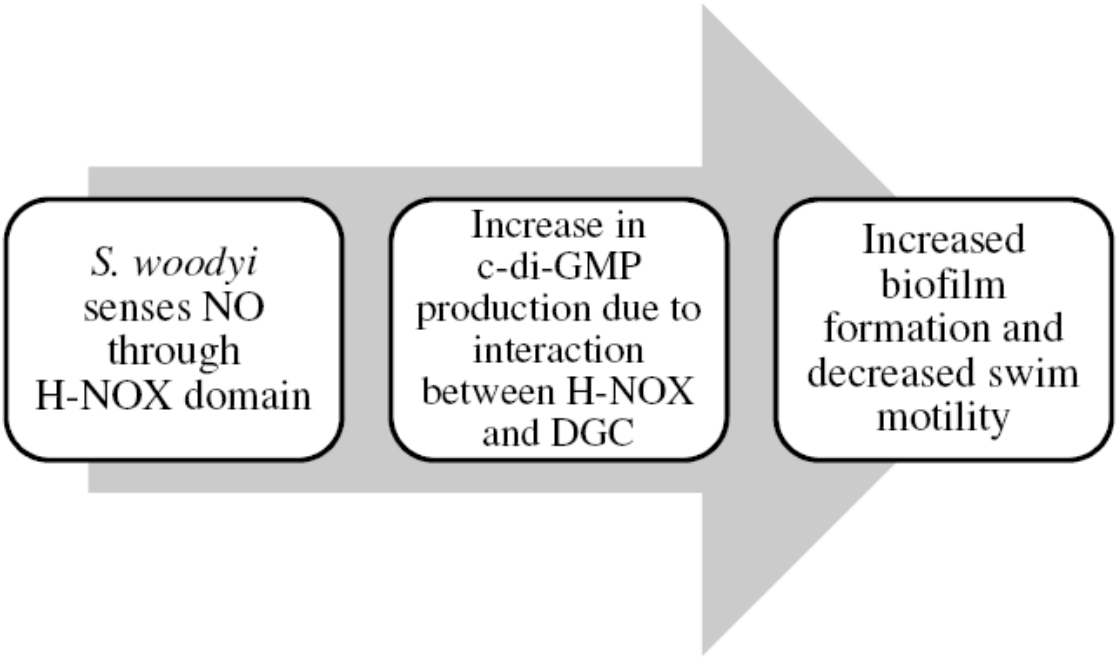

Figure 1-7 Current hypothesis for the biological role of *Sw* H-NOX due to its location directly upstream from an annotated DGC and its ability to bind NO in the ferrous state.

Chapter 2: Phenotypic Changes Observed in Response to Nitric Oxide

2.1 Summary

Previous results obtained in the group suggested a change in protein expression and phosphorylation in response to nitric oxide. A hypothesized nitric oxide sensor in *S. woodyi* is the H-NOX domain, and its location directly upstream of a DGC led us to determine whether a phenotypic effect on cellular movement would be seen upon treatment with the small molecule. A titratable increase in biofilm development was observed after 24 hours when static cultures were treated with DPTA/NO. A corresponding decrease in swim motility was recorded upon addition of another NO donor, DETA/NO, when a semi-solid swim assay was utilized. These coupled results indicate a definite relationship in cellular motility to NO and a possible relationship to the *Sw* H-NOX/DGC operon.

2.2 Introduction

The discovery of a nitric oxide sensing protein in bacteria is extremely peculiar: what evolutionary advantage would these few bacteria have if their genomes contained the conserved H-NOX domain? The first clue to the cellular funcation of H-NOX lies in the genes around the gene encoding for the H-NOX. For the majority of facultative aerobes sequenced thus far containing the H-NOX domain, H-NOX is in the same predicted operon as either a histidine kinase or a diguanylate cyclase[1]. Both proteins are extremely important in eliciting cellular responses, either through a phoshorelay signaling cascade or a second messenger molecule, respectively[2,3]. In the case of *S. woodyi*, as stated above, the DGC directly downstream from the H-NOX lacks an annotated sensory region. Phosphorylation, in conjunction with dimerization, has been shown to play an important role in other characterized DGCs[2]; however, due to gene topology, phosphorylation is not believed to significantly affect DGC activity, unless the phosphorylation is due somehow with NO binding to H-NOX. The DGC does, however, contain a PAS domain; these structurally homologous domains have been shown to play a significant role in detecting changes in redox potentials, forming protein-protein and dimerization interfaces, and sensing small molecules when a cofactor is present[4,5]. We

predict, based on the models for characterized DGCs with and without associated PAS domains, that the PAS domain in *Sw* DGC is assisting in protein-protein interactions between H-NOX and DGC[6].

According to our model, NO bound H-NOX will alter the interaction between the DGC and H-NOX. If the H-NOX is acting as a regulatory protein, DGC activity will be up- or down-regulated and the concentration of intracellular c-di-GMP will fluctuate accordingly. The concentration change in the second messenger molecule will regulate downstream cellular motility proteins, which can be visualized by a phenotypic change in motility. The effect of NO on *Sw* DGC can therefore be determined by utilizing two motility assays: biofilm development and swim plates. Here, a static biofilm assay was employed, in which a culture was grown without agitation over 24 hours; if cell attachment occurs, the amount of cells in the biofilm can be determined using a dye-based approach. An increased amount of cell contained within the biofilm will correspond to more crystal violet (CV) being absorbed by the adhered cells; when resolubilized with a decolorizing solution, the relative amounts of CV absorbed by the biofilms can be compared to determine whether the NO had an effect on biofilm formation. The swim plates would in theory provide a reciprocal result to any biofilm data since the soft agar plates are developed to mimic a planktonic environment. If a particular species shows an increase in biofilm development, then a corresponding decrease in swimming should be observed; the bacterial cells are producing more exopolysaccrahide (EPS) and adhesion molecules, making it more difficult for the cells to freely swim in the soft agar. Therefore, both assays were performed to provide solid evidence that nitric oxide was affecting the cellular motility of *S. woodyi*.

This model is also consistent with work completed by a previous rotation student, Ming Yang. In using DPTA NONOate (DPTA/NO), she was able to determine that the growth rate of *S. woodyi* was not significantly affected upon addition of the NO-donor molecule up through an initial concentration of 2mM; therefore, NO at these concentrations does not appear to alter the planktonic growth of the bacteria in culture (Figure 2-1). In addition, two-dimensional PAGE was performed on the cleared cell lysate from *S. woodyi* with and without the addition of 1mM DPTA/NO (Figure 2-2). A multitude of proteins appeared on the gel upon addition of the nitric oxide donor,

indicating that protein expression was activated in the different cellular environment. Furthermore, the results suggested that some of the proteins were phosphorylated in the NO treated samples, as evidenced by a small shift in pI, but not in molecular weight, of the protein spots. The transition between the phosphorylated and dephosphorylated state often triggers the activation or deactivation of proteins; therefore, the altered expression and evidence in change in phosphorylation was promising in that *Sw* H-NOX, along with the DGC, might be affecting the cellular physiology of *S. woodyi*.

2.3 Materials and Methods

2.3.1 Bacteria and Media

S. woodyi (ATCC 51908) was grown at 25°C in 2216 Marine Media (MB; 28g/L, BD Difco). All agar plates contained 28g/L MB; solid agar plates were prepared with 1.5% (w/v) Bacto Agar (BD Difco), whereas swim motility plates contained 0.3% (w/v) Bacto Agar.

2.3.2 Biofilm Formation in Microtiter Plates

Biofilms were statically grown at room temperature on polyvinyl chloride (PVC) microtiter plates for 24 hours[7]. The plates were prepared from a tenfold serial dilution of overnight cultures with fresh MB, with 100µL culture added to each well. To quantify biofilm formation after growth, the supernatant was removed and placed in polystyrene (PS) 96-well plate. The PVC wells were washed with 100µL fresh MB and removed to a PS plate. Both the supernatant and wash PS plates were analyzed at 600nm to determine cell density after each step. The PVC plate was then inverted and blotted on a paper towel to remove residual media. 200µL 0.1% crystal violet was added to each well and allowed to incubate at room temperature for 30 minutes. The residual crystal violet was then removed by vigorous shaking over a waste container, followed by two successive rinses with deionized water (diH_2O). The plates were allowed to air dry and the absorbed CV in the biofilms was solubilized with 200µL DMSO for 15 minutes.125µL of the resulting solution was removed to another PS plate and the absorbance was taken at 570nm to quantify the amount of adhered cells.

2.3.3 Swim Motility Assays

Swim plates were prepared in the cold room after the agar solution was cooled in a 37˚C water bath. Various concentrations of DPTA/NO and DETA/NO were added to reach the indicated starting concentration. After cooling to solidify, swim plates were stab inoculated with fresh single colonies and allowed to sit at room temperature for at least 24 hours. The swim diameter was measured at the largest zone of motility with a metric ruler at the time points indicated.

2.4 Results

In order to determine optimal assay times, biofilm quantification utilizing the CV method was employed over a course of three days. The microtiter plate was cut prior to sample addition so that quantification could be performed each day without disturbing the remaining samples. A MB control was brought through the trial to determine any background CV staining of the wells from the media and to check the possibility that the MB was contaminated with another sample. However, no exogenous contamination was observed in the supernatant or CV absorbance in the MB control, indicating that biofilm development is due to *S. woodyi* alone. Distinct biofilms at the air-broth interface in the PVC microtiter plates were observed after 24 hours of static growth(Figure 2-3A). An increase in CV absorbance was seen after three days, but a slight increase in CV staining was noted at this time point. Since a robust biofilm was observed over the background staining, the time course for the subsequent experiments was set at 24 hours.

The static biofilm assay was repeated over three independent experiments to verify that the results were repeatable and reliable. Consistent staining in both the negative MB control and *S. woodyi* sample wells was recorded. It is important to note here that all triplicate experiments were done with the same 0.1% CV stock solution. Invariably between different CV solution preparations, a change of absorption at 570nm was observed.

In order to supplement any media used with NO, specific NO donors termed NONOates were utilized; NONOates are parent compounds that decompose according to first-order kinetics to liberate typically two NO molecules in a neutral or slightly acidic solution. To test our hypothesis that NO affects biofilm formation, *S. woodyi* biofilm

cultures were then supplemented with various concentrations of the NO donor DPTA/NO, ranging from 100μM to 2mM (Figure 2-4B). DPTA/NO was chosen due to its half-life of five hours at room temperature; over the course of the experiment, a steady amount of NO should be liberated by the first-order degradation of the parent NONOate. Other NONOates with shorter or longer half-lives may have been harder to ensure that an effective amount of NO remained in the overnight biofilm cultures. For three independent trials, a significant increase in cellular attachment was observed when the concentration of nitric oxide was increased in the starting culture. Again, no additional staining was noted in the negative MB control, indicating that no exogenous species are present in the culture. The normalized percent increase in biofilm formation over the untreated WT *S. woodyi* (0μM DPTA/NO) was calculated as follows and represented in Figure 2-4A:

$$\text{Increase} = [(\text{Mean } A_{570} \text{ NO culture})/(\text{Mean } A_{570} \text{ WT})] * 100\% \qquad (2\text{-}1)$$

To our best approximation, the CV staining of the attached cells suggests an approximate twofold increase in the amount of cells in the biofilm after treatment with 2mM DPTA/NO for 24 hours.

Any amount of DPTA/NO over 100μM appears to be able to elicit a cellular response towards biofilm formation; therefore, we ought to see a corresponding, proportional decrease in the swim motility assays at similar NONOate concentrations. Therefore, all swim plates were supplemented with approximately 600μM NONOate, unless otherwise noted in the experimental description put forth hereafter, since we are certain an increase in cell attachment is occurring at this particular concentration. Swim plates were initially attempted with solely DPTA/NO; however, daily inconsistencies between plate formations made quantifying swim behavior difficult. To that end, the longer half-life DETA/NO was employed first in conjunction with DPTA/NO to show that similar responses would be obtained; in theory, the slower DETA/NO would supply a reduced amount of nitric oxide to the semi agar over a longer period of time when compared to DPTA/NO. Swim plates prepared with equivalent amounts of the two NO donors suggested a larger phenotypic response associated with DETA/NO after 24 hours (Figure 2-5). The response observed is probably due to a consistent NO concentration equilibrium during the entire course of the experiment; if any amount of DPTA/NO was

lost during plating as a result of the high agar temperature, then the degradation would occur at a faster rate than DETA/NO. Therefore, the amount of actual NO in the semi agar treated with DPTA/NO might be in actually less than those plates treated with DETA/NO.

The experimental time was increased to determine the effect of NO over time on the ability of *S. woodyi* to effectively swim (Figure 2-6); DETA/NO at equivalent starting concentrations again showed a larger decrease in swim motility even when considered for longer time points. Finally, a titratable increase in concentration of DETA/NO was added to the swim plates in an effort to show a corresponding decrease in swimming at higher NO concentrations. Indeed at concentrations equal to and higher than 125μM, a significant decrease in the swim diameter is observed (Figure 2-7) when compared to WT untreated. These results parallel the data obtained with DPTA/NO in the static biofilm assays.

2.5 Discussion

We predict that the *Sw* H-NOX domain, when bound to NO, is involved in modulating DGC activity and c-di-GMP production through protein-protein interactions. If our hypothesis is correct, a phenotypic response to NO should be observed in the form of a change in cellular motility (Figure 1-8). If H-NOX serves to activate the DGC activity, then a response indicative of an increase in c-di-GMP should be observed; if the heme domain is down regulating the production of c-di-GMP or up regulating the PDE activity, then a decrease in c-di-GMP is expected in *S. woodyi*. The results obtained in the static biofilm and swim motility assays suggest that an increase in c-di-GMP is observed in response to nitric oxide. Increased biofilm formation indicates that an increased amount of c-di-GMP is present within the cell, which would in turn theoretically up regulate exopolysaccharide and adhesion molecule production; if genes responsible for these products are activated, then an analogous decrease in swimming behavior should be observed. The cells are activating protein expression to irreversibly attach to a solid surface and start secreting copious amounts of sticky extracellular components; this would therefore make *S. woodyi* less able to freely swim in the soft agar assay.

Unfortunately, it is impossible to make a definitive conclusion as to what is causing this observed relationship between biofilm formation and swim motility. Other unknown factors may be contributing to the response to nitric oxide not involved in c-di-GMP production, such as phosphorelay signaling or defense mechanisms in response to the reactive nitrogen species present within the cell; however, no decrease in cell viability has been noted over the NO concentrations utilized. Advanced genetic manipulations are required in order to demonstrate that the response is due to regulation of the DGC by the purported H-NOX sensor in *S. woodyi*. No known resistance has been reported for *S. woodyi*, which makes a knockout mutation more difficult to obtain. However, one avenue that has been attempted, to minimal results thus far, is a knockin based approach (see Chapter 3). Even with the knock-in approach, work towards an antibiotic resistant strain and H-NOX and DGC gene manipulations in *S. woodyi* should be investigated as well. The results obtained thus far could be verified if biofilm formation and swim motility is no longer affected by increasing levels of NO in the absence of either one of these proteins.

Finally, the association between the two proteins should also be investigated *in vitro* to determine if and how the two proteins interact. How is the H-NOX affecting the activity of the DGC? Are the DGC or PDE domains being activated by a conformational change initiated by a change in the conformation of H-NOX when bound to NO? What role does the PAS domain have in DGC activity? Where are the two proteins interacting? Multiple random mutants could be employed to determine if *in vitro* activity is affected; *in vivo* assays could complement these results if H-NOX is shown to modulate c-di-GMP production. Nevertheless, an important step forward was taken to further support previous work which indicated that NO had an effect on the proteome of *S. woodyi*. If and how H-NOX is involved in these effects would provide the first insight as to the biological role of these purported NO sensors in bacteria.

2.6 References

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2.7 Tables and Figures

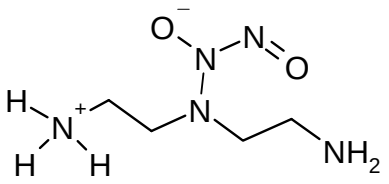
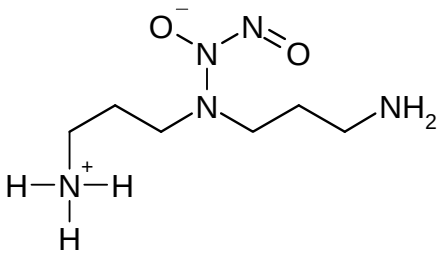
Specific NONOate	$t_{½}$ at 25°C	Structure
DETA/NO	37 hours	
DPTA/NO	5 hours	

Table 2-1 Specific NO donor compounds utilized over experiments. Compounds liberate two molecules of NO upon degradation of the parent compound according to a first-order, pH-dependent mechanism.

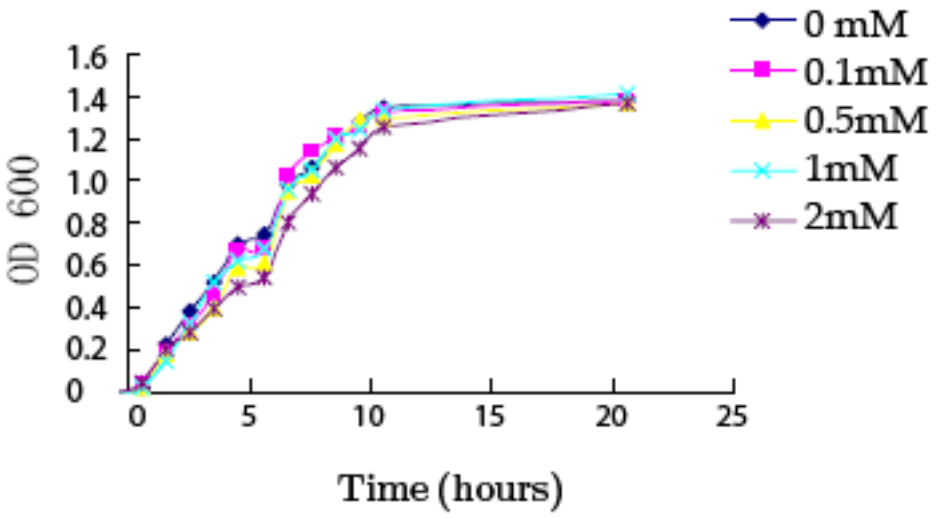


Figure 2-1 Planktonic cell growth was determined over approximately 20 hours, with or without supplemented DPTA/NO. All concentrations indicated refer to effective concentration of DPTA/NO at the beginning of the trial. No significant effect on cell growth was observed with concentrations through 2mM of the NO donor.

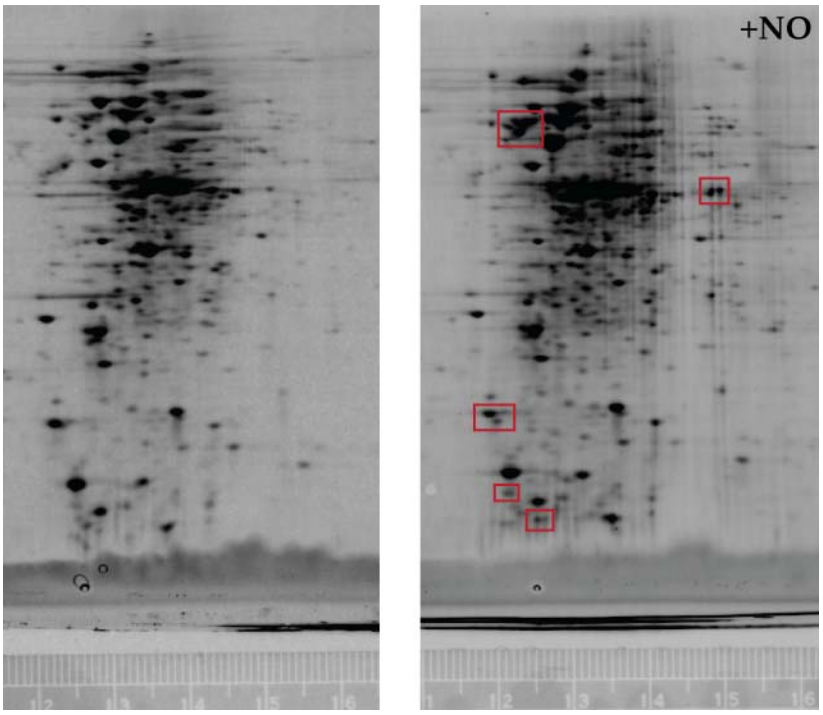


Figure 2-2 Two-dimensional PAGE comparing protein expression with (1mM) or without supplemented DPTA/NO. Equal protein concentrations were utilized in both assays. Several proteins appeared upon addition of nitric oxide to the culture medium, indicating that these proteins are up-regulated in response to NO. In addition to increased expression, the gels provide probable evidence for changes in the state of phosphorylation in the proteome.

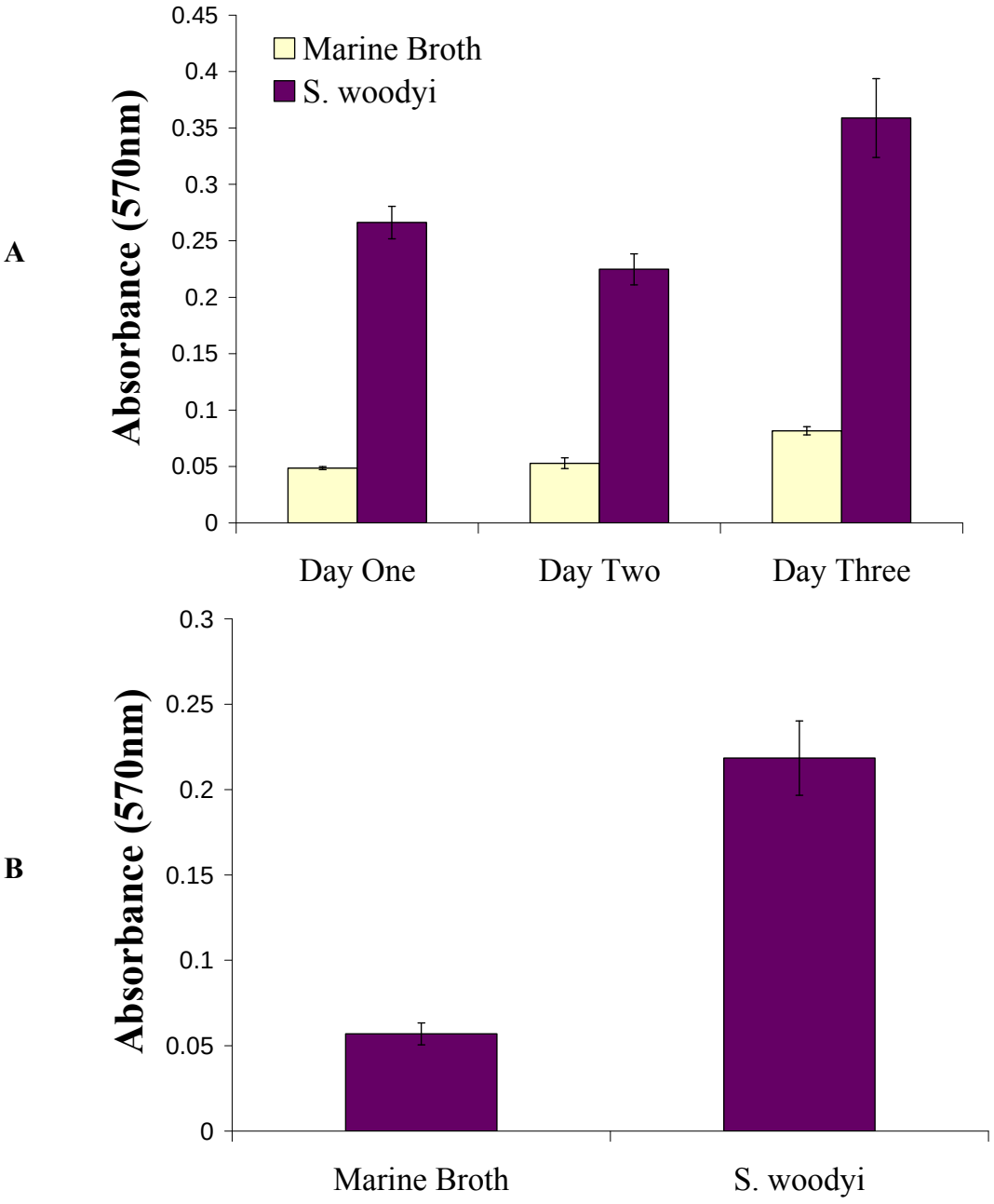


Figure 2-3 Initial static biofilm experiments to determine appropriate time course for the subsequent assays involving nitric oxide. (A) After one day, considerable cellular attachment was observed over the MB negative control not containing *S. woodyi* culture. (B) The reliability of the assay was confirmed in three independent experiments performed in triplicate.

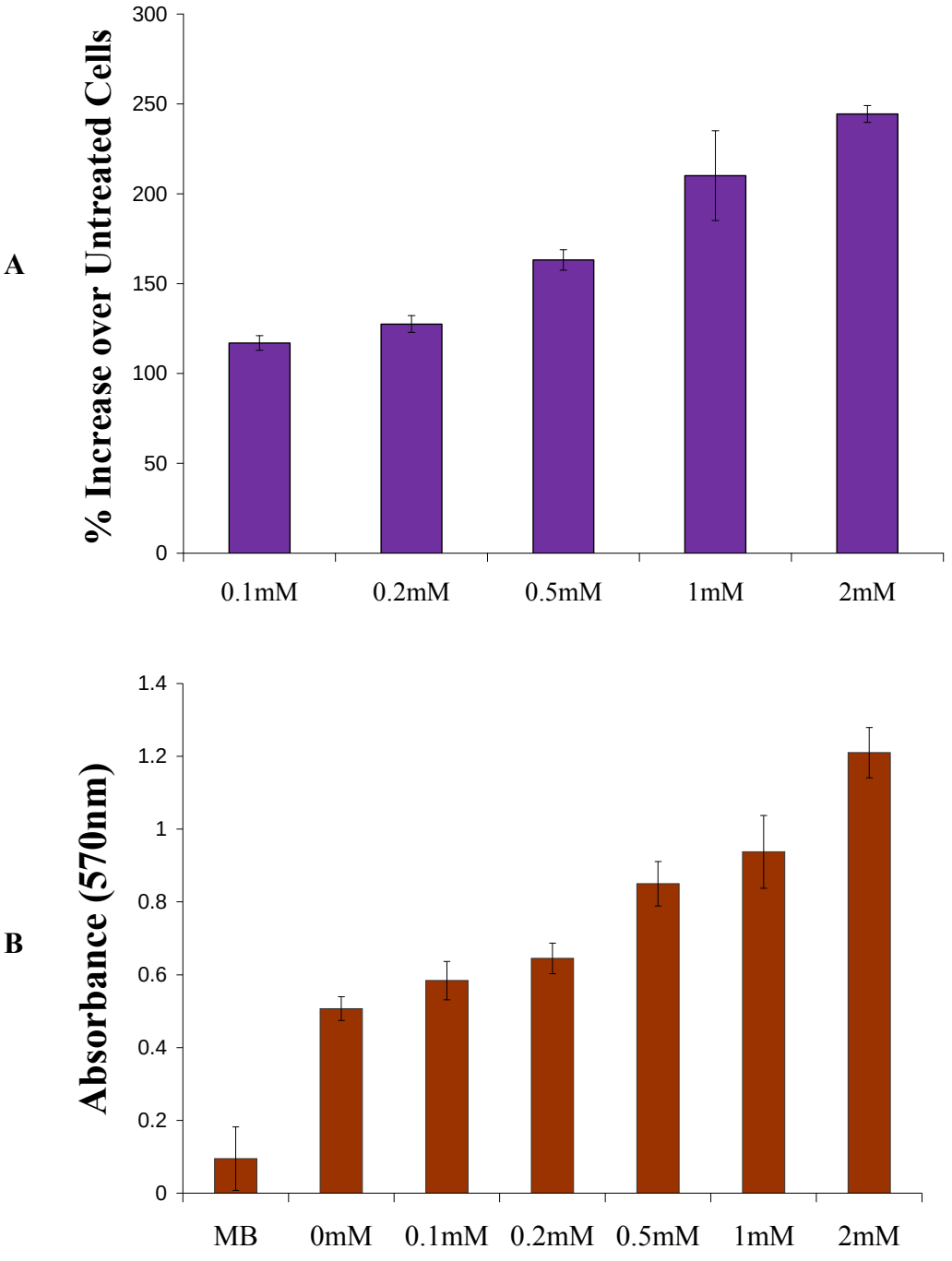


Figure 2-4 Static biofilm development in response to increasing concentration of DPTA/NO. (A) Attachment was augmented nearly 2.5 fold when the MB is supplemented with 2mM DPTA/NO compared to the untreated WT *S. woodyi*. Mean absorbance normalized to untreated culture for each trial. (B) Results from one of the three independent experiments. All concentrations indicate starting concentration of DPTA/NO.

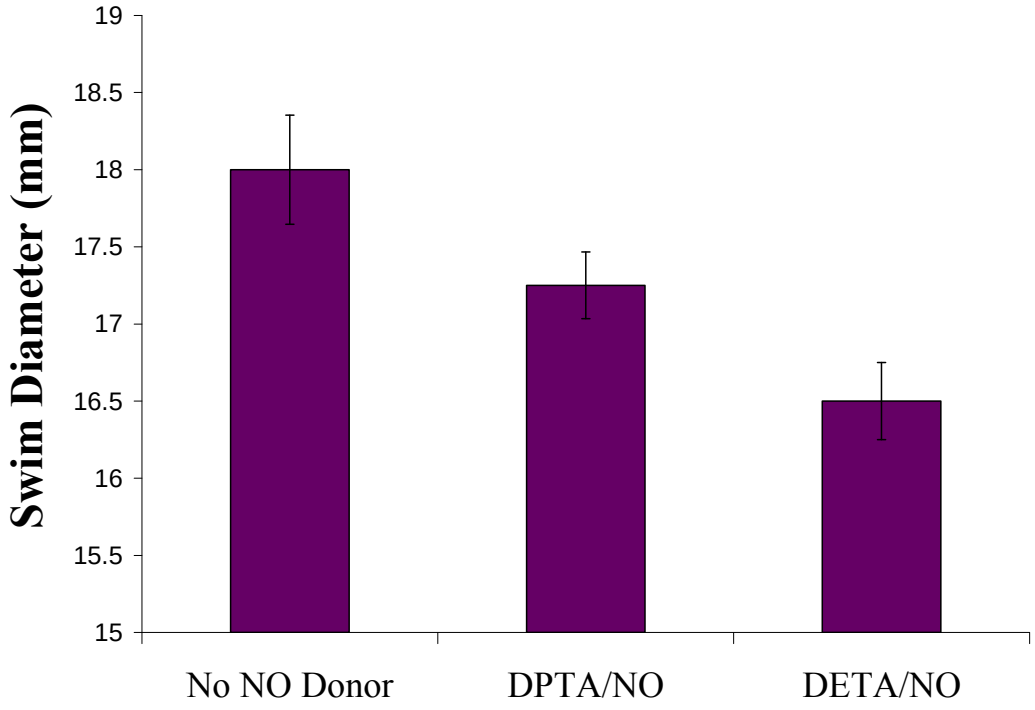


Figure 2-5 Swim diameter (mm) of untreated and treated *S. woodyi* single colonies. A consistently decreased swim rate was recorded for DETA/NO in all experiments. Results above indicate one independent trial done in quadruplicate after 24 hours at room temperature. Concentration of both NO donors in agar upon pouring of plates is approximately 600µM.

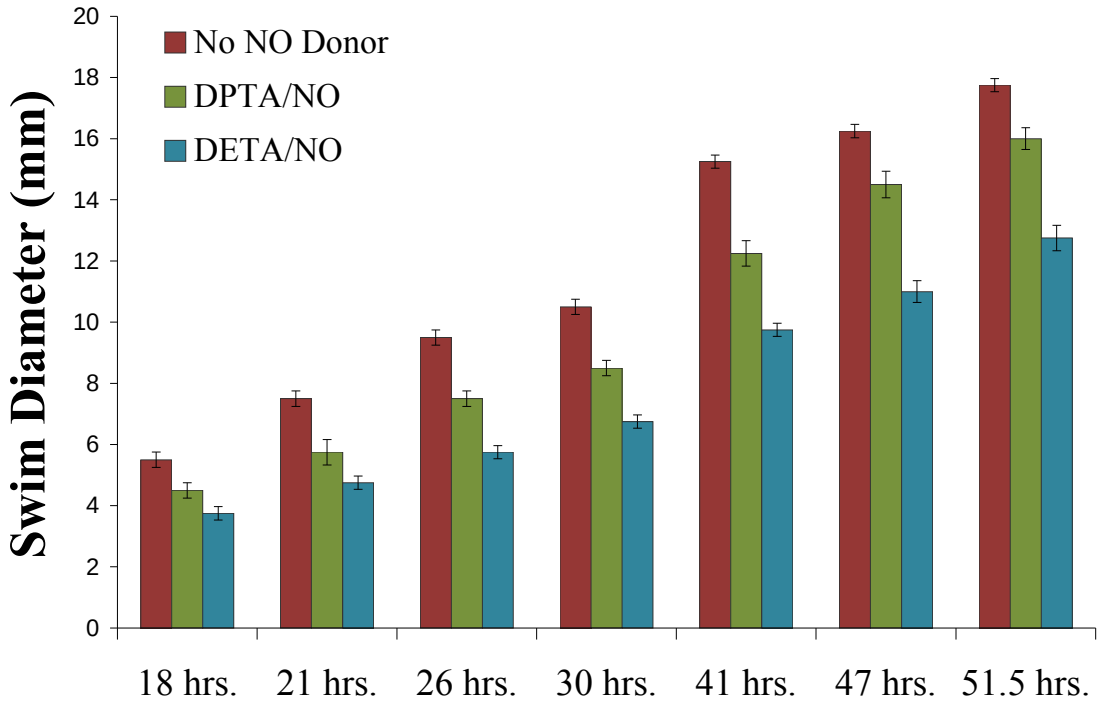


Figure 2-6 Swim motility assay comparing DPTA/NO and DETA/NO to untreated WT over time. Results above indicate one independent trial done in quadruplicate after 24 hours at room temperature. A considerable decrease was again observed in the swim diameter (mm) in the DETA/NO treated cells. The effective concentration of the swim plates when poured was approximately 600µM.

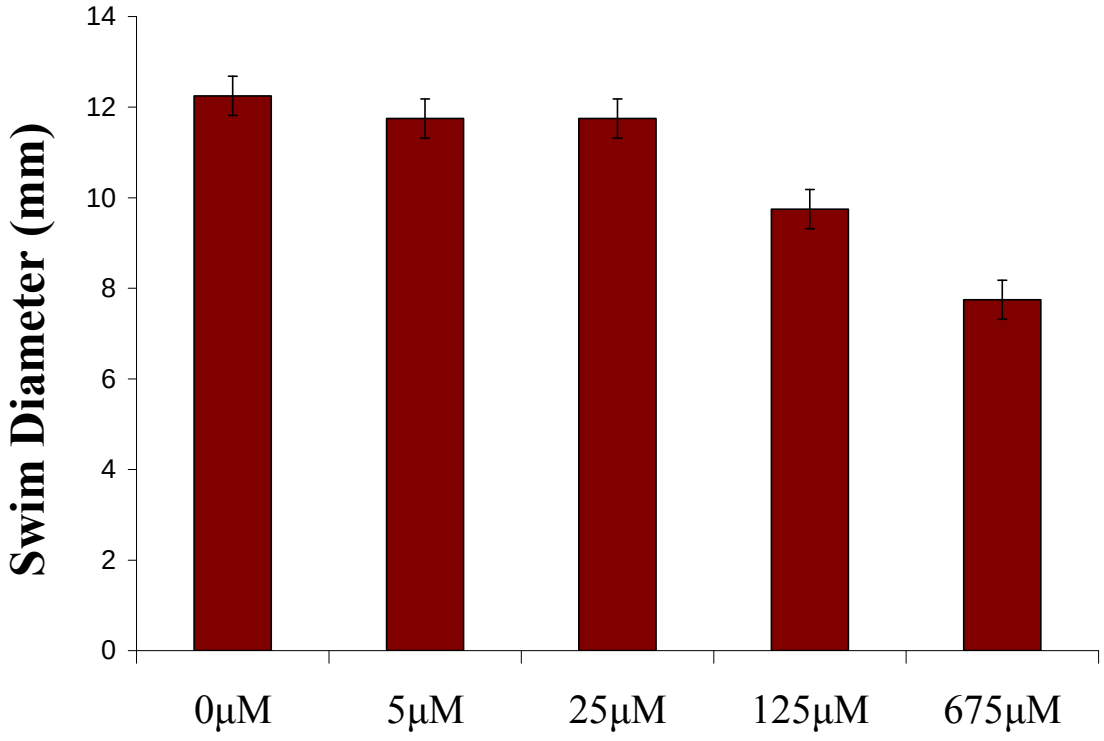


Figure 2-7 Increasing the concentration of DETA/NO effectively decreased the ability of *S. woodyi* to freely swim in the swim motility assay. The swim diameter (mm) was measured after 24 hours for all samples and the results above indicate one independent trial done in quadruplicate.

Chapter 3: Knockin progress

3.1 Summary

Two vector-based knockin approaches were employed in order to demonstrate the effect of NO ligated H-NOX on *Sw* DGC activity. To date, the vectors have been developed, and preliminary results indicate that the annotated diguanylate cyclase downstream of *Sw* H-NOX is in fact catalyzing the formation of c-di-GMP in the orthogonal *E. coli* strain. Cell motility assays developed previously for the *S. woodyi* wild type strain have been utilized in the knockin *E. coli* strains, in addition to Congo Red staining techniques, in an effort to elicit the relationship between H-NOX and DGC. Initial results are thus far inconclusive, yet the project appears to be promising in our studies toward understanding the biological role of the H-NOX domain in bacteria.

3.2 Introduction

According to our hypothesis outlined in Figure 1-7, upon nitric oxide binding to *Sw* H-NOX, the enzymatic activity of the downstream DGC is altered. Based on the cellular motility assays described in Chapter 2, an increase in intracellular c-di-GMP appears to be occurring due to the increase in biofilm formation and the corresponding decrease in planktonic cell movement. Due to the lack of an antiobiotic resistant *S. woodyi* strain, a knockout of either *Sw* H-NOX or *Sw* DGC is impossible as of this time; this inherent problem disallows us from testing a change in cell motility phenotype between mutant knockout strains and wild-type *S. woodyi*. However, a knockin-based approach has been developed in an effort to still elicit a definitive relationship between NO and biofilm formation in *S. woodyi*.

E. coli bacterial strains do not contain an H-NOX encoding gene in their genomes. If *Sw* H-NOX is subcloned after an inducible promoter in a plasmid, it could then be introduced into *E. coli* and its expression controlled by the particular inducer. However, the *E. coli* strains harboring these plasmids would not be able to respond to NO through the H-NOX domain since the purported downstream target, *Sw* DGC, is not present. If the *Sw* DGC gene is also contained on the plasmid, then both proteins would be present within the cell when expression is induced. Therefore, any deviations in

cellular motility from the wild type *E. coli* strain when NO is added to the culture media would be directly attributed to the H-NOX/DGC protein pair. This conclusion can be made due to the fact that *E. coli* has been shown to be able to respond to the universal second messenger molecule c-di-GMP. The exact stage in which the H-NOX/DGC contributes to *S. woodyi* biofilm formation could not be discerned; however, the direct interaction between the two proteins can be recreated within the orthogonal *E. coli* strain.

Two vector based systems were developed in attempts to recreate the protein-protein interaction in *E. coli*, in which the genes were subcloned into vectors containing two different inducible promoters. The pBAD/Myc-HisA vector contains the tightly controlled *ara*BAD promoter, which is supressed in the absence of L-arabinose[1]. Therefore, gene transcription can be titratably induced in the presence of increasing concentrations of L-arabinose. Furthermore, the number of plasmids contained within in any one cell is limited due to the pBR322 origin of replication. The low copy number nature of the pBAD based vector was appealing in our efforts to physiologically recreate the H-NOX/DGC interaction. If the two proteins are massively overexpressed in the *E. coli* strains, we may force a non-native interaction between H-NOX and DGC and elicit a phenotypic response to NO which is not physiologically relevant to *S. woodyi*.

The other vector constructed for the knockin studies is based on the pETDuet-1 plasmid system. This particular vector contains two multiple cloning sites downstream of an inducible promoter, which allows for the separate subcloning of the two genes of interest into the plasmid. In the pBAD vector described above, the entire operon was cloned downstream of the *ara*BAD promoter; therefore, the mRNA transcript would contain the H-NOX and DGC genes as processed by *S. woodyi.* The pETDuet-1 vector allows for the induction of both genes under IPTG. Two different mRNA constructs will be simultaneously processed by the *E. coli* cellular machinery; this will overcome concerns with the pBAD system in which the second gene in the mRNA transcript may not be processed normally in the orthogonal *E. coli* strain. Nonetheless, the IPTG promoter is not a tightly controlled as *ara*BAD, leading to the possibility of leaky expression of the two gene products. In addition, the relative copy number of pET based plasmids in higher than the pBAD plasmids; thus, at any one time, the expression of *Sw* H-NOX and DGC would be greater in the pET Duet vectors.

Since we hypothesize that H-NOX will alter DGC enzymatic activity, any changes in c-di-GMP levels within the *E. coli* knockin strains will alter cellular motility. The same cell motility assays utilized in the wild-type *S. woodyi* experiments can be theoretically applied to the knockin constructs. Since fluctuations in c-di-GMP levels have also been linked to changes in EPS secretion[2], assays utilizing Congo Red dye (CR) can also be employed. Congo Red nonselectively binds to long fibers and has been used in other bacteria to visualize changes in EPS secretion[3,4]. Hence, if CR is added to the culture media, we can quantify the relative amount of EPS secretion by the *E. coli* strains by simple electronic absorption of the CR at 500nm. The CR in the culture media will bind to the EPS secreted; after a set amount of time, the cells are centrifuged and the EPS-CR complexes are brought down with the cell pellet. As more c-di-GMP is produced within the cell, more CR will be pulled out of solution and a smaller absorbance at 500nm will be recorded. Therefore, the larger the difference in absorbance between the supernatants of the knockin and control strains would correlate directly to an increased amount of EPS and intracellular c-di-GMP. As less quantitative approach can also be employed in which CR is added to solid agar plates[3]. If the cell culture added to the CR agar plates is able to secrete EPS, then the colonies will appear red after an extended period of incubation; cells deficient in intracellular c-di-GMP will remain white.

Specific mutants were constructed for the two vector knockin systems in order to have controls for the different cell motility assays utilized (Table 3-1). Vectors containing the GGAA/EF mutation have either one or two of the key catalytic residues removed from the active site; therefore, this mutant will not be able to catalyze the formation of c-di-GMP and no phenotypic results should be observed. The AAL mutation removes the glutamate residue involved in the hydrolysis of c-di-GMP, which would remove any regulation from the phosphodiesterase domain and would theoretically have a higher intracellular c-di-GMP concentration that the wild-type. The PAS domain at the N-terminus of *Sw* DGC is hypothesized to be integral in the interaction between H-NOX and DGC; thus, the removal of this domain would allow us to determine the role of this particular domain in *S. woodyi*. The H104A mutation in *Sw* H-NOX renders the protein unable to bind the heme moiety and therefore unable to respond to nitric oxide; no significant difference should be observed in the cell motility assays when the culture

media is supplemented with a NO donor molecule. Finally, the P117A mutation in H-NOX has been shown previously to flatten the heme and cause an overall conformational change within the domain[5]; therefore, the cell motility assays can be utilized to elicit any possible biological relevance to the severely distorted heme moiety purported to be found ubiquitously in all H-NOX domains.

3.3 Materials & Methods

3.3.1 Bacteria and Media

E. coli strains XL1-Blue [*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)*], TOP10 [*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*] and BL21 (DE3) pLys [*F$^-$ ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3) pLysS(cm^R)*] were grown in 20g/L LB media at 37°C, unless otherwise noted. Solid agar plates were prepared with 20g/L LB media and 15g/L BactoAgar, with the appropriate antibiotic resistance, if necessary. CR agar plates and CR media were supplemented with 25μg/mL of the dye. XL1-Blue and BL21 cells were maintained with 20μg/mL tetracycline and 33μg/mL chloramphenicol, respectively. Vector constructs were maintained with either 100μg/mL ampicillin or 15μg/mL kanamycin.

3.3.2 Cloning and Site-Directed Mutagenesis

All primers utilized in cloning and site-directed mutagenesis have been catalogued in Appendix II; all plasmid constructs have been catalogued in Appendix III. pDGC01 was previously constructed by E.M. Boon. pSW01 and pKI01 were constructed by Y. Altshutter at the Stony Brook Molecular Cloning Service (SBMCS). For pSW01, the entire H-NOX/DGC was amplified with Herculase II polymerase from *S. woodyi* genomic DNA using the SWFor and SWRev cloning primers. The polymerase chain reaction (PCR) product was submitted to SBMCS for subcloning into the pBAD/Myc-HisA parent plasmid. For pKI01, the H-NOX and DGC genes were amplified from lab plasmids using PfuUltra AD polymerase and the primers listed in Appendix II.2.1. The H-NOX gene was first subcloned into the parent plasmid pETDuet-1 using the NdeI and

XhoI restriction enzymes and T4 DNA ligase. The DGC PCR product was then submitted with the pETDuet/H-NOX plasmid to SBMCS to obtain the pKI01 construct.

Site-specific mutations were performed by the protocols listed in Table 3-2 and Table 3-3. For pKI02, pKI03, and pKI04, the DGC genes were amplified from pDGC02, pDGC03, and pSW04, respectively, using PfuUltra AD polymerase, and these PCR products were submitted to SBMCS for cloning. All other site-specific mutations were performed in lab using the protocols highlighted in the tables. pSW knockin vectors were transformed into XL1-Blue cells for the knockin assays since the strain cannot metabolize the inducer molecule L-arabinose; pKI knockin vectors were transformed into BL21 cells for the knockin assays since the strain contains T7 RNA polymerase.

3.3.3 Congo Red Binding Assay

Overnight cultures of the *E. coli* strains were reinoculated and grown in LB at 37°C and 250rpm until an approximate OD_{600} of 0.4-0.5 was obtained. 2.5µL of cell culture was placed slightly below the agar in CR agar plates supplemented with a final concentration of 50µM to 400µM IPTG. The plates were incubated at 30°C for 24 hours, then removed and placed at room temperature for 48 hours. Cells positive for cellulose production will be red in appearance; cells negative for cellulose will remain opaque[3].

3.3.4 Congo Red Liquid Cultures

Overnight cultures of the *E. coli* strains were diluted tenfold in CR media. 1mL cultures were grown in plastic culture tubes for 1 hour at 37°C and 250 rpm, and then supplemented with a final concentration of 100µM to 400µM IPTG. Cultures were then grown for 2 hours at 30°C and 200rpm. The cell cultures were spun down at 13,000xg for 15 minutes to remove cells and bound CR[4]. 100µL of the supernatant was removed and placed in a 96-well plate. The absorbance at 500nm was recorded for each sample.

3.3.5 Cellular Motility Assays

Biofilm and swim motility assays were performed as described in Section 2.3, with the following exceptions. For biofilm assays, the *E. coli* strains were statically grown in LB media for 24 hours at 37°C. Swim motility plates were prepared with 20g/L

LB media and 0.3% BactoAgar. Single colonies were stab inoculated, and the plates were allowed to incubate at 37°C for 24 hours.

3.4 Results

Before proceeding with knockin strategies for H-NOX and the diguanylate cyclase, we first verified that the gene annotated as the DGC in fact catalyzes the formation of c-di-GMP. The CR binding assay was utilized in which two samples, containing either pDGC01 or a pET negative control vector, were spotted on plates containing concentrations of IPTG ranging from 50μM to 400μM. After 24 hours of incubation at 30°C, the cells containing the control vector were all white and opaque in appearance; however, at higher concentrations of IPTG, the *E. coli* cells harboring the diguanylate cyclase turned ragged and red (Figure 3-1). This morphology is indicative of CR binding to the EPS secreted by the cell in response to increased levels of c-di-GMP[3].

To determine whether a possible increase in biofilm formation would result from DGC expression, pDGC01 was transformed into BL21 cells and induced with various concentrations of IPTG. The preliminary results from this particular assay indicate that a small increase in biofilm formation was observed with higher concentrations of IPTG on a PVC solid surface (Figure 3-2); however, biofilm results from the AAL and GGAAF mutants were not taken at the time of the assay since they had not been developed.

Initial attempts to quantify the relative amounts of c-di-GMP produced within the knockin strains have been inconclusive. Knockin TOP10 strains containing pSW01 and its derivatives have been preliminarily tested for exopolysaccharide production through the Congo Red liquid culture assay (Figure 3-3). Gene transcription was induced with 0.2% L-arabinose. The small decrease in the absorbance at 500nm upon induction indicates that more EPS could be produced in these DGC constructs. In this study, the GGAEF mutant serves as a negative control since one of the key residues (D295A) involved in c-di-GMP formation has been removed. As shown in Figure 3-3, the decrease in absorbance at 500nm for the induced GGAEF knockin strain was within error of the uninduced strain, indicative of little or no c-di-GMP produced. However, with these preliminary results, it is hard to determine whether the vector construct is in fact producing a definite phenotypic response indicative of increased c-di-GMP. The largest

decrease was observed for the AAL knockin strain, which was expected: if the DGC is producing basal amounts of c-di-GMP in these cells, then the AAL mutation would eliminate the phosphodiesterase activity of the enzyme. This assumption is predicated on the fact that both enzymes would be active at once; since we do not understand how the enzymatic activity of the two enzymes is regulated, it is hard to speculate what may be occurring with these results alone. Results from the PAS deletion and P117A mutant knockins exhibited a small decrease in absorption when induced with L-arabinose; however, with the associated error, a definitive conclusion cannot be made at this time. The site-specific mutation H104A in H-NOX does not appear to drastically alter DGC activity in the absence of nitric oxide; further tests are required to determine the significance of this specific mutation. The CR binding assay was attempted to verify DGC activity with these vectors in TOP10 cells, but initial attempts were unsuccessful.

An initial swim motility assay with the pSW01-based knockin TOP10 strains was performed in addition to the Congo Red trials (Figure 3-4). Upon induction with L-arabinose, a decrease in the swim diameter is observed after 24 hours; however, no negative control was performed with this assay, so the results may or may not indicate a considerable difference in swim motility due to the presence of the H-NOX/DGC operon. As with the other data presented thus far, more work is needed to verify these results; however, these models appear to show a small, but significant change in phenotypic upon induction, which is promising for future studies on the effect of NO in the H-NOX/DGC *E. coli* knockin strains.

3.5 Discussion

Due to the lack of an H-NOX domain in all *E. coli* strains, it provides a promising vehicle to measure the effect of H-NOX function upon NO binding. If the diguanylate cyclase in the same predicted operon is controlled by *Sw* H-NOX, then we would theoretically be able to measure any changes in enzymatic activity through phenotypic assays. In the preliminary results obtained with the knockin strains containing either the pSW or pDGC vectors, the DGC appears to produce c-di-GMP. An increase in biofilm formation using pDGC01 indicates that an increase in c-di-GMP is produced upon induction with IPTG. Further evidence for DGC enzyme activity was observed with the

Congo Red assays, in which increased CR binding was noted when the media was supplemented with either IPTG or L-arabinose, depending on the vector system utilized in the trials. Since c-di-GMP has been shown to be involved in EPS production in different bacteria species, an increase in CR binding over the controls and induced knockin strains provides initial evidence for c-di-GMP production by *Sw* DGC.

An increase in c-di-GMP level shown by biofilm and EPS secretion should correspond to a downregulation of proteins involved in planktonic motility. The swim motility assay would therefore provide further evidence for the knockin strains to respond to the inducer. Preliminary results from the swim plates using TOP10 and the pSW01 based vectors suggest that an increase in c-di-GMP is produced upon induction with L-arabinose; since c-di-GMP is one of the molecules involved in the transition from single, freely swimming planktonic cells to biofilm macrocolonies, a decrease in swimming was expected and observed when gene expression was initiated. These results, combined with the biofilm and EPS results, imply that c-di-GMP levels are increasing in response to induction with the specific inducer molecule; however, further tests must be done with the different vectors to verify these results. No definitive results concerning the P117A mutation or PAS deletion knockin strains could be discerned with the Congo Red liquid assay; further studies utilizing these particular mutants would provide evidence for the biological roles of the heme distortion and PAS domains, respectively, in the H-NOX/DGC operon. Additionally, the pKI based vectors have not been tested to date and would provide additional information on these mutants and on DGC activity in the presence of nitric oxide.

It was first important to determine the appropriate assay conditions for the *E. coli* strains used before the addition of a NO donor molecule. Nevertheless, toxicity problems should be investigated to determine the appropriate NONOate concentrations to utilize for the assays. Once the enzymatic activity and NO donor concentration is determined, the actual experiments detailing the biological role of H-NOX in DGC function can be tested. The results from the *S. woodyi* experiments indicate that in the knockin strains, nitric oxide would increase biofilm formation and EPS production over the baseline values recorded thus far in the absence of NO. If this phenotypic response is not observed, overall protein expression should be verified to ensure that the lack of response

is due to the vector system, not a lack in expressed H-NOX or DGC in the cell. The pKI vector system was developed in an effort to eliminate a problem with protein expression, and as stated before, knockin strains containing this vector system should be investigated further. Other *E. coli* laboratory strains may produce better results than the strains found in the lab, so changing strains may provide another potential avenue to scrutinize if the orthogonal vector system does not work as well in TOP10 or BL21 cells. Finally, affinity labels or antibody epitopes contained within the open reading frames for these proteins may inhibit protein function or protein-protein interactions; further site-directed mutagenesis might be required for the proper interactions to occur within the constructed *E. coli* knockin strains.

If the knockin strains produce a considerable difference in phenotype upon NO supplementation, then the first steps in a definitive biochemical pathway for nitric oxide signaling would be determined. Further genetic studies within *S. woodyi* would be able to determine the possible downstream targets for the c-di-GMP produced by the DGC characterized in our experiments. Elucidating how biofilm formation and maturation occurs, especially in the response to small molecules, is increasingly important in drug discovery and understanding the diseased states of many bacteria species. How the H-NOX domain fits into the overall picture remains to be seen, but the results thus far are promising for this relatively uncharacterized protein family in bacteria.

3.6 References

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3. Goymer, P. *et al.* Adaptive divergence in experimental populations of Pseudomonas fluorescens. II. The role of the GGDEF regulator WspR in evolution and development of the wrinkly spreader phenotype. *Genetics*, genetics.106.055863 (2006).
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3.7 Tables and Figures

Specific Mutation	Domain	Mutation Result
GGAAF	diguanylate cyclase (GGDEF)	active site cannot catalyze formation of c-di-GMP
AAL	phosphodiesterase (EAL)	disrupts the hydrolysis of intracellular c-di-GMP
PAS Deletion	PAS domain	purported to disrupt interaction between H-NOX and DGC
H104A	H-NOX	domain unable to bind heme
P117A	H-NOX	purported to cause a conformational change within the H-NOX domain

Figure 3-1 Specific mutations performed by site-directed mutagenesis.

Reaction Mixture Component	Amount
PfuUltra AD polymerase	1µL
PfuUltra AD buffer	5µL
dNTPs (100mM)	1µL
Forward **or** reverse primer (10µM)	1µL
Plasmid (50-150ng/µL)	1µL
mqH_2O	41µL
Total Volume	**50µL**

Table 3-2 Site-directed mutagenesis reaction mixture components. Two separate PCR tubes were set up for each specific mutant desired, each one only containing either the forward or reverse primer. After the first cycle, the two reaction mixtures were mixed together to obtain a 100µL mixture. The second cycle was then commenced to complete the protein mutant.

Step	First Cycle (50µL)	Second Cycle (100µL)
1	95°C for 2 minutes	95°C for 2 minutes
2	95°C for 30 seconds	95°C for 30 seconds
3	50°C for 1 minute	50°C for 1 minute
4	68°C for 10 minutes[§]	68°C for 10 minutes[§]
5	Repeat Steps 2-4 *three* times	Repeat Steps 2-4 *eighteen* times
6	68°C for 10 minutes	68°C for 10 minutes

Table 3-3 Cycling parameters for site-directed mutagenesis. The first cycle contains only one of the primers needed for the mutagenesis reaction. The second cycle is began when the two reaction mixtures from the first cycle are mixed together, giving the 100µL reaction mixture; therefore, both the forward and reverse primers are present at this point so that the site-directed mutagenesis may occur on both strands of the plasmid.

§ Note: When deletion mutations were performed, the extension time was increased from 10 minutes to 15 minutes.

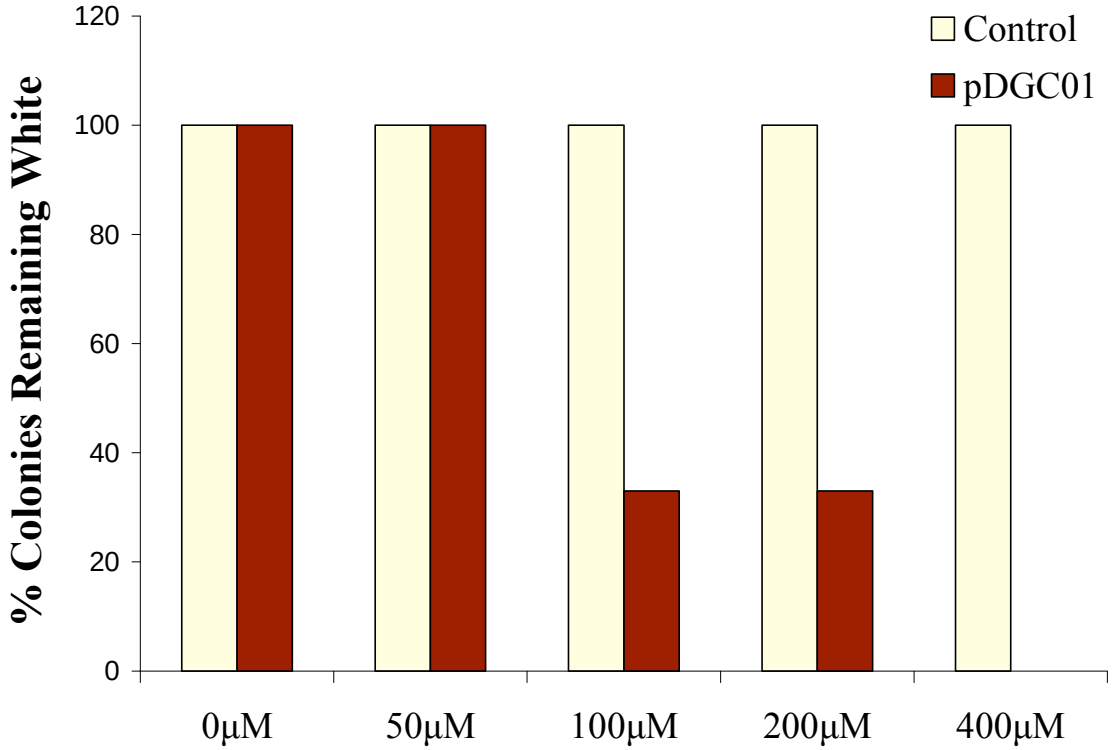

Figure 3-1 Percentage of colonies which remained white after 24 hours of incubation at 30°C and then 48 hours at room temperature. As the IPTG concentration is increased, the percentage of colonies which turned from white to red increased for *E. coli* strain containing the pDGC01 vector. The negative control lacking a transformed vector remained white as the amount of IPTG was increased. Results after one initial trial.

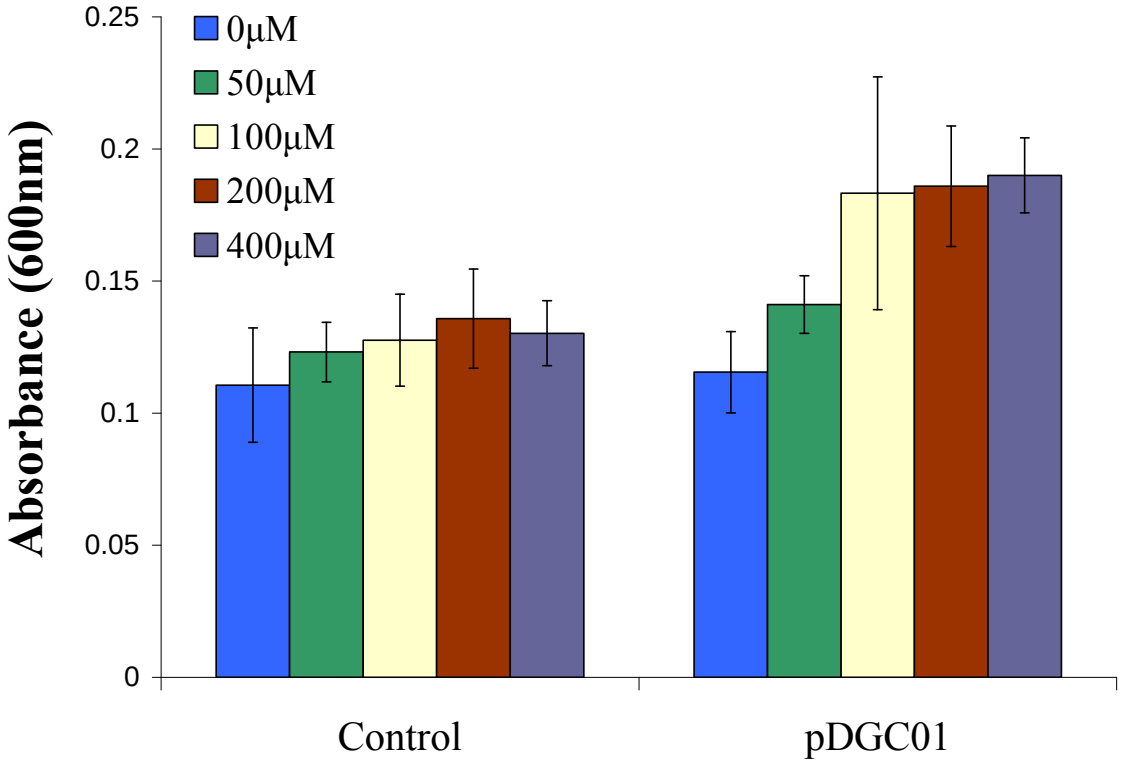

Figure 3-2 Initial biofilm results from BL21 *E. coli* knockin strains. When the IPTG concentration is increased, an increase in crystal violet absorption is observed above the pET-28b negative control. An increase in crystal violet staining indicates an increase in biofilm formation in these knockin strains, which corresponds to an increase in c-di-GMP production due to the *Sw* DGC. Further trials are needed with the other knockin strains, including the GGAAF negative control. Results after one initial trial.

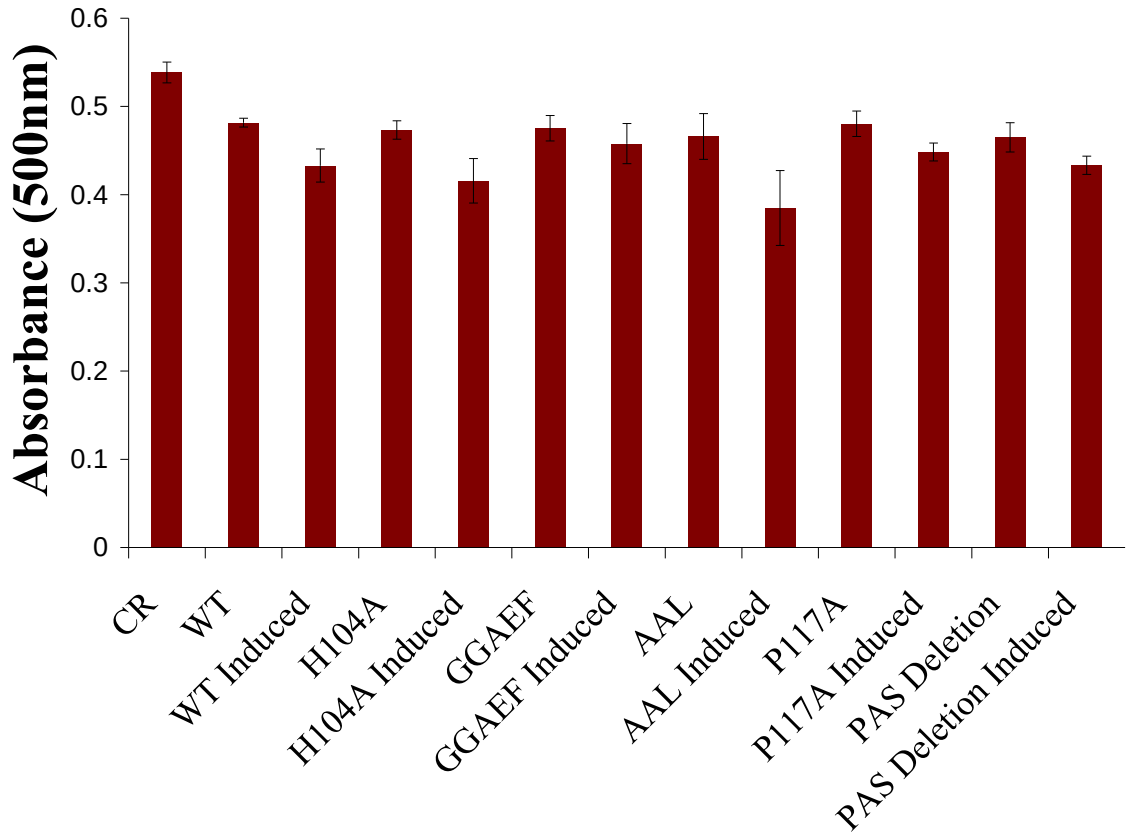

Figure 3-3 A decrease in absorption indicates more CR binding to the EPS in liquid culture, which can be correlated to an increase in intracellular c-di-GMP. Congo Red liquid assay results suggest a c-di-GMP increase upon induction with 0.4% L-arabinose in pSW01 (wild type), H104A, and AAL TOP10 *E. coli* knockin strains. The negative control GGAEF showed no significant difference in absorption upon induction. A small decrease was observed with the P117A and PAS deletion mutant knockin strains, but with the preliminary results, no trend can be put forth. Results of one trial.

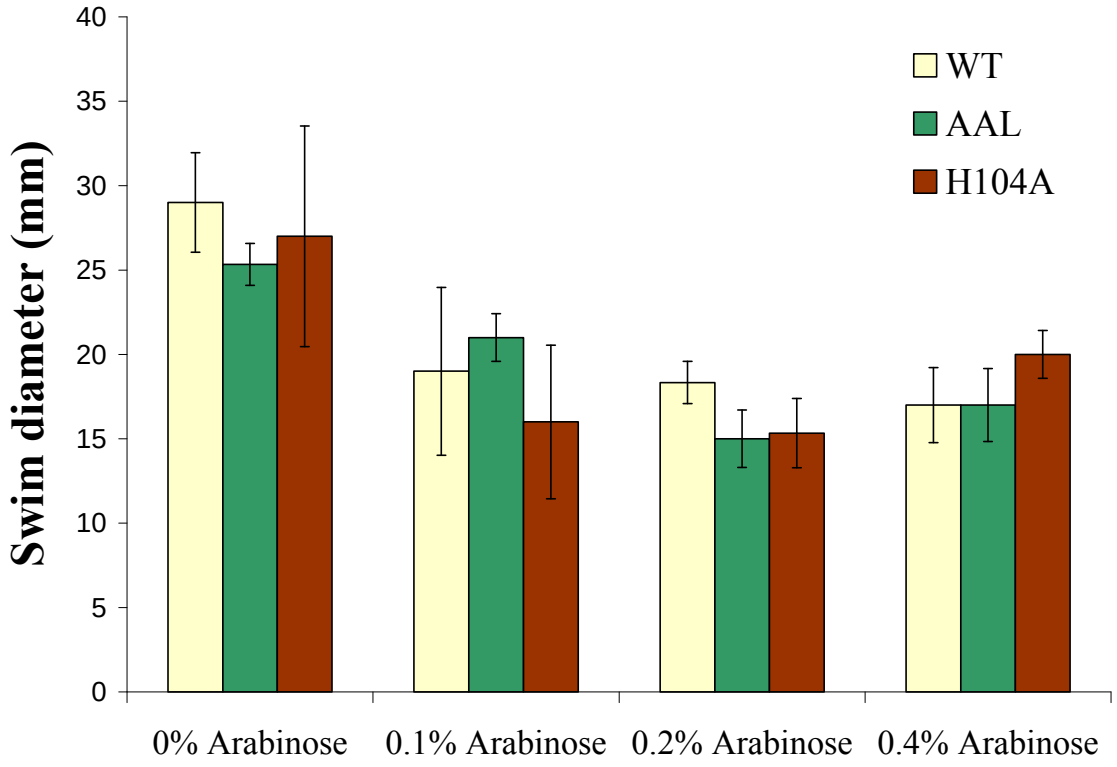

Figure 3-4 Preliminary swim plate results indicate a decrease in swim motility in TOP10 *E. coli* knock in strains upon induction with L-arabinose. A decrease in swim diameter is indicative of an intracellular c-di-GMP increase. However, further studies are needed with the GGAEF knockin strains to ensure that the decrease in swim motility is not a consequence of the pBAD vector system. Results after one initial trial.

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Appendix I: Gene Sequences

I.1: *Shewanella woodyi*

I.1.1: *Sw* H-NOX (gi:6117059)

atgatgggcatggttttcacagggttaatggagttgattgaagatgagttcggatatgaaaccttagatactttacttgag
tcatgtgagttacaaagtgaagggatatatacatctgttggcagttatgatcaccaagagttacttcagttagtggttaag
ctcagtgaggtttcatcggtacctgtgacagagcttgttaggttatttggcaaaaaactgtttgtggagttgattgagggg
caccctgaaattgctaatgagatgaaagactctttcgatctgctttctaaaatagacagctttattcatgtggaggtttata
agctttatcctcaagccgaattacccaaatttacctgtgatcgtttaggcgataatgacattagactgcattatcaatctaa
aaggccatttgcatcgtttgctgaaggcttgctcgatggctgtgcagagtattttaaagaggactttactattagccgga
caccggaaactcaagattccgagaccgacgttatttttaatattacacgagcgcctcgttag

I.1.2: *Sw* DGC (gi:6117058)

atgagtgcacttgaggacagagaacaagtttttctgcgaagaatagacagagagaagcgtgctcggcagcaagcgg
agcagatccttaatgaaaaaagtaaggagttatggaacaccaatcagaaattacagcagatcaatgagcaacttgatc
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ctaaggagaggtttcagttggccatgcgggcgaccagtgctggcgtctgggaaaggaatattgacgaggatgcttg
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gctgaccagtgaagggcgctataaatggttttggattgtcggccaagcggtatggaacgacaagggcgaagtagttc
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ctgactatggtgccgaatcgtgttttatacaatcaaaagctggatattgcactctctcttgccgataaagaacagagcca
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tgatggttaatgccgatctcgctatgtataaagctaaggggggagaaacacatgggcagtggctttcagttttgtgagca
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taaaggggtactgcttcctgaaaagttcatttcgatagctgaggatagtgggttaattactcggttaggcaagctctctat
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gagatcactgaagtggtatttttactcaatatggaggtggcgaaaaaaacggtgattgagctccatgatatggggatca
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I.2: *Pseudoalteromonas atlantica*

I.2.1: *Pa* H-NOX (gi:4172981)

atgaaaggcattatttttgtaaagttgaatcagtttgttgatgaattatggggcgatgagttttgggacgaattgttgcaag
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ttgaacaaccagaatgtgagcatgatggcgacgcacgatgtgtgataaaggttaccaaagttggctaa

I.2.2: *Pa* HK (gi:4172982)

ttggctaaatcgaacgaggaattagaggcaaggatcgccgttttagagcgtaaagtcgagcgagaaaagaaagccc
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acgtgcatacacaagtgcaatctactgtaggtaaaggaactcaattctctcttagttttcctctacctcagtag

Appendix II: Primer Sequences

II.1: pKI cloning primers

DGC: 5'-catgccatggatgagtgcacttgaggac-3'
DGC-R: 5'-aaggaaaaaagcggccgcctatttagcgggtaaagagatac-3'
HNOX: 5'-ggaattccatatgatgggcatggttttcacagggttaatggag-3'
HNOX-R: 5'-ccgctcgagacgaggcgctcgtgtaatattaaaaataacgtcggtc-3'

II.2: pSW cloning and mutagenesis primers

SWFor: 5'-caagccatggatgatgggcatggttttcacagg-3'
SWRev: 5'-catgctcgagttatttagcgggtaaagagatacagcgc-3'
InFrame: 5'-taacaggaggaattaaccatgatgggcatggttttcacaggg-3'
InFrame-R: 5'-ccctgtgaaaaccatgcccatcatggttaattcctcctgtta-3'
StopArg: 5'-ctgtatctctttacccgctaaacgactcgagatctgcagct-3'
StopArg-R: 5'-agctgcagatctcgagtcgtttagcgggtaaagagatacag-3'

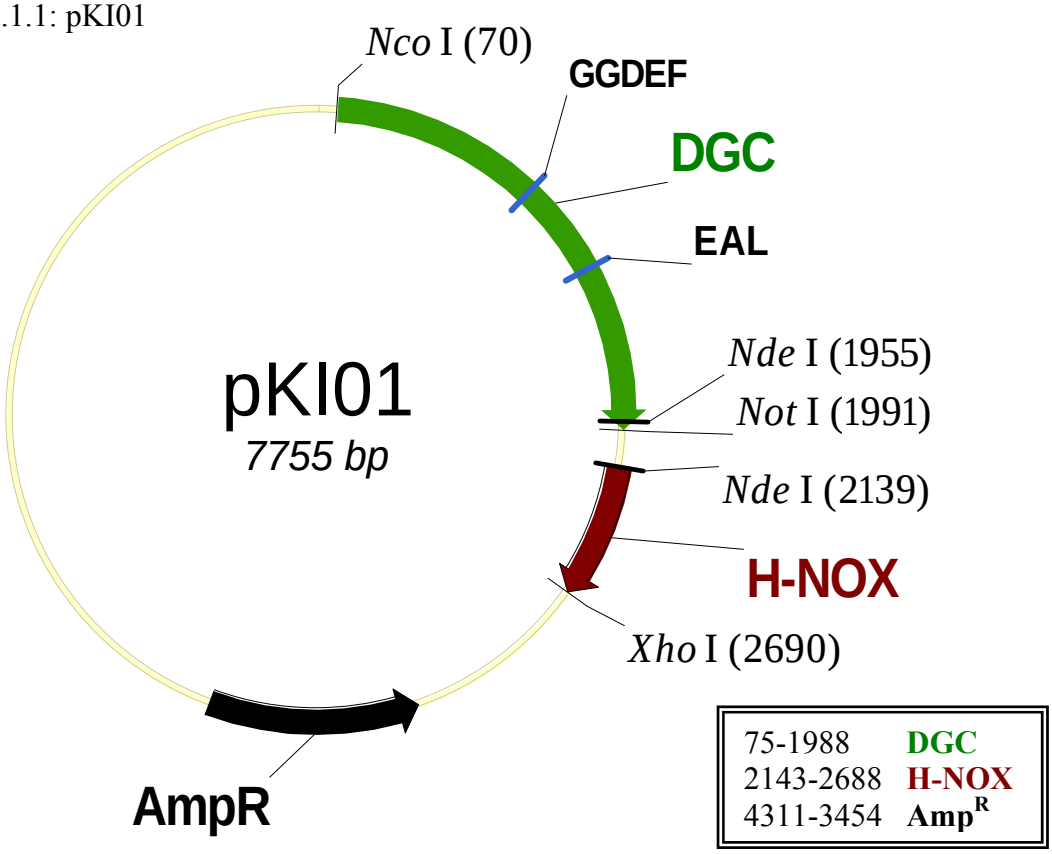
II.3: Conserved mutagenesis primers

AAL: 5'-ccaacggatcaaggctgcagcaaaggtcacttc-3'
AAL-R: 5'-gaagtgacctttgctgcagccttgatccgttgg-3'
GGAAF: 5'-gttaacacaatagagaacgcagctccccccaacctggcaa-3'
GGAAF-R: 5'-ttgccaggttgggggggagctgcgttctctattgtgttaac-3'
GGAEF: 5'-gttaacacaatagagaactcagctccccccaacctggcaa-3'
GGAEF-R: 5'-ttgccaggttgggggggagctgagttctctattgtgttaac-3'
H104A: 5'-aggataaagcttataaacctccacagcaataaagctgtctattttagaaagc-3'
H104A-R: 5'-gctttctaaaatagacagctttattgctgtggaggtttataagctttatcct-3'
P117A: 5'-cgatcacaggtaaatttggctaattcggcttgaggata-3'
P117A-R :5'-tatc ctcaagccgaattagccaaatttacctgtgatcg-3'
PAS: 5'-aatgagccatcttctcaaccaacttggagttctcaaccgcccgcatggccaactgaaacctctccttag-3'
PAS-R: 5'-ctaaggagaggtttcagttggccatgcgggcggttgagaactccaagttggttgagaagatggctcatt-3'

Appendix III: Vectors & Vector Maps

III.1: pKI vectors

III.1.1: pKI01



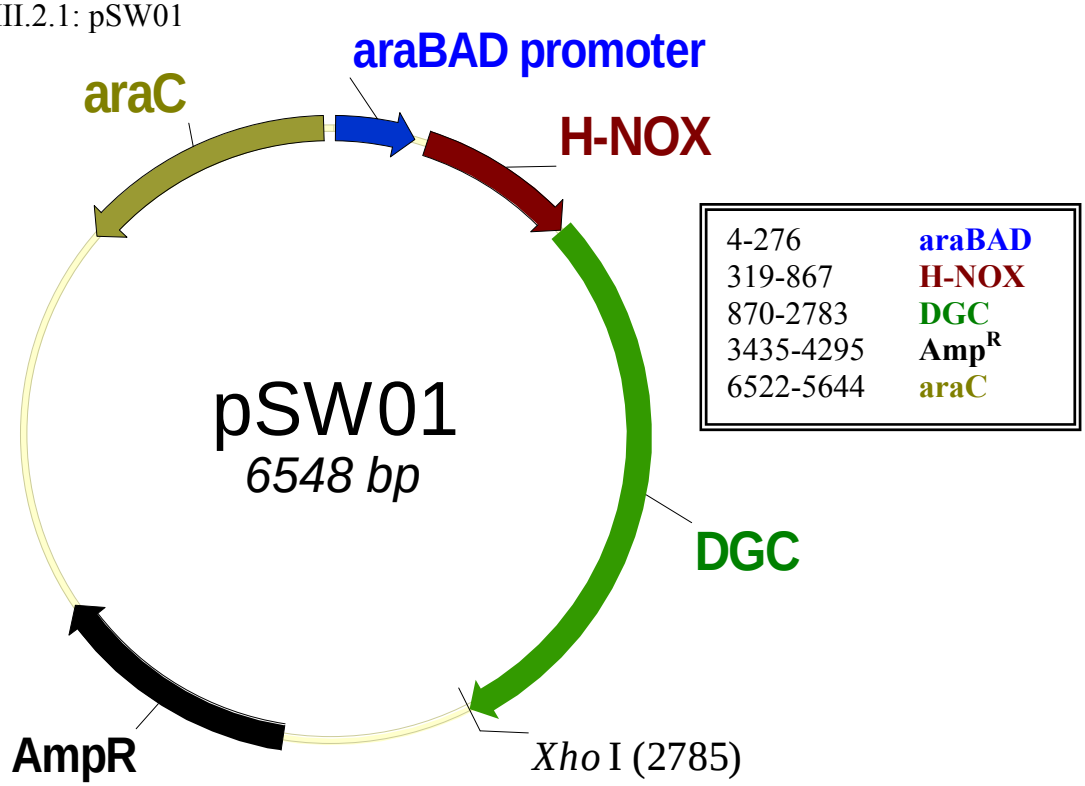
Parent plasmid: pETDuet-1

III.1.2: pK01 Derivatives

Name	Mutagenesis	Primers Used
pKI02	AAL: E415A (DGC)	AAL/AAL-R
pKI03	GGAAF: D295A,E296A (DGC)	GGAAF/GGAAF-R
pKI04	PAS Deletion, Residues 89-195 (DGC)	PAS/PAS-R

III.2: pSW vectors

III.2.1: pSW01



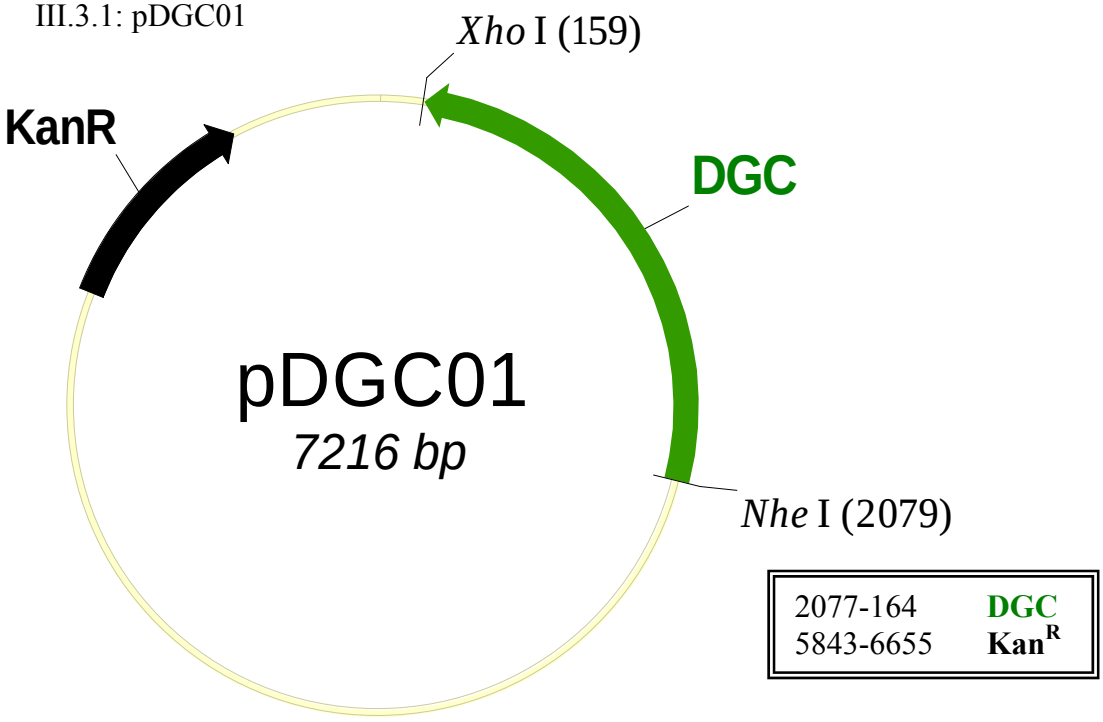
Parent plasmid: pBAD/Myc-HisA

III.2.2: pSW01 Derivatives

Name	Mutagenesis	Primers Used
pSW02	AAL: E415A (DGC)	AAL/AAL-R
pSW03	GGAEF: D295A	GGAEF/GGAEF-R
pSW04	PAS Deletion, Residues 89-195 (DGC)	PAS/PAS-R
pSW05	H104A (H-NOX)	H104A/H104A-R
pSW06	P117A (H-NOX)	P117A/P117A-R

III.3: pDGC vectors

III.3.1: pDGC01



Parent plasmid: pET-28b

III.3.2: pDGC01 Derivatives

Name	Mutagenesis	Primers Used
pDGC02	AAL: E415A (DGC)	AAL/AAL-R
pDGC03	GGAAF: D295A,E296A (DGC)	GGAAF/GGAAF-R

Appendix IV: *Pseudoalteromonas atlantica* as an H-NOX model

IV.1 Summary

The primary biofilmer *Pseudoalteromonas atlantica* contains the conserved H-NOX domain directly upstream of an uncharacterized histidine kinase[1]. Due to the ability of *Pa* H-NOX to bind nitric oxide, the H-NOX domain is purported to serve as a nitric oxide sensor in *P. atlantica* and numerous other bacteria[2, 3]. Due to the unknown nature of the downstream histidine kinase, we first attempted to establish a change in phenotype, namely cellular motility, in response to increasing concentrations of NO. However, the affect of nitric oxide on cellular motility in *P. atlantica* was inconclusive based on the evidence obtained thus far. Biofilms were attempted on glass and PVC solid surfaces with varying results, and swim and twitch motility assays were performed with limited success. Further work ought to be performed in conjunction with a genetic knockout of either the H-NOX or HK domain in an attempt to elicit the cellular function of H-NOX in bacteria. Finally, initial attempts to determine whether *P. atlantica* could produce NO endogenously were inconclusive with the engineered protein *Tt* H-NOX Y140F[4]; more sensitive methods of detecting NO are needed to elicit the probable source of nitric oxide which binds to the H-NOX domain. Even though the initial results were not as promising as expected, the cellular motility assays that were used later for *S. woodyi* were worked out while using *P. atlantica*, so the work below was essential for the results highlighted previously in this thesis.

IV.2 Introduction

Pseudoalteromonas atlantica is a marine γ-proteobacterium which contains the conserved H-NOX domain (gi: 4172981) and adjacent histidine kinase (gi: 4172982). Histidine kinases (HK) are known to be involved in a variety of cell signaling mechanisms, most notably coupled with response regulators (RR) in phosphorelay signal transduction[5]. The eventual cellular effect of the HKs, predominantly activation or repression of gene transcription, is a fundamental reason why these sensor kinases have been linked to detecting changes in the external environment[6]. If the HK found directly downstream from the H-NOX in *P. atlantica* or other facultative aerobes affects gene

transcription (Figure IV-1), then the downstream effects could be monitored as well. Unfortunately, the particular downstream targets of these HKs are not known in these particular facultative aerobes. Nonetheless, the H-NOX domain for *P. atlantica* has been previously cloned, purified, and characterized; the protein has been shown to selectively bind NO in the presence of an oxic environment[2]. These results indicate that *Pa* H-NOX can serve as selective sensor of nitric oxide at low concentrations.

P. atlantica is one of the first bacteria present in complex multi-species biofilms and are therefore referred to as a primary biofilmer[1]. In order to sequester the additional species to the biofilm, *P. atlantica* secretes extracellular polysaccharides (EPS)[7], characteristic of the mucoid morphology of the species[8]. *P. atlantica* can also exist as translucent or crenated morphologies, but these cell types are either partially or completely deficient in EPS production, respectively. The regulation between mucoid and crenated is controlled by a controlled phase variation in which a transposable element in inserted into *epsG*, rendering the cell incapable of production of EPS[9,10]. The exact mechanism of this variation is under investigation, but the transposition has a reported effect on biofilm development[11].

HKs and RRs have been shown to be integral in the early stages of biofilm development[12,13]; transcription of many important biofilm genes is altered when the specific HK sensors detect its corresponding signal molecule. Many RR are in fact transcription factors which directly effect which regions of the genome will be promoted for transcription. Numerous biochemical pathways are known to influence different stages of biofilm development, including diguanylate cyclases as highlighted earlier[14]. Due to the proximity of H-NOX to methyl-accepting chemotaxis proteins and diguanylate cyclases in other bacteria[15], we hypothesize that the H-NOX domains upstream of HKs serve as universal NO sensors that either (a) promote changes in the transcription of cellular motility genes or (b) change the phosphorylation state of key proteins involved in cellular motility. In either case, we predict in response to NO, a shift in biofilm phenotype would be observed. To test our hypothesis, numerous cellular motility assays were performed to determine if a phenotypic change was observed in response to nitric oxide.

IV.3 Materials and Methods

IV.3.1 Bacteria and media

P. atlantica strains T6c and DB27 (*hsd-1*, Rif^r) were grown at either room temperature or 25°C in crenated cell solution. The crenated cell solution (CCS) is comprised of 28g/L MB and supplemented with $CaCl_2$ to a final concentration of 10mM Ca^{2+}. Minimal growth media contained 2.3% (w/v) sea salts (Instant Ocean, Aquarium Systems), 0.05% NH_4Cl (w/v), 50mM Tris-HCl[16], and varying concentrations of yeast extract (YE), including 3M-YE (0% YE), 3M/01 (0.01% YE), 3M/05 (0.05%YE), and 3M (0.1% YE). The crenated cell morphology (C) is obtained as previously described[839], in which two serial dilutions taken from the air-broth interface were reinoculated in 10mL of CCS in 125mL Erlenmeyer flasks for 1 day at 25°C and 200rpm. The mucoid cell morphology (M) is obtained by using larger cell cultures in CCS without serial dilutions. Cell morphology was verified by plating serial dilutions on MB plates, and DB27 cultures were maintained on 100μg/mL rifampicin.

IV.3.2 Biofilm Formation

Biofilms were grown on 17mL Hungate glass anaerobic tubes for three to six days, either statically at room temperature or under gentle aeration at 25°C and 40rpm. Controls were maintained at room temperature and 250rpm to confirm growth when aerated biofilms were grown. Overnight cultures were grown in CCS at 25°C and 200rpm, and a 1mL cell culture with an optical density (OD) of approximately 0.20 at 600nm was obtained through appropriate dilutions to start each assay.

Biofilm quantification utilized either 0.1% crystal violet or colony formation units, as modified from O'Toole[17,18]. At the end of biofilm formation, the supernatant was removed and OD_{600} measured. The tubes were subsequently washed with 1mL of fresh CCS, again recording the OD_{600} of the solution. 2mL of 0.1% crystal violet solution was then added, and the tubes were allowed to incubate at room temperature for 45 minutes. After crystal violet staining, the tubes were rinsed vigorously with diH_2O, with moderate vortexing to remove any loosely adhered cells. 2mL of DMSO was subsequently added to each tube to solubilize the CV. After incubation at room temperature for 20 minutes in DMSO, the absorbance of the DMSO/CV solution was taken at 570nm.

Colony formation units were analyzed by growing the biofilm as described above, removing the supernatant, and rinsing the tubes with diH_2O repeatedly with moderate vortexing to remove planktonic and loosely attached cells. 2mL of fresh CCS was added to each tube after the rinse, vortexed for 1 minute, and 100µL of various dilutions were plated on CCS plates. After incubation at room temperature for 48 hours, the number of colonies was counted.

Biofilms in PVC microtiter plates were performed under the same conditions as *S. woodyi*, with the exception of duration of static growth and culture media. Biofilms were first grown in CCS media for three to six days for both *P. atlantica* strains; subsequent trials were performed after either three to four day periods to eliminate contamination.

IV.3.3 Cell Growth

The growth rates of *P. atlantica* in CCS or minimal media were determined in either duplicate or triplicate using 5mL of culture in 17mL Hungate anaerobic tubes. Aerobic growth was measured using test tube plugs to close the tube opening, whereas anaerobic growth was characterized using the supplied anaerobic top assembly. Flushing with argon was not completed for anaerobic growth. Sodium nitrate, sodium nitrite, and sodium thiosulfate were added in various concentrations to serve as terminal electron acceptors. DETA/NO was also utilized at various concentrations to serve as a NO donor to the cell culture[19]. Growth was measured over time using cell density readings on the Ultrospec 10 Cell Density Meter (Biochrom).

IV.3.4 Swimming and Twitching Motility

The various methods of determining whether *P. atlantica* was able to swim or twitch are listed in Table IV-1[16,18,20,21]. All swim and twitch plates were poured in 100x15mm Petri dish; twitch plates were poured to a height of 3mm, whereas approximately 20mL agar solution was added to produce the swim plates. Two methods of inoculating the cells to the agar medium were employed: liquid or single colony. For liquid inoculation, a few microliters of cell culture in the exponential phase were placed slightly below the agar layer for the swim plates only; for single colony inoculation, a single colony was introduced to the plate by either a sterile pipette or a sterile toothpick.

The placement in the agar depended on the motility test being employed. If swim plates were being studied, then the single colony was placed just below the agar layer, whereas for twitch plates, the stab was placed at the bottom of the plate, in the interface between the semisolid agar and the plate bottom. Crystal violet quantification on twitch plates was attempted by removing the solid agar with a spatula, pouring 0.1% crystal violet into the dish, and allowing it to incubate at room temperature for 15 minutes. The crystal violet was removed and rinsed thoroughly with diH_2O to determine if cells had adhered to the plate.

IV.3.5 Y140F Purification

The pET-20b vector containing *Tt* H-NOX Y140F was transformed into DH5α component cells and grown overnight in 50mL LB media at 37°C and 250rpm. Overnight culture was prepared from single colony in 50mL LB media, shaking at 37°C and 250rpm. 15mL of the overnight culture was placed in 1L fluted flask with 45g yeast extract and a final concentration of 10mM sodium phosphate. Three such protein expression systems were prepared under ampicillin and chlorampenicol selection, and the cultures were shaken at 250rpm and 37°C until the OD_{600} reached approximately 0.40. The temperature was reduced to room temperature, and after shaking for an additional 30 minutes, protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration 10μM. After incubation for 18 hours at room temperature, the cultures were removed from the incubator and spun down at 5500rpm for 18 minutes. The cell pellet was removed and resuspended in 30mL loading buffer (50mM NaH_2PO_4 and 300mM NaCl). The slurry was sonicated for 10 minutes to lyse the cells until a homogenous mixture was observed. The sample was spun down for 15 minutes at 10000xg and 4°C. The supernatant was then removed and heat shocked at 65°C for 20 minutes. The resulting solution was spun down for 45 minutes at 10000xg and 4°C. After centrifugation, the supernatant was loaded onto a HisTrap column (GE Healthcare) and washed with 15mL and 7mL loading buffer supplemented with 20mM and 40mM imidazole, respectively. The elute the *Tt* H-NOX Y140F protein, loading buffer with 250mM imidazole was applied to the column. The eluted protein was desalted over a salt

column with loading buffer plus 5% glycerol and 5mM dithiothreitol (DTT). 100µL aliquots were stored at -80°C for later usage.

IV.3.6 Y140F Assay

P. atlantica T6C and DB27 strains were grown overnight in CCS at room temperature and 200rpm[4]. The culture was diluted with fresh CCS until a final OD_{600} of approximately 0.40 was obtained in the 17mL Hungate anaerobic tubes. The anaerobic tubes were capped and incubated at 25°C and 200rpm. After 2.5 and 5 hours, the optical densities were taken to ensure continual growth, and 2.5mL of headspace was removed to be injected in anaerobic 2mL ReactiVials (Pierce). After the headspace was removed at the 5 hour time point, *Tt* H-NOX Y140F aliquots were removed from the -80°C freezer and dethawed at 4°C. The protein was desalted to remove residual DTT in 50mM NaCl and 50mM NaH_2PO_4. The desalted protein was diluted with the buffer listed above until a final volume of approximately 10mL was obtained; the final volume of the protein depended on the number of samples analyzed, but the Soret peak absorbance was targeted to be between 0.20 and 0.30 after dilution. 500µL of protein was injected into the ReactiVials using a sterile 1mL syringe, and the ReactiVials were placed at 4°C for 20minutes. One ReactiVial contained solely the Y140F protein without injected headspace to serve as maximum expected Soret absorbance. The baseline was established on the Cary UV/Vis (Varian) with 350µL of 50mM NaCl and 50mM NaH_2PO_4, and the absorbance spectrum of 350µL of Y140F protein samples was taken.

IV.4 Results

To first determine the source of NO, the *Tt* H-NOX Y140F protein was utilized to determine if the nitric oxide is being produced endogenously through denitrification. Since the integral tyrosine residue responsible for O_2 binding has been mutated to a phenylalanine, oxygen can no longer serve as a ligand for the Y140F protein; the engineered protein can therefore be used to quantify the amount of nitric oxide in a solution through changes in Soret band absorption at 424nm[4]. Numerous attempts were made to utilize this protein to detect NO; baseline issues and protein handling were troubleshot in an effort to optimize the assay. For time points of 2.5 hours and 5 hours,

NO concentrations of 120(±248) pM and 303(±422) pM, respectively, were detected with the Y140F assay in the best trial (Figure IV-2). The error associated with these values makes this unreliable for determining the amount of NO produced in *P. atlantica* to this date. In addition, the detection limit of the assay is reported to be between 300nm and 30μM. The picomolar NO concentrations obtained from these trials are clearly outside of the assay limits and are probably associated with error in the assay itself. Initial attempts to utilize a NO electrode to measure NO concentration in anaerobic *P. atlantica* cell cultures supplemented with nitrate and nitrite were similarly inconclusive.

Since the nitric oxide was assumed to be coming endogenously through denitrification, we had to concurrently determine the characteristic growth rates of *P. atlantica*. If NO is derived from the denitrification pathway, then the *P. atlantica* cultures must be able to grow anaerobically in the presence of either nitrate or nitrite, the compounds which are reduced in denitrification to form nitric oxide. To that end, a relevant minimal growth media is necessarily required in order to control the terminal electron acceptors present during cell growth studies. Therefore, the growth of *P. atlantica* aerobically and anaerobically was determined in rich and minimal media with different nitrogenous terminal electron acceptors. Under anaerobic conditions, both T6c and DB27 are able to grow to similar cell densities as aerobic cultures when supplemented with nitrate in rich media (Figure IV-3). However, when the yeast extract was removed from the sea salt minimal media, the cultures were unable to grow as dense, which indicates that the minimal media utilized thus far is lacking key nutrients for the bacteria to thrive (Figure IV-4).

The effect of nitric oxide on cell motility is ultimately in question, but we want to study these changes under conditions that will not drastically alter normal cellular activity. If the intracellular physiology of the organism was severely affected, then a noticeable change in cell growth would be noted. Therefore, in order to determine if nitric oxide had a negative effect on normal cell function, the nitric oxide donor molecule DETA/NO was added in various concentrations to culture media. In DETA/NO concentrations less or equal to 1mM, cell growth was unaffected; however, a decrease in growth was observed at 5mM (Figure IV-5). The decrease in cell growth might be attributed to the relatively high concentration of nitric oxide present, which would lead to

cell death after the concentration reached a level to which the cells cannot survive. Since concentrations of DETA/NO above 5mM appear to be toxic, concentrations below this value were used in subsequent cellular motility assays.

The downstream histidine kinase led us to propose that nitric oxide bound to the H-NOX leads to a change in either cell motility or adhesion; to that end, three different assays were prepared to test this hypothesis. First, in an effort to quantify changes in planktonic cell motility, various media swim plates were used with agar concentrations ranging from 0.2% to 0.8%; however, insignificant cell swimming was observed in *P. atlantica* with the conditions attempted thus far (Table IV-1). Small speckling was observed <1cm from the point of inoculation using single colony stabs, whereas no swimming was observed using liquid culture inoculation. The addition of nitrate, nitrite, and DETA/NO to the agar medium produced similar results. The twitch motility, a method of quantifying solid surface adhesion, was also not detected by CV staining; likewise, supplementation of the agar medium of nitrogenous terminal electron acceptors or DETA/NO did not produce a change in cell motility in *P. atlantica*.

The final cellular motility studied was static biofilm formation. Reproducible biofilm data for static cultures could not be obtained due to formation of a solid mass on the bottom of some glass test tubes after staining with CV. Constant aeration at 40rpm did not eliminate this inherent problem with the CV staining. Furthermore, contamination was observed in CCS controls in T6c, so the data obtained for these trials are difficult to assign to biofilm formation exclusively to *P. atlantica* without considering the impact of a potential contaminating cell line. Colony formation unit quantification was equally as difficult since no selection method could be used to eliminate cross contamination. Therefore, the effect of nitric oxide on T6c biofilms could not be adequately identified utilizing the glass tube method, as evidenced by the huge error seen in many trials (Figure IV-6). In an effort to eliminate the solid mass formation and increase the sample size of our experiments, we began utilizing another method of biofilm quantification: the microtiter plate. However, contamination was observed for T6c biofilms in the microtiter plates as well, leading to the utilization of DB27 strain to eliminate any potential exogenous species.

To determine the correct time period to quantify biofilm formation with DB27, static biofilms were studied between three to six days; as seen with previous biofilms using the T6c strain, no consistent biofilm formation was measured before three days. An increase in DB27 biofilm development was observed from day three to day four (Figure IV-7); however, contamination in the CCS control wells was observed after this time point. Spillage between the CCS control wells and adjacent experimental wells was believed to be the culprit of this contamination, so the control wells were cut away from the remaining wells. The removal of these wells appears to eliminate the contamination problem; nonetheless, the formation of solid masses has sporadically appeared on the bottom of the wells, reminiscent of the problem with the glass tubes. Therefore, the effect of NO on static biofilm formation in *P. atlantica* has eluded us to this date.

The cellular motility assays described above utilized the mucoid morphology of both *P. atlantica* strains. Initial results utilizing crenated DB27 cells did not improve the irreproducibility of biofilm development in *P. atlantica* or the apparent inability of the organism to freely swim in the soft agar swim plates. Further genetic work is needed in order to better determine the possible biological role of H-NOX in *P. atlantica*.

IV.5 Discussion

Pseudoalteromonas atlantica is reported to a robust, primary biofilmer, but we have not been able to reproduce these findings in the lab thus far. The crenated cell morphology is deficient in EPS production, which should provide us with more conclusive results if H-NOX is playing a role in the initial attachment of *P. atlantica* to a solid surface. It is quite feasible that the NO sensed by the H-NOX protein in *P. atlantica* plays a significant role further in the biofilm life cycle, which we could not reliably determine with the current motility assays. It is also a possibility that our hypothesis for the HK playing a role in biofilm formation is invalid, and in fact, NO is critical for gene regulation in another important cellular function. For this reason, a genetic knockout of H-NOX would be beneficial as a screening method for normal cell function deficiency. Initial attempts at a genetic knockout were unsuccessful through a *sacB*-based approach due to problems in subclone preparations[22]; therefore, additional work on this or additional knockout protocols ought to be developed for *Pa* H-NOX. Changes in protein

expression could be monitored between the mutant and wild type to determine what biochemical pathway is being adversely affected by H-NOX upon NO binding.

The numerous agar preparations for swim and twitch motility assays were unable to demonstrate planktonic motility and initial solid surface attachment, respectively. Other members of the *Pseudoalteromonas* family have been determined to be swim-deficient in various media[23], so the deficient swimming in *P. atlantica* may be a common feature with the Pseudoalteromonads. Twitch assays in *S. woodyi* were inconclusive as well, so additional optimization of this cellular motility assay is warranted to ensure that the results obtained are not due to the assay itself.

The source of NO must be considered as well, as to whether the bacterium is responding to an intracellular product of the denitrification pathway or if the NO is arising from the immediate environment. The assumption that *P. atlantica* is able to reduce nitrite to NO is valid if and only if the bacteria contains a nitrite reductase. According to present annotation, *P. atlantica* does contain two genes which encode for a large and small subunit nitrite reductase. Unfortunately, with the information provided, it is hard to determine whether the organism will utilize the denitrification or assimilatory pathway for nitrite reduction. However, based on gene annotations and homology searches, it does not appear as if *P. atlantica* can reduce nitrite to nitric oxide. If this assertion is valid, then inability of the Y140F assay to detect NO could be explained. More sensitive detection methods, such as fluorescence-based copper complex, could be employed to definitively determine whether the source of NO is endogenous to *P. atlantica*. The bacteria are commonly found in crab lesions[24], so the nitric oxide may be coming from an immune response to the invading pathogen as well. Nonetheless, the biological role of H-NOX in *P. atlantica* remains to be determined, but the foundation of that work has been established in this thesis.

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IV.7 Tables and Figures

Media Type	BactoAgar (w/v%)	Method of Inoculation
Swimming		
Marine Media	0.2, 0.35, 0.5, 0.65	Liquid (10µL)
1% Tryptone, 0.5% NaCl	0.3, 0.4	Liquid (5µL)
8% Nutrient Broth	0.5	Liquid (3µL)
Marine Media	0.2	Stab
3M-05 (2.3% Instant Ocean, 0.05% NH_4Cl, 0.2% D-glucose, 0.05% Yeast Extract, 50mM Tris-HCl)	0.2	Stab
3M-YE (2.3% Instant Ocean, 0.05% NH_4Cl, 0.2% D-glucose, 0% Yeast Extract, 50mM Tris-HCl)	0.2	Stab
3M-YE + 10µM NO_2^-	0.25	Stab
3M-YE + 100µM NO_2^-	0.25	Stab
3M-YE + 2.5mM NO_3^-	0.2	Stab
3M-YE + 25mM NO_3^-	0.2	Stab
3M-YE + 2.5mM NO_2^-	0.2	Stab
3M-YE + 25mM NO_2^-	0.2	Stab
3M-YE + 250µM DETA/NO	0.2	Stab
Twitching		
2% LB	1.5	Stab
2% LB + 10µM NO_2^-	1.5	Stab
2% LB + 100µM NO_2^-	1.5	Stab
1% Tryptone	1.0	Stab
1% Tryptone + 2.5mM NO_3^-	1.0	Stab
1% Tryptone + 25mM NO_3^-	1.0	Stab
1% Tryptone + 2.5mM NO_2^-	1.0	Stab
1% Tryptone + 25mM NO_2^-	1.0	Stab
1% Tryptone + 250µM DETA/NO	1.0	Stab

Table IV-1 Various agar conditions utilized in swim and twitch motility plates. The agar concentrations were varied between 0.2%-0.8% BactoAgar in order to produce different agar consistencies and optimize planktonic swim behavior. Stab method of inoculation entailed introducing a single colony to the agar plate, whereas a fixed volume of cell culture was ulilized for liquid inoculation.

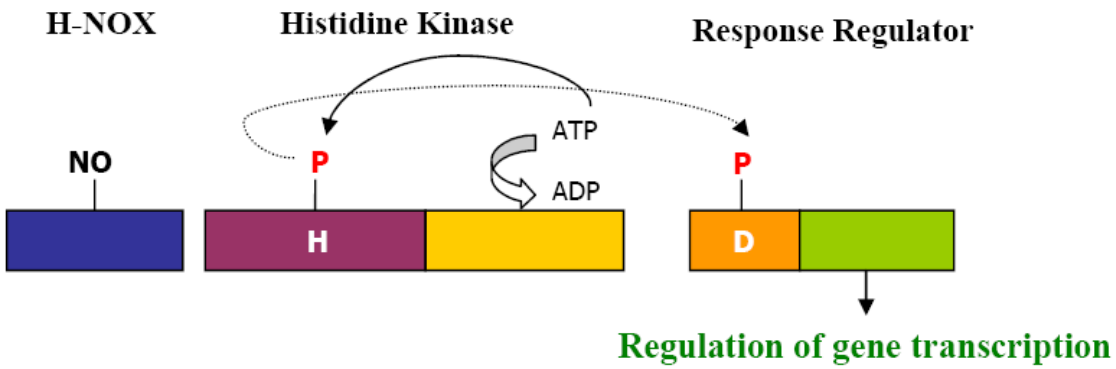

Figure IV-1 The proposed H-NOX/HK/RR phosphorelay system in *P. atlantica*. The H-NOX domain and HK are predicted to be in the same operon, whereas the exact response regulator associated with the HK has yet to be determined. Upon binding of NO to the heme center of H-NOX, the protein-protein interaction between H-NOX and HK is altered, which in turn changes to phosphorylation state of the histidine residue. If the phosphorylation rate increases, then the corresponding transfer from the histidine residue of the HK to the aspartate residue of the RR will increase. This change in phosphorylation of the RR will then change the activity of the enzyme. We predict that the change in phosphorylation state in response to NO will affect gene transcription for biofilm-related genes.

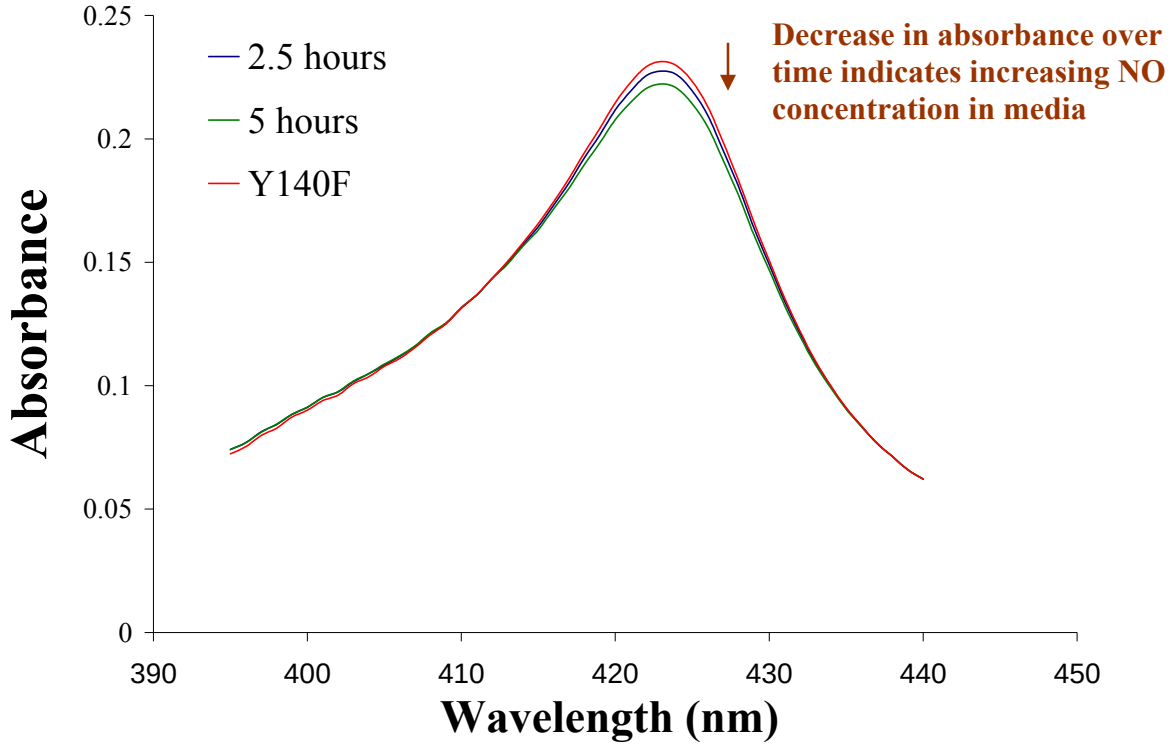

Figure IV-2 Detection of NO in *P. atlantica* was attempted utilizing the engineered *Tt* H-NOX Y140F protein. Upon NO binding to the protein, a decrease in absorbance is observed at 424nm, which can be related back to the NO concentration in the headspace. Due to either the detection limit of the Y140F assay or error in experimental protocol, no consistent NO concentration could be established for *P. atlantica*.

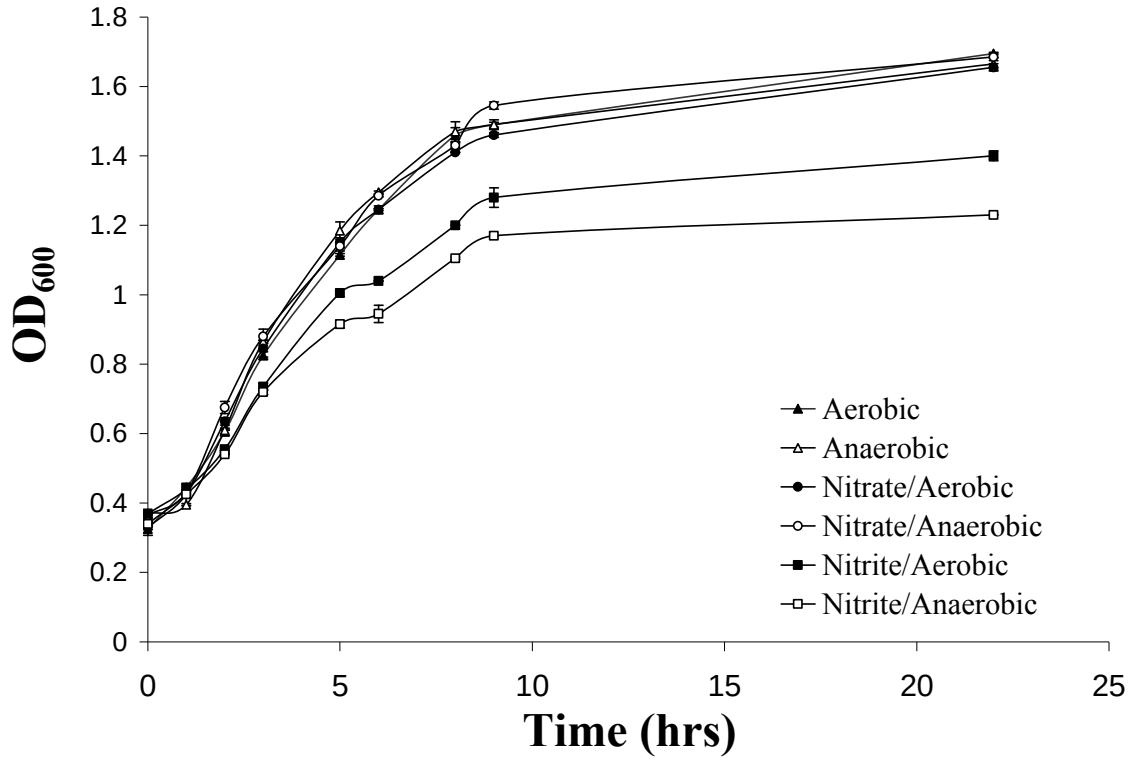

Figure IV-3 Aerobic and anaerobic growth of *P. atlantica* was followed over a 22 hour time period. The effective concentration of the nitrogenous terminal electron acceptors at the beginning of the trial was approximately 25mM. The untreated and nitrate supplemented cultures grew at the same appreciable rate, whereas cells treated with nitrite grow more slowly and to a reduced cell density.

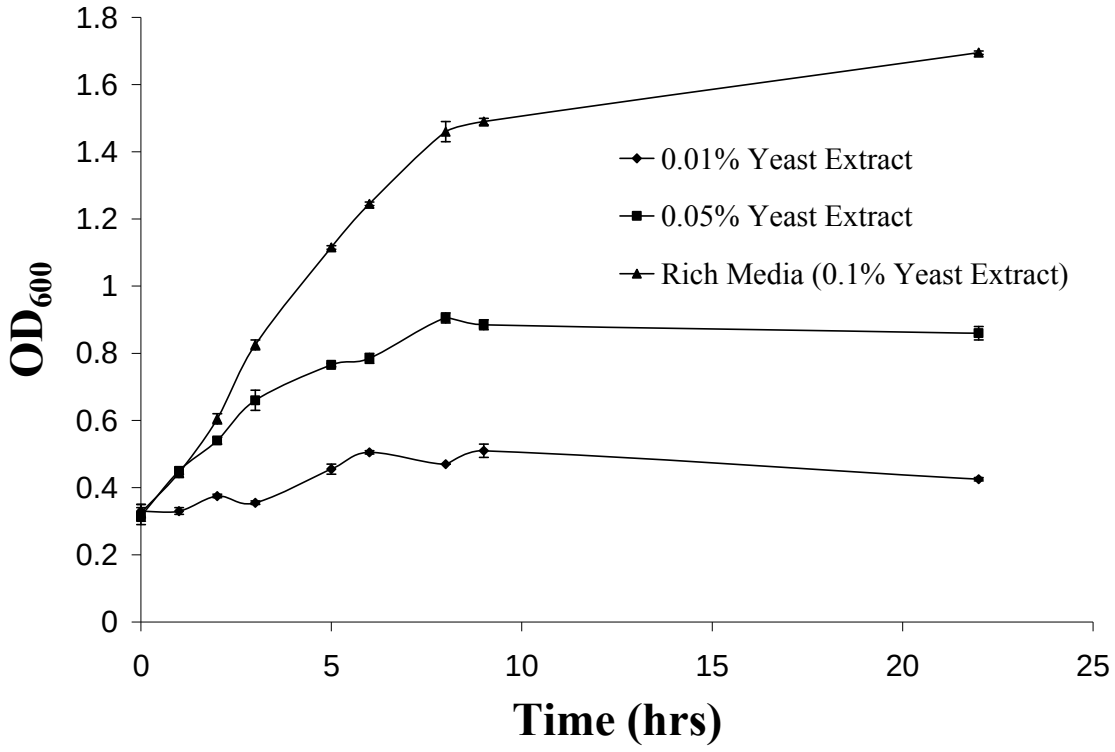

Figure IV-4 The concentration of yeast extract in the cell culture drastically affected *P. atlantica* cell growth in minimal media. Previous experiments indicated that sea salt minimal media cultures grew at the same rate as those grown in 2216 media, as both contain the same amount of yeast extract. The rich media indicated above is 3M, the sea salt minimal media supplemented with 0.1% yeast extract.

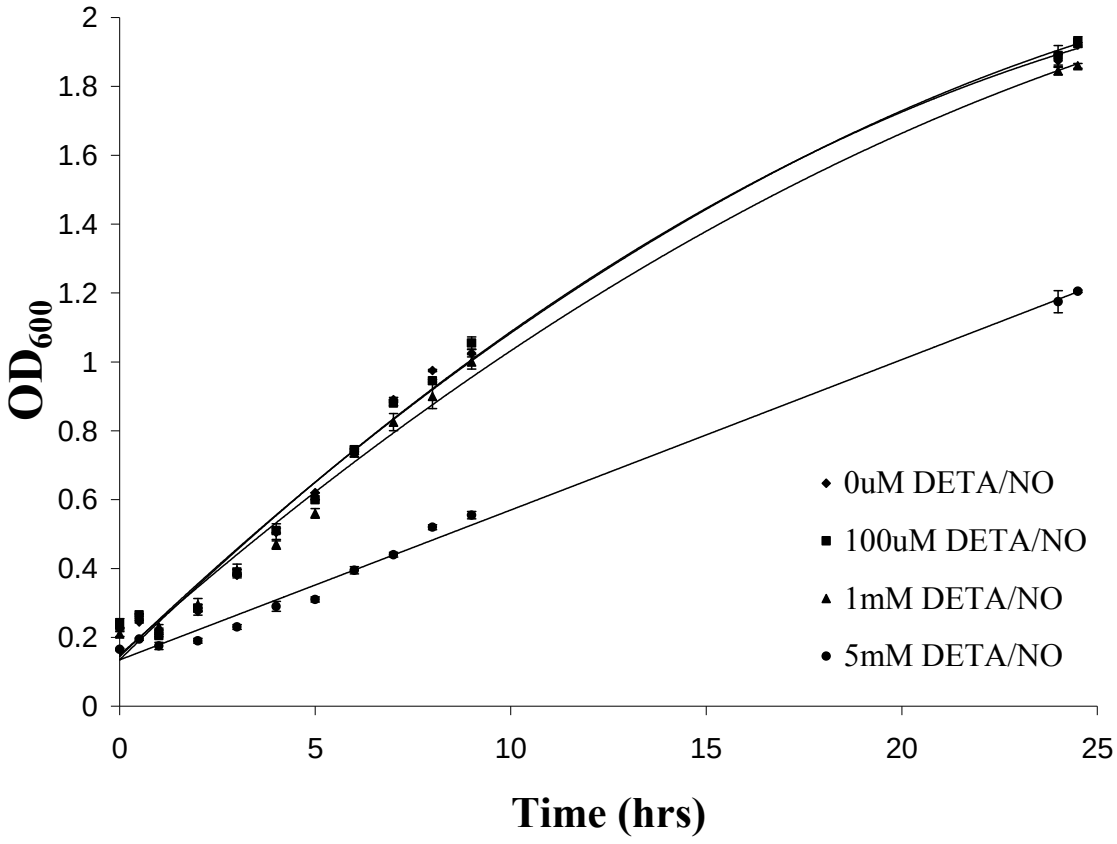

Figure IV-5 The effect of DETA/NO on *P. atlantica* cellular growth. At concentrations less than 1mM DETA/NO, the normal cellular function of the bacteria does not appear to be drastically affected; the cells are still able to grow at the same rate in the presence of NO. However, at concentrations above 1mM DETA/NO, a noticeable change in the growth rate is observed, indicating a possible toxic threshold of the NO donor molecule that *P. atlantica* can withstand.

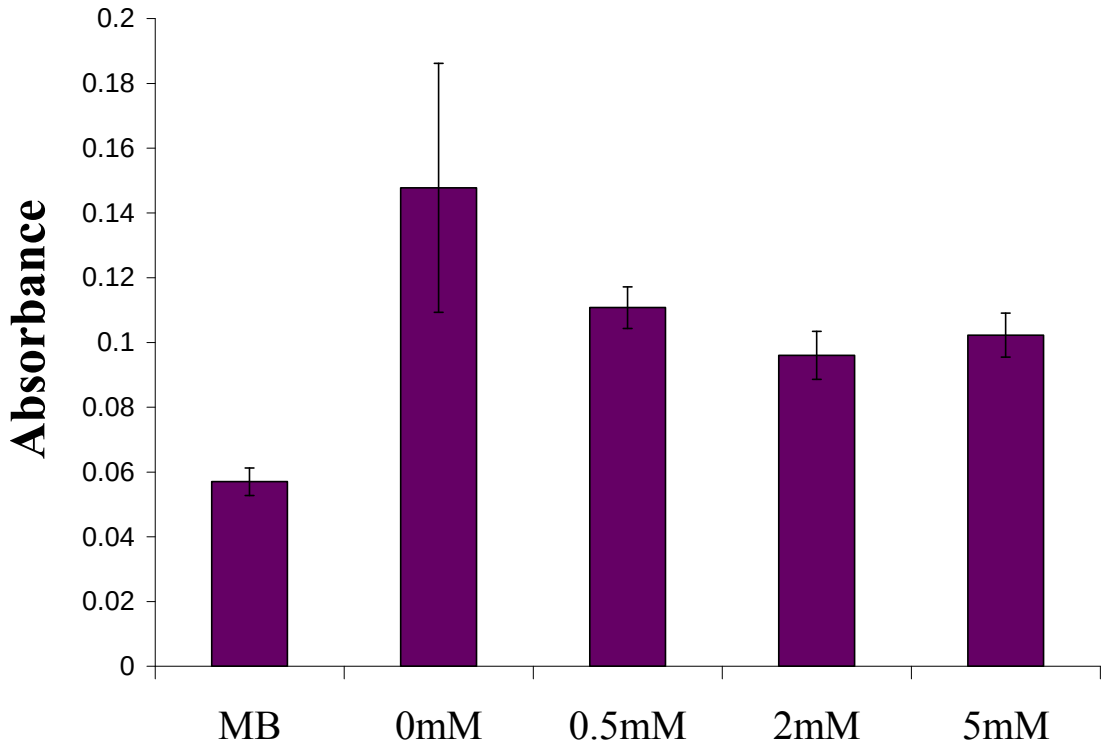

Figure IV-6 Biofilm formation of *P. atlantica* strain T6c after 3 days. Initial concentration of DETA/NO are indicated. Negative control lacking *P. atlantica* is indicated as MB. As shown with the untreated biofilm data above, the inconsistencies of biofilm formation across different trials make it difficult to determine the effect of NO on cellular motility.

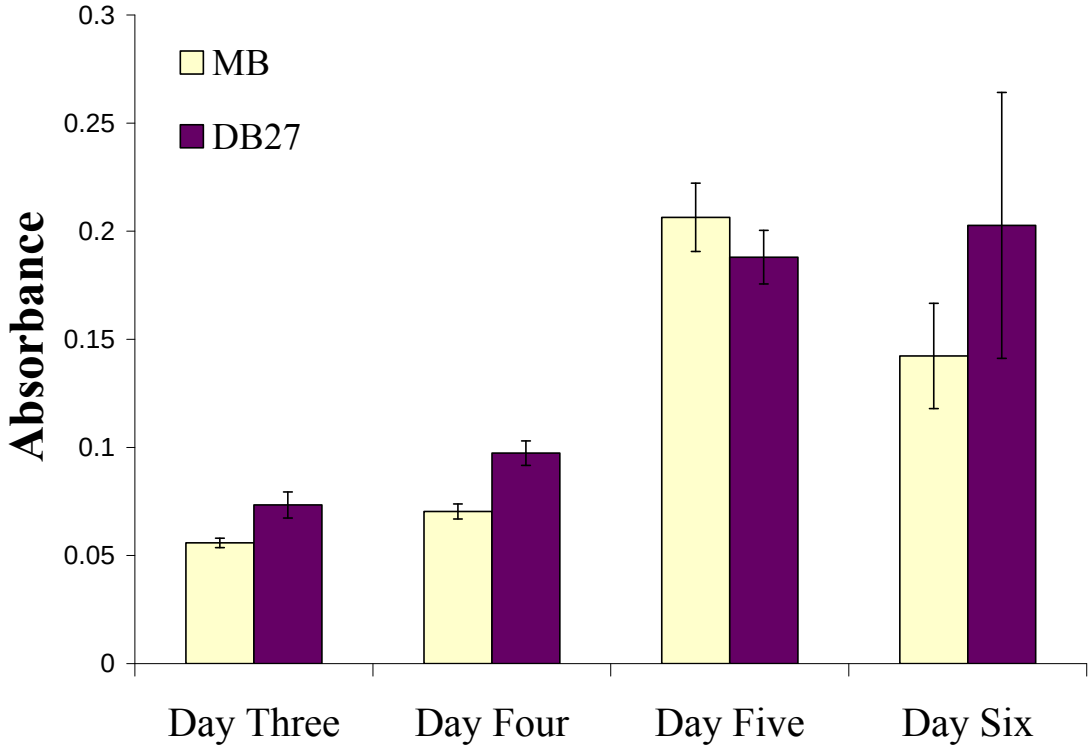

Figure IV-7 Biofilm development over time for *P. atlantica* DB27 strain. A slight increase in CV quantification can be observed between day three and day four; however, cross contamination between wells and solid mass formation disallow biofilm quantification after day four. Media not inoculated with DB27 is indicated as MB.