

A NOVEL NORSPERMIDINE RESPONSIVE SIGNALING  
PATHWAY IN VIBRIO CHOLERAЕ AFFECTING BIOFILM FORMATION

A Thesis  
by  
STEVEN RANDOLPH COCKERELL

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APPROVED BY:

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Dr. Ece Karatan  
Chairperson, Thesis Committee

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Dr. Ted Zerucha  
Member, Thesis Committee

---

Dr. Sue Edwards  
Member, Thesis Committee

---

Dr. Sue Edwards  
Chairperson, Department of Biology

---

Dr. Edelma D. Huntley  
Dean, Cratis Williams Graduate School

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## Abstract

### A NOVEL NORSPERMIDINE RESPONSIVE SIGNALING PATHWAY IN VIBRIO CHOLERAE AFFECTING BIOFILM FORMATION

Steven Randolph Cockerell  
B.S., University of North Carolina Asheville  
M.S., Appalachian State University

Chairperson: Ece Karatan

Polyamines are ubiquitous molecules characterized by the presence of one or more amine groups on a simple carbon chain. As a general class of molecules, polyamines are utilized by nearly every living organism. *Vibrio cholerae* for example is capable of producing the polyamines norspermidine and cadaverine. The polyamine norspermidine has been shown to be a positive regulator of biofilm formation. This positive regulation of biofilm formation was found to be dependent on the presence of the proteins MbaA and NspS. When NspS is removed from the cell, there is a decrease in biofilm formation suggesting that NspS is a promoter of biofilm formation. Further, NspS shows sequence similarity with a polyamine transport protein in *V. cholerae* and preliminary data suggest that it has the capacity to bind polyamines. Strains of *V. cholerae* lacking MbaA, however, show increased biofilm formation suggesting it as a repressor of biofilm formation. MbaA has sequence similarity with the GGDEF/EAL family of proteins. These proteins function in the production and degradation of the bacterial second messenger cyclic diguanosine monophosphate (c-di-GMP).

Given the location of NspS (periplasm) and MbaA (inner membrane) and sequence similarity to other proteins, the following model is proposed: Norspermidine binds NspS in the periplasm which in turn associates with MbaA and attenuates its function, allowing an increase in biofilm formation. To show evidence for this model, the binding ability of NspS was assessed by Thermal Shift Assay (TSA). In this experiment, addition of norspermidine to pure NspS led to an increase in thermal stability over NspS alone. The next goal was to show that MbaA is capable of functioning as a phosphodiesterase (PDE). PDE proteins degrade c-di-GMP into phosphoguananylyl- (3'-5')-guanosine (pGpG), and that activity can be assessed via *in vitro* enzymatic assay and analysis by High Performance Liquid Chromatography (HPLC). HPLC data showed that MbaA functions as a PDE protein. The following study provides evidence for the first norspermidine-responsive signaling system that regulates biofilm formation.

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## Introduction

The aquatic microorganism *Vibrio cholerae* is a human pathogen and the causative agent of the disease cholera. Cholera is a devastating diarrheal disease primarily affecting countries and communities without sufficient water or sewage treatment. The disease cholera is initiated by the ingestion of *V. cholerae* which then colonizes the human gut. There are up to five million cases of cholera reported annually with about 100,000 infections resulting in death (1). While cholera is potentially deadly, the treatment for cholera is relatively simple and is primarily a matter of replacing lost fluids and electrolytes (2). The mechanism of dehydration in cholera is based on the presence and action of cholera toxin and toxin co-regulated pilus (TCP). Not all strains of *V. cholerae* produce cholera toxin and TCP, but when the two are present the cells have the capacity to cause cholera. Cholera toxin acts by causing an increase in intracellular concentrations of cyclic adenosine monophosphate (cAMP) and the over activation of Cystic fibrosis transmembrane conductance regulator channels (CFTR) also referred to as chloride channels (3, 4). The overactive chloride channels allow large amounts of Cl<sup>-</sup> and other ions out of the cell causing water in the cells to move out into the intestinal lumen. This movement of water is the cause of the voluminous diarrhea associated with the disease. The characteristic diarrhea also contains large numbers of *V. cholerae* cells, which can then re-enter their natural reservoirs to cause disease. *V. cholerae* can be spread by ingesting tainted water or underprepared and tainted food.

As an aquatic organism, *V. cholerae* can survive in the environment indefinitely, making repeated outbreaks a common occurrence (5). A feature that makes *V. cholerae* so persistent is its ability to form biofilms. A biofilm is a multicellular community of bacterial cells that secrete an extracellular matrix of carbohydrates, protein and DNA that can protect member cells from environmental stresses like fluctuations in pH, antibiotics and host immune response (6, 7). In the environment, *V. cholerae* forms biofilms on most aquatic surfaces, and while *V. cholerae* cells that are not part of a biofilm can cause disease, recent evidence has linked biofilms with hyperinfectivity. An epidemiological study in India asked individuals to filter their water using their scarves (sari) folded over four times for an effective pore size of 20  $\mu\text{m}$ . The study found that among individuals who filtered their water this way there was a 50% decrease in the incidence of *V. cholerae* infections (8). This was interesting as the folded scarves did not form a pore size small enough to filter out individual *V. cholerae* cells, but it was sufficient to filter aggregates of the cells. Aggregates of *V. cholerae* could be comprised of biofilm fragments or cells associated with small particles that are commonly chitinous material. This suggests indirectly that *V. cholerae* biofilms are associated with the disease cholera. Animal model studies have shown more directly that *V. cholerae* cells that are in or were previously a part of a biofilm were hyperinfectious compared to cells not part of a biofilm (9). This implicates the formation of biofilm as being important to the disease cholera and thus justifies further study.

Biofilm formation in *V. cholerae* begins with receiving a signal from the environment that triggers certain proteins and genes to become active. There are many different signals that a cell will react to by forming a biofilm, and while there are

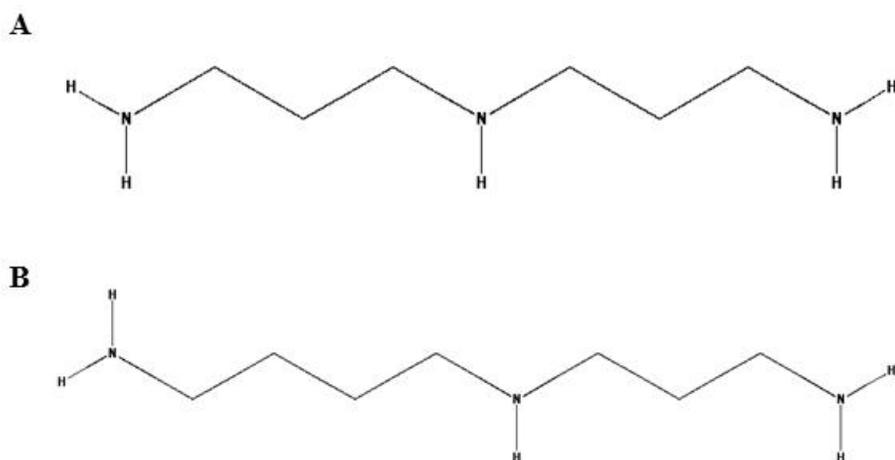
commonalities between species each individual species reacts to different stimuli. These stimuli can be classified as those having an effect on the establishment of a biofilm or those that can affect the growth or dispersal of a biofilm once established. *V. cholerae* can use their flagella to sense a surface, which is an example of mechanical sensing that leads to biofilm formation (10). There are also host-derived signals that affect *V. cholerae* biofilm formation such as bile. Exposure of bile has been shown to increase the biofilm formation in *V. cholerae*. This exposure to bile can influence both the initial formation of a biofilm or increasing the number of member cells (11, 12). Autoinducer molecules are compounds produced and excreted by the cell and are important in quorum sensing. When concentrations of autoinducer reach a certain level, the cell can react to the molecule as an indication of cell density. Increases in autoinducer concentration have an inhibitory effect on *V. cholerae* biofilm formation (10, 13). Once a signal is received, it must be transduced from the cell surface to the cellular machinery responsible for changing the cell's behavior. Once cells are attached to a surface, the biofilm can begin to grow and mature by recruiting new cells and by producing an extracellular matrix composed mostly of polysaccharide, which surrounds the cells and offers a more stable environment. The primary component of *V. cholerae* biofilms is *Vibrio* polysaccharide (VPS) (14, 15).

Two clusters of genes control the production of VPS: *vpsA-K* and *vpsL-Q*. The *vps* genes encode several types of proteins: those that produce VPS, transport VPS or regulate the production of VPS. To date, there are four proteins shown directly to be capable of regulating expression of *vps* genes. VpsR and VpsT are both positive regulators and HapR and CytR are negative regulators (16-19). The production of VPS is

necessary for biofilm formation; therefore, finding signals that affect VPS production is important to better understand the biofilm forming process.

Signals that can affect biofilm formation are diverse and include a class of simple organic molecules called polyamines. Polyamines are a broad class of organic molecules characterized by a carbon chain with terminal and/or internal amine groups. Polyamines (Figure 1) are nearly ubiquitous in life and are synthesized by most organisms.

Polyamines play a role in normal cell growth, signaling and gene expression as well as modulate several cellular activities by associating with RNA, DNA and proteins (20). *V. cholerae* has the capacity to synthesize several polyamines, namely, norspermidine, putrescine, and cadaverine, and can import spermidine. In *V. cholerae*, the polyamine norspermidine was identified to have a significant effect on biofilm formation (21).

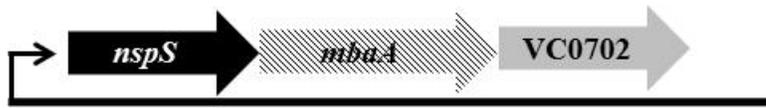


**Figure 1. The polyamines norspermidine and spermidine.** (A) Norspermidine (B) Spermidine. Molecular models were sourced from the online database <http://pubchem.ncbi.nlm.nih.gov/>.

Addition of norspermidine to cultures of *V. cholerae* has been shown to increase biofilm formation up to two fold (21). The increase in biofilm formation signaled by norspermidine was also found to be dose dependent, with higher concentrations

increasing biofilm to a greater degree. Further study of the norspermidine-based biofilm response found that the increase in biofilm formation was dependent on two proteins, MbaA and NspS. When the *nspS* gene was deleted from *V. cholerae* there was a decrease in biofilm formation, but not in a norspermidine dependent manner as in wild-type cells. This suggests that NspS is an enhancer of biofilm formation and that it is necessary for *V. cholerae* to respond to norspermidine. When *mbaA* was removed from *V. cholerae* there was an increase in biofilm formation indicating it is a repressor of biofilm formation. Also, like the *nspS* deletion, the *mbaA* deletion caused an effect on biofilm formation independent of norspermidine addition, indicating MbaA is involved in norspermidine signaling (21).

The genes *nspS* and *mbaA* occur on the same operon along with a third gene VC0702 and are all co-transcribed (Figure 2) (21, 22). When genes occur on the same operon, the encoded proteins are often involved in the same process. The predicted location of NspS is the periplasm indicated by the presence of a signal sequence. MbaA is predicted to be an integral membrane protein with a periplasmic domain and three cytoplasmic domains. Given the putative location of the two proteins and their involvement in biofilm formation, it was hypothesized that the two interact. Evidence supporting the interaction of NspS and MbaA was provided by research in our lab (23). Co-immunoprecipitation experiments showed that NspS and MbaA interact in the cell, indicated by formation of a complex containing both of these proteins.



**Figure 2. Genomic view of *nspS* and *mbaA* with the gene VC0702.** Arrows depict the genes and their orientation on the genome. Arrows also indicate the direction of RNA polymerase when transcribing the operon.

NspS shares sequence similarity with other periplasmic proteins, specifically PotD from *Escherichia coli*. PotD is a component of the PotABCD transport system, which is an ABC type transporter shown to import spermidine in *E. coli* (24). In *V. cholerae* there is an ABC type transporter homologous to the PotABCD system in *E. coli* (24). The difference between the *E. coli* and *V. cholerae* PotABCD systems is that there are two homologues of PotD, PotD1 and PotD2. PotD1 in *V. cholerae* has been shown in our lab to be responsible for transporting norspermidine in addition to spermidine (24, 25). Given the ability of PotD1 to transport norspermidine and the sequence similarity of PotD1 and NspS, it is highly likely that NspS is capable of binding norspermidine. Further, studies in our lab have shown that NspS is not responsible for norspermidine transport. The *potD1* and *nspC* genes were removed from *V. cholerae* producing mutants incapable of synthesizing or importing norspermidine. This mutant was unable to import exogenous norspermidine, despite the fact that it still contained the *nspS* gene, indicating that NspS could not support norspermidine transport (25).

The second protein to be implicated in norspermidine based regulation of biofilm is MbaA. MbaA is a hybrid protein consisting of a periplasmic portion, transmembrane domain, HAMP domain, GGDEF domain and EAL domain. The periplasmic portion of MbaA is the predicted site of a NspS/MbaA interaction that allows norspermidine to transduce a signal in *V. cholerae*. The HAMP domain is commonly associated with

signaling proteins especially those with transmembrane domains. The HAMP domain is named for being common in several different types of signaling protein: histidine kinases, adenylate cyclases, methyl-accepting chemotaxis receptors and phosphatases (26). The HAMP domain is more specifically thought to be a linker domain associated with environmental signaling, so its presence in MbaA is appropriate for the model proposed in this study. The GGDEF and EAL domains of MbaA indicate the possible function of MbaA as affecting the intracellular concentration of the bacterial second messenger bis(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP).

C-di-GMP regulates phenotypes associated with biofilm formation, motility, virulence in pathogenic species, and life-cycle transitions as in *Caulobacter crescentus* (10). The phenotypes regulated by c-di-GMP are triggered by changes in the intracellular concentration of the messenger. In *V. cholerae* and many other bacteria, increased c-di-GMP concentrations tend to increase biofilm formation whereas low levels of the messenger decrease biofilm formation. Changing the concentration of c-di-GMP involves synthesizing more of the messenger or degrading it. These activities are accomplished through two types of proteins; the diguanylate cyclases (DGC) which synthesize c-di-GMP from two molecules of GTP and the phosphodiesterases (PDE) which degrade c-di-GMP to phosphoguanylyl (3'-5') guanosine (pGpG) (27). DGC proteins are identified by the GGDEF amino acid motif and PDE proteins are identified by the EAL motif. GGDEF and EAL amino acid motifs characterize the enzymatic domains of DGC and PDE proteins, respectively. MbaA contains both a GGDEF and an EAL domain, making it a hybrid domain protein, which is common (28). Often, in hybrid domain proteins the GGDEF domain is inactive and the EAL domain is active. The GGDEF domain of MbaA

contains a substitution of glycine to serine in the GGDEF motif, making it SGDEF.

Mutation of the glycine residues in GGDEF domains has been shown to result in a loss of function (28, 29). The EAL domain, however, has the amino acid motif known to be active in other PDE proteins, EVL (30). The alanine to valine substitution is a conservative substitution that has been shown to be tolerated and allow for production of pGpG. The regulation of intracellular pools of c-di-GMP is the proposed mechanism of regulation events involved in biofilm formation in *V. cholerae*.

Based on the observation that MbaA and NspS interact, that NspS putatively binds norspermidine and that MbaA may be a functional phosphodiesterase, the following model is proposed. NspS detects norspermidine which regulates the interaction of NspS and MbaA and this interaction attenuates the PDE activity in MbaA. An interaction between MbaA and NspS could then affect cellular behavior by modulating intracellular pools of c-di-GMP. As concentrations of norspermidine remain low NspS would be unable to attenuate PDE activity in MbaA causing c-di-GMP levels to remain high. Further, removal of *mbaA* confers a consistently elevated level of biofilm formation (21). In this case, absence of MbaA allows c-di-GMP pools in the cell to rise and thus biofilm formation to increase. The objective of this study was to provide *in vitro* evidence of both norspermidine/NspS binding and MbaA PDE activity. The data obtained supports a model of polyamine based signaling, transduced through the bacterial second messenger c-di-GMP. This is the first polyamine-based signaling system elucidated to date and potentially constitutes a new class of bacterial signaling.

## Materials and Methods

### Bacterial strains, media and solutions

Bacterial strains used in this study are detailed in Table 1. *E. coli* strains carrying pMAL-c5x or its derivatives were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract and 1% NaCl) with 0.2% glucose supplemented with 100 µg/mL ampicillin and incubated at 37°C with shaking at 200 rpm. *E. coli* strains carrying pET28b constructs were grown in LB supplemented with 50 µg/mL kanamycin and incubated at 37°C with shaking at 200 rpm.

### Plasmid constructs

#### *nspS* plasmid

The NspS expression plasmid, pET28b::*nspS*, was constructed previously and the pET28b::*nspS* plasmid was isolated from strain AK223 (31). The *nspS* fragment carried in the pET28b plasmid lacks its signal sequence, which causes the resulting NspS protein to remain in the cytoplasm as opposed to being moved to the periplasm. Further, the pET28b plasmid encodes a 6-Histidine tag that is added to the end of the protein, which can be used in protein purification. The *lac* promoter on the plasmid allows control over expression of the inserted gene by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). This molecule is structurally similar to allolactose and removes repression of the *lac* promoter, allowing transcription of the inserted gene. The plasmid pET28b::*nspS* was transformed directly into the SHuffle® T7 Express from New England Biolabs (Ipswich,

MA) chemically competent cells by heat shock. SHuffle™ T7 Express cells were chosen because the cells have been optimized for cytoplasmic production of periplasmic proteins.

**Table 1. Bacterial strains used in this study**

Strain	Genotype	Source
<i>Escherichia coli</i>		
DH5α™	F– Φ80 <i>lacZ</i> ΔM15 Δ( <i>lacZYA-argF</i> ) U169 <i>recA1 endA1 hsdR17</i> (rK–, mK+) <i>phoA supE44 λ– thi-1 gyrA96 relA1</i>	Invitrogen
NEB Express	<i>fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10--Tet<sup>S</sup>)2 [dcm] R(zgb-210::Tn10--Tet<sup>S</sup>) endA1 Δ(mcrC-mrr)114::IS10</i>	New England Biolabs
SHuffle® T7 Express	<i>fhuA2 lacZ::T7 gene1 [lon] ompT ahpC gal λatt::pNEB3-r1-cDsbC</i> (Spec <sup>R</sup> , <i>lacI<sup>q</sup></i> ) Δ <i>trxB sulA11 R(mcr-73::miniTn10--Tet<sup>S</sup>)2 [dcm] R(zgb-210::Tn10 --Tet<sup>S</sup>) endA1 Δgor Δ(mcrC-mrr)114::IS10</i>	New England Biolabs
AK223	pET28b plasmid carrying <i>nspS</i> gene lacking the signal sequence, Kan <sup>r</sup>	(31)
<i>Vibrio cholerae</i>		
PW249	MO10, clinical isolate of <i>V. cholerae</i> O139 from India, Sm <sup>r</sup>	(32)

The genome of SHuffle™ cells includes a gene encoding DsbC, a disulfide bond isomerase, as well as deletions of genes encoding cytoplasmic reductases. These modifications encourage disulfide bond formation in the cytoplasm, which helps with

correct folding of some periplasmic proteins. Cells were recovered for 1 hour in pre-warmed SOC (2% tryptone, 0.5% yeast extract, .05% NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub> and 20 mM glucose) media at 37°C with shaking at 180 rpm. Cells were then plated on LB plates supplemented with 50 µg/mL kanamycin and grown overnight at 37 °C. Presence of *nspS* was confirmed by colony PCR using T7 promoter and T7 terminator primers (Table 2).

**Table2. Primers used in this study.**

Primer	Primer Sequence	Description
PA211	GATAAGCATATGGTGA TCAATCCGA	Forward primer for pMAL <i>mbaA</i> insert
PA212	ACTTGGATCCCTAACGG CATTCACTTTG	Reverse primer for pMAL <i>mbaA</i> insert
PA215	GTGGGCTTCGCAGTATT GCTGCG	Forward primer for E553A mutagenesis
PA216	CGCAGCAATACTGCGA AGCCAC	Reverse primer for E553A mutagenesis
PA226	GCATCGGTTCGACACGG CATTCACTTTGGC	Forward primer for pFLAG <i>mbaA</i> insert
PA227	CAGCGTCTCGAGAAGC TAAACCATAGAAT	Reverse primer for pFLAG <i>mbaA</i> insert
T7 promoter	TAATACGACTCACTATA GGG	Forward primer for detecting insert in pET28b plasmid
T7 terminator	GCTAGTTATTGCTCAGC GG	Reverse primer for detecting insert in pET28b plasmid

### *mbaA* plasmids

In order to express the MbaA protein, a plasmid carrying the *mbaA* gene was constructed. Since MbaA has an N-terminal transmembrane domain, we decided to only express the C-terminus of the MbaA protein to avoid complications resulting from

solubility issues with membrane proteins. Along with the *mbaA* fragment encoding the C-terminus of the protein a point mutant was constructed (MbaA E553A), which had a single nucleotide substitution that altered the glutamate at position 553 on the protein to an alanine. To assemble the plasmids for expressing the cytoplasmic C-terminal portion of the MbaA protein, DNA sequences were PCR amplified using genomic DNA from *V. cholerae* and cloned into an expression plasmid. The expression plasmid used for MbaA WT and MbaA E553A expression was pMAL-c5x (New England Biolabs). The pMAL-c5x plasmid has the nucleotide sequence for maltose binding protein (MBP) 5' of the multiple cloning site. When transcribed, the gene of interest and the MBP gene will form one transcript and when translated form a fusion protein. Like pET28b, pMAL-c5x utilizes the *lac* promoter which can be activated by IPTG addition.

All of the primers used in this study are in Table 2. Phusion High Fidelity polymerase enzyme (New England Biolabs) was used to amplify all *mbaA* gene fragments used in this study. For amplification of the C-terminal *mbaA* fragment, the forward primer PA211 and the reverse primer PA212 were used. The forward primer added an *NdeI* site to the 5' end of the fragment whereas the reverse primer added a *BamHI* site to the 3' end. For the C-terminal *mbaA* fragment, an initial cycle of 98°C for 10 seconds was followed by annealing for 15 seconds at 56°C and then a 45 second extension period at 72°C for 30 cycles with a final extension of 7 minutes at 72°C. The nucleotide sequence for MbaA E553A was constructed using Overlap Extension PCR (33). Phusion polymerase enzyme (New England Biolabs) was used to first construct the “up” and “down” fragments which include the E to A substitution. The “up” fragment was constructed using the forward primer PA211 and reverse primer PA216 that coded

for the A to C nucleotide substitution. The “down” fragment was constructed using the forward primer PA215, which was complementary to PA216, and the reverse primer PA212. Thermo cycler settings for the “up” and “down” fragments began with an initial denaturation at 98°C for 30 seconds followed by 30 cycles beginning with 98°C for 10 seconds, 59°C and 62°C for 15 seconds for annealing, extension for 1 minute at 72°C and a final extension of 7 minutes at 72°C. Both the up and down fragments were run on a gel, then excised and purified from the gel. The “up” fragment was 853 bp and the “down” fragment was 728 bp; these fragments were spliced together in another PCR reaction using PA211 and PA212. Thermo cycler settings for this reaction were identical to those used in amplifying the nucleotide sequence for MbaA WT. The last PCR reaction yielded a 1.5 kbp DNA fragment with a substitution of A to C at 1659 bp from the start of *mbaA*.

All fragments were first cleaned using the GE Healthcare illustra™ GFX™ PCR DNA and Gel Band Purification Kit (Buckinghamshire, UK). The fragments were then digested with *NdeI* and *BamHI* restriction enzymes for 3 hours at 37°C. After digestion the fragments were separated from digested DNA by gel electrophoresis and then gel purification using the GE Healthcare illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE). The digested and cleaned fragments were then ligated into pMAL-c5x which had been digested with *BamHI* and *NdeI*. Ligation was carried out at 16°C overnight using T4 DNA ligase enzyme (New England Biolabs). After overnight incubation, the ligation mixture was dialyzed against water using a Supor® - 100, 0.1 µm, 25mm membrane filter (Ann Arbor, MI) for 20 minutes. After dialysis, all of the ligation reaction was electroporated into NEB express cells, a protein expression strain of

*Escherichia coli* from New England Biolabs. Transformed NEB cells were plated on LB plates supplemented with ampicillin (100 µg/mL) and incubated overnight at 37°C. Colonies were checked for the presence of the insert in the pMAL-c5x plasmid using colony PCR. Cells were transferred from a plate to 100 µL nuclease free water and heated to 95 °C for 5 minutes. Lysed cells were centrifuged at 8000 x g for 10 minutes and the resulting supernatant was the template for a PCR reaction. OneTaq® (New England Biolabs) polymerase enzyme was used to amplify the template in colony PCR reactions. The primers PA211 and PA212 were used to detect presence of the *mbaA* insert and were used with the following thermo cycler settings: initial denaturation at 94 °C for 30 seconds followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing for 30 seconds at 56°C, extension at 68 °C for 45 seconds and a final extension at 68 °C for 7 minutes. Once a colony with the insert was identified, a sample of the plasmid was extracted and purified using the Wizard® Plus SV Minipreps DNA Purification System (Madison, WI). A sample of the plasmid was submitted for sequencing to the Biotechnology Resource Center: DNA Sequencing Facility, at Cornell University (Ithaca, NY). Sequencing was used to verify that the fragment had been inserted into the plasmid and that the insert was free of errors.

#### *mbaA* in pFLAG-CTC

To express the MbaA protein in *V. cholerae*, the full length *mbaA* gene was amplified for cloning into the pFLAG-CTC™ vector from Sigma-Aldrich (St. Louis, MO). The pFLAG-CTC vector adds a FLAG tag to the sequence cloned into it, which can be used to detect the protein produced. The full-length *mbaA* gene fragment was amplified using Phusion DNA polymerase. The full-length *mbaA* fragment was amplified

from *V. cholerae* genomic DNA using primers PA226 and PA227. PCR settings for the amplification of the full *mbaA* were: initial denaturation at 98 °C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 1.5 minutes and a final extension of 10 minutes at 72°C. PCR products was purified using the GE Healthcare illustra™ GFX™ PCR DNA and Gel Band Purification Kit (Buckinghamshire, UK). After purification, the PCR fragments were digested with *XhoI* and *Sall* (New England Biolabs) for 3 hours at 37°C. Two separate attempts were made to ligate the full-length *mbaA* insert into pFLAG-CTC but were unsuccessful.

### **NspS production and purification**

SHuffle® T7 Express (New England Biolabs) *E. coli* were used to express and produce NspS protein lacking the signal sequence. NspS cultures (1 L) were grown in LB broth at 37°C and shaking at 200 rpm to mid log-phase indicated by an OD<sub>655</sub> measurement of 0.3 - 0.4 as described above. At mid log-phase IPTG (Gold Biotechnology, St. Louis, MO) was added to a final concentration of 0.1 mM and cells were induced at 30°C overnight with shaking at 200 rpm. After induction, cells were harvested by centrifugation at 5,000 x g for 10 minutes and the pellet was resuspended in Lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride and 10 mM imidazole, pH 7.4). Cell suspension was frozen overnight at -20°C and then thawed in cold water. PMSF was added to the cells immediately before lysis to a final concentration of 1 mM. After freezing and thawing, the cell suspension was lysed by sonication. Sonication was carried out with the cell suspension in ice water for 2 minutes. After sonication, the cell debris was separated by centrifugation at 16,000 x g for 20 minutes at 4°C. The

supernatant was then added to a separate tube along with 500  $\mu$ L of prepared HisPur™ Cobalt Resin (Thermo Scientific, Rockford, IL). Cobalt resin was prepared by briefly spinning at 700 x g and discarding the supernatant. The resin was then washed three times with lysis buffer. Cleared lysate was then incubated with cobalt resin overnight with gentle rotation at 4°C.

After the cobalt resin was allowed to incubate with cell lysate, the solution was loaded onto a column and the flowthrough was collected for analysis by SDS-PAGE. The NspS-bound resin bed was washed twice. The first wash was with 10 mL of the Lysis Buffer and the second wash was 3 mL of Wash Buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 8.0). After washing, NspS was eluted from the column using a 250 mM imidazole solution (50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, pH 8.0) and collected in 1.5 mL fractions. All elution fractions were analyzed by SDS-PAGE before further processing.

### **Thermal shift assay**

To determine the binding ability of NspS to various ligands, a thermal shift assay was performed essentially as previously described (34). The Thermal Shift Assay (TSA) relies on the principle that a protein bound to a ligand has a higher thermal stability than the ligand-free protein. Purified NspS was buffer exchanged into the TSA buffer (150 mM NaCl, 100 mM HEPES) using a Nanosep 10 kDa Omega centrifugal filter (Pall Life Sciences, Ann Arbor, MI). The TSA reaction mixture contained NspS at a concentration of 20  $\mu$ M, spermidine and norspermidine at 100  $\mu$ M or 1 mM, and SYPRO® Orange (Invitrogen) at a 5x concentration. The protein, ligand and dye were combined and transferred to an Optical 96-well reaction plate from Applied Biosystems (Grand Island,

NY) and covered with an optical adhesive cover from Applied Biosystems. Negative controls were contained SYPRO® Orange, assay buffer and polyamines to ensure no reactions were occurring between the polyamines and SYPRO® Orange indicator. A well with NspS protein, SYPRO® Orange and binding buffer was assembled to determine a baseline for the thermal stability of the protein. The experimental tubes were the same as the NspS only with the addition of the polyamines norspermidine, spermidine, cadaverine or putrescine. The reaction plate was then analyzed in an Applied Biosystems 7300 Real Time PCR System (Grand Island, NY).

The TSA used the detection filters for the TAMRA dye that can be found in the options for the Applied Biosystems 7300 machine. The excitation and absorption spectra for TAMRA overlap with the spectra of SYPRO® Orange. The instrument was then set to increase the temperature of the wells by 1°C every cycle, starting at 25°C and ending at 95°C. The binding assays were performed in triplicate with two biological replicates. The data was analyzed in SigmaPlot where the first derivative of the raw fluorescence values was taken. The converted fluorescence values were graphed and the graphs were used to calculate the average shift in thermal stability of NspS.

### **MbaA production and purification**

NEB express *E. coli* was used to produce MbaA and E553A. Both MbaA variants were purified from cells grown in 1 L of LB + 0.2% glucose supplemented with 100 µg/mL ampicillin. A single colony was used to inoculate an overnight culture in 25 mL of LB + 0.2% glucose supplemented with 100 µg/mL ampicillin. After overnight growth, 1 L of LB + 0.2% glucose supplemented with 100 µg/mL ampicillin was inoculated with 10 mL (1% of 1 L volume) of the 25 mL overnight. Cultures were grown at 37°C and

shaking at 200 rpm, to mid log phase, indicated by an optical density reading of  $OD_{655} \sim 0.3 - 0.4$ . Optical density measurements were taken in a Bio-Rad Model 680 microplate reader (Hercules, CA) by transferring 150  $\mu\text{L}$  of culture to a microplate and analyzing using the 595 nm filter. Once cells were in mid log phase, IPTG was added to a final concentration of 0.3 mM and the culture was grown overnight at 30°C with shaking at 200 rpm. After induction, cells were harvested by centrifugation at 5000 x g for 10 minutes and resuspended in Column buffer (20 mM Tris, 200 mM NaCl, and 1 mM Ethylenediaminetetraacetic acid (EDTA)). Cell suspension was transferred to a 50 mL conical tube and was frozen at -20°C overnight. After freezing, the cell suspension was thawed by placing the conical tube in cold tap water. Once thawed, phenylmethanesulfonylfluoride (PMSF) (Amresco, Solon, OH) was added to a final concentration of 1 mM and the cell suspension was lysed by sonication (Heat Systems Ultrasonics W-380, Farmingdale, NY). Sonication was performed in ice water for a total of 2 minutes. After lysis, the solution was transferred to two 50 mL Nalgene centrifugation tubes. Lysis solution was centrifuged at 16,000 x g for 20 minutes at 4°C, the supernatant was then split evenly into two separate 15 mL conical tubes. To each of the conical tubes containing the cleared cell lysate, 500  $\mu\text{L}$  of prepared amylose column resin was added and the volume was raised in each tube to 15 mL. Amylose resin was prepared by centrifugation at 700 x g for 30 seconds followed by removal of the supernatant. The resin was then washed three times with 1 mL of column buffer.

The amylose resin/cell lysate solution was rotated gently at 4°C overnight. After incubation of the lysate with the resin, the solution was transferred to a column. The flowthrough was collected and saved to be analyzed by SDS-PAGE to evaluate the

amount of protein that did not bind to the resin. The MbaA fusion protein bound resin was washed on the column with 20 mL of column buffer which was saved for analysis by SDS-PAGE. Finally, MbaA fusion protein was eluted from the column using column buffer + 10 mM maltose. The elution was collected in 1 mL fractions and analyzed by SDS-PAGE before further processing. To prepare the MbaA/MBP fusion protein for use in enzymatic assays, the elutions were collected and dialyzed using a 3500 MWCO, 0.5 mL – 3 mL Slide-A-Lyzer® dialysis cassette from Thermo Scientific (Rockford, IL) overnight in a 50 mM Tris solutions pH 8.5. Protein solution was dialyzed against 500 ml of buffer for 1 hour at room temperature, then overnight at 4°C after transferring the cassette to another 500 mL of fresh buffer.

### **Phosphodiesterase assays**

#### *Bis(pNPP) assay*

Bis(p-nitrophenyl) phosphate is a molecule that has been shown to be able to serve as substrate that is available to c-di-GMP PDE proteins. An assay for PDE activity using Bis(pNPP) was carried out essentially as previously described (35). After MbaA and MbaA E553A production, a buffer exchange into a 50 mM Tris, pH 8.5 solution was performed. Buffer exchange involved concentrating the elutions in a Nanosep 10 kDa Omega centrifugal filter from Pall Life Sciences (Ann Arbor, MI). After concentration Tris buffer was added to the concentrated protein and was centrifuged again. After three subsequent concentration and dilutions with the Tris solution, the fusion protein was in the correct buffer for the pNPP assay. For this study 10 µg of fusion protein was used in each reaction. To determine the necessity for cations to the function of MbaA: MnCl<sub>2</sub>, MgCl<sub>2</sub>, and CaCl<sub>2</sub> were added to the assay at a final concentration of 2 mM. Further, as

Mn<sup>2+</sup> will form oxides at pH 8.5, all cations were added at a final concentration of 2 mM just prior to the start of the assay. The assay was carried out in triplicate at 37°C for 2.5 hours. After 2.5 hours, the reaction mixture was transferred to a 96 well microplate and analyzed using a microplate reader at 415 nm. Results from the spectrophotometer were averaged and the data were analyzed using Microsoft Excel.

Three negative controls were performed for the pNPP experiment; they consisted of one of the three cations combined with the pNPP substrate and assay buffer and were incubated alongside the experimental groups. The values of the negative controls were used to adjust the experimental assays that contained MbaA to control for any effect by pNPP or cations alone.

#### *HPLC Assay*

The HPLC assay conducted was based on a previously described method (36). Fusion proteins were purified and dialyzed overnight into 50 mM Tris pH 8.5. The reaction mixture consisted of 20 µg (2.5 µM MbaA) total of MBP fusion protein, 100 µM nucleotides, 2 mM cations, 50 mM Tris pH 8.5 at a final volume of 100 µL. To determine specificity, c-di-GMP and c-di-AMP (BioLog Life Science Institute, Germany) were used in separate reactions. All nucleotides were used at a final concentration of 100 µM in the reaction mixture. The reactions were incubated for 2.5 hours at 37°C. After incubation, reactions were centrifuged through a Nanosep 10 kDa Omega centrifugal filter from Pall Life Sciences for 2 minutes at 14000 x g. The reaction products were separated using a SUPELCOSIL™ LC-18 column (Sigma-Aldrich, St. Louis, MO) with a Waters 1525 Binary HPLC pump and analyzed using a Waters 2487 Dual λ Absorbance Detector (Milford, MA). Each enzymatic reaction had a total volume of 100 µL, which allowed for

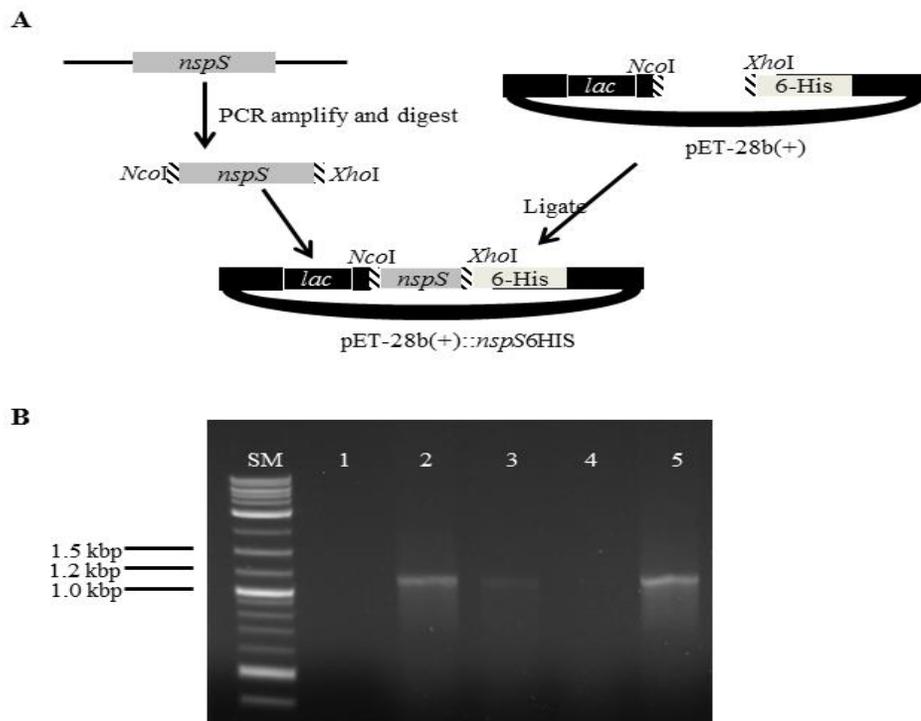
two 40  $\mu\text{L}$  injections per reaction tube. The identity of the peaks was determined by comparison of the reaction products to purified c-di-GMP, pGpG and c-di-AMP standards (BioLog Life Science Institute).

The MbaA PDE assays were carried out with two technical replicates for each biological replicate. MbaA PDE activity was tested in the presence of the same three cations listed for the pNPP assays. The HPLC traces were used to quantify the apparent activity of MbaA. Extensive analysis of MbaA enzymatic parameters were not evaluated due to time constraints and lack of enzyme. Apparent activity was calculated by comparing the area under the c-di-GMP standard peaks with the area under the pGpG peaks in the experimental runs. That ratio was used to determine roughly the number of nanomoles of pGpG produced by MbaA over the 2.5 hour assay. That information was then compared to known  $k_{\text{cat}}$  values in order to compare MbaA activity with other known PDE proteins.

## Results

### Transformation of *pET28b::nspS* into Shuffle

An expression plasmid of *nspS* lacking its signal sequence was previously constructed (31). The signal sequence was removed so that NspS could be expressed in the cytoplasm of the *E. coli* expression strain. Cytoplasmic production of NspS is likely to lead to a greater yield of product than periplasmic preparations. The *pET28b::nspS* plasmid was isolated from *E. coli* BL21 and transformed into Shuffle® T7 Express (Figure 3A).

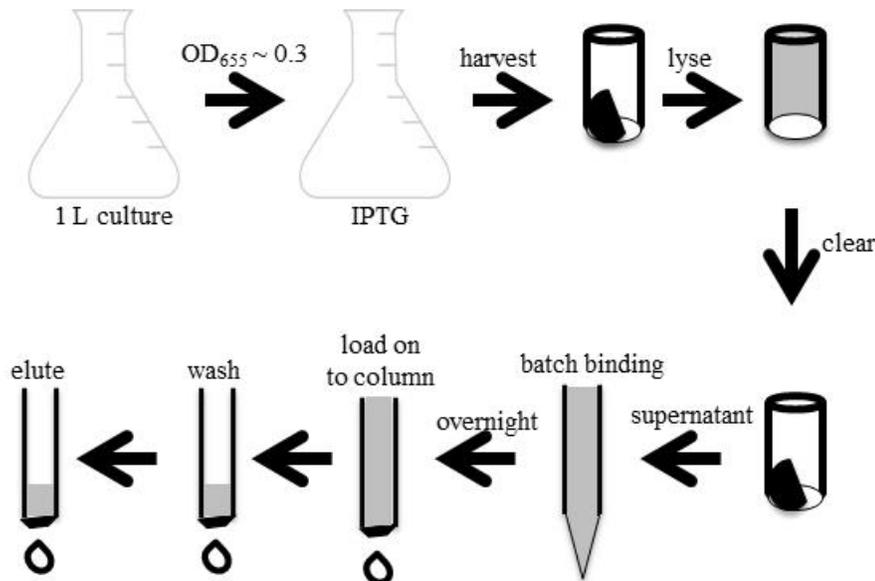


**Figure 3. *nspS* cloning and transformation.** (A) The cloning procedure used by Zayner, 2008 to insert *nspS* into pET28b lacking its signal sequence. (B) The plasmid containing *nspS* was detected in SHuffle® cells after transformation. Lanes 2, 3 and 5 are positive for *nspS*.

SHuffle® T7 Express cells were chosen because they have been optimized for the production of periplasmic proteins in the cytoplasm, giving a higher yield of correctly folded protein. The plasmid pET28b::*nspS* was confirmed to be in SHuffle® transformants by colony PCR (1 kbp fragment) (Figure 3B) and by sequencing.

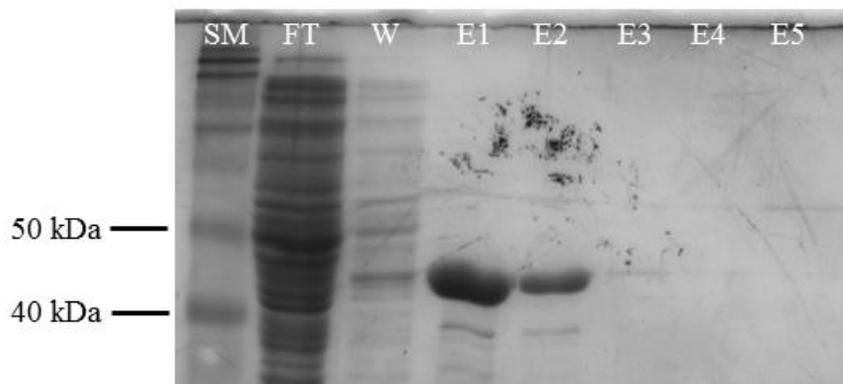
### Production of NspS in SHuffle™

In order to determine the ability of NspS to bind norspermidine, pure samples of the protein were required. Small scale optimization experiments were conducted to determine the ideal conditions for the production of NspS from SHuffle™ cells. The optimal induction conditions for production of NspS were 100  $\mu$ M IPTG for approximately 18-20 hours at 30°C (Figure 4).



**Figure 4. Production and purification of protein.** Diagram depicting production and purification protocol for NspS, MbaA and MbaA E553A. NspS purification is achieved through use of a cobalt resin which the 6 histidine tag on NspS has an affinity. For MbaA, purification is achieved by selective binding of Maltose Binding Protein to amylose immobilized to resin.

Further, to increase the amount of protein from each purification experiment, two 1 L cultures were grown simultaneously. The protein was affinity purified using a cobalt resin which has an affinity for the 6His tag on the NspS protein. The protein could then be washed of contaminating proteins and eluted using a solution containing 250 mM imidazole, which outcompetes the 6His tagged NspS for binding to the cobalt immobilized on the resin. The flowthrough, wash and elutions were collected and analyzed by SDS-PAGE before using the protein in any assay (Figure 5). After the presence and purification of NspS was determined the protein was dialyzed against the TSA buffer.

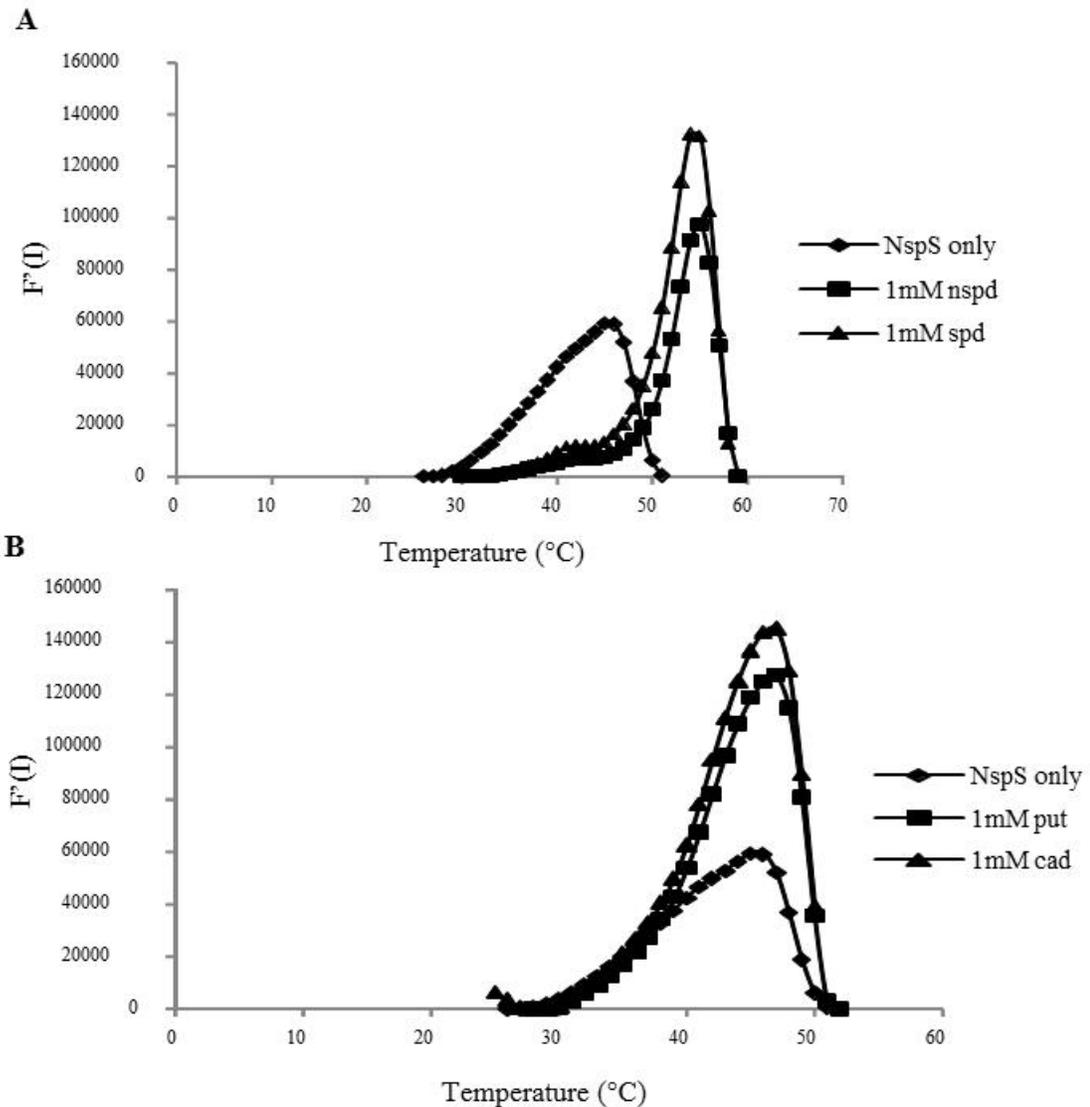


**Figure 5. NspS purification.** The dark band in lanes marked 1 and 2 represent the NspS protein which has a molecular weight of 41 kDa. The smaller and lighter bands represent contaminating protein. Though contamination was present, purity of NspS in this preparation was estimated as >90%. FT – flowthrough, W – Wash, E1-E5 – elutions 1 through 5.

### **NspS binds norspermidine and spermidine, but not cadaverine or putrescine**

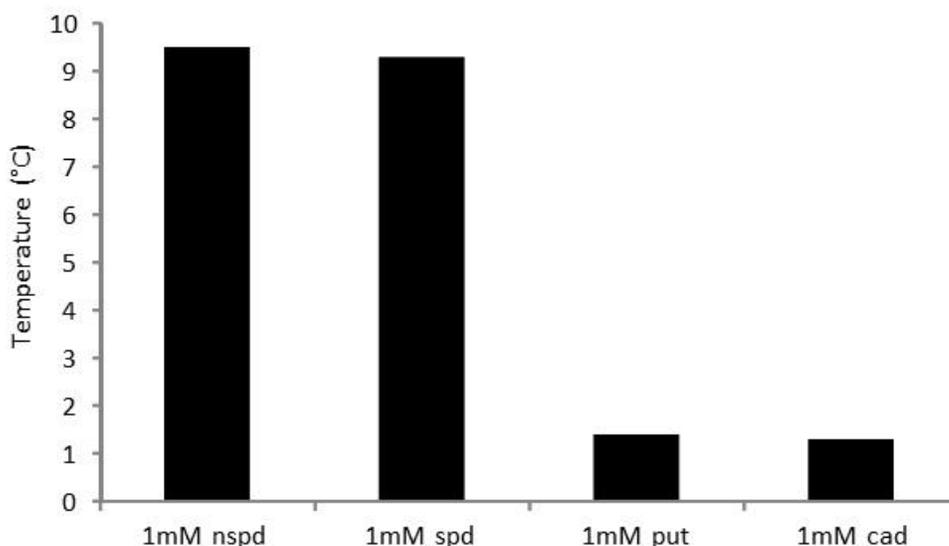
The periplasmic protein NspS is predicted to bind norspermidine based on similarity to other periplasmic polyamine binding proteins and its effect on biofilm formation. The ability of NspS to bind norspermidine was evaluated by a Thermal Shift Assay (TSA), which operates on the principle that a ligand binding protein will be more

thermally stable when bound to its ligand. The TSA was performed using purified samples of NspS at a final concentration of 5  $\mu$ M. TSA experiments were set up with a NspS-only control to determine the melting temperature of the protein. The experimental wells contained polyamines at 1 mM concentrations (Figure 6).



**Figure 6. Thermal Shift Assay of NspS 6His.** Thermal Shift Assay presented as the first derivative of total fluorescence values. Peaks of the curve represent the point at which half of the protein has denatured. (A) Thermal shift assay of NspS 6His with the polyamines norspermidine (nspd) and spermidine (spd). (B) Thermal Shift Assay of NspS 6His with the polyamines putrescine (put) and cadaverine (cad).

There is a pronounced shift in the thermal stability of NspS after the addition of both norspermidine and spermidine at concentrations of 1 mM, which indicates a binding event. Further, the binding of NspS to polyamines is specific to norspermidine and the structurally similar spermidine. The addition of norspermidine and spermidine resulted in an average 10°C shift in thermal stability (Figure 7).

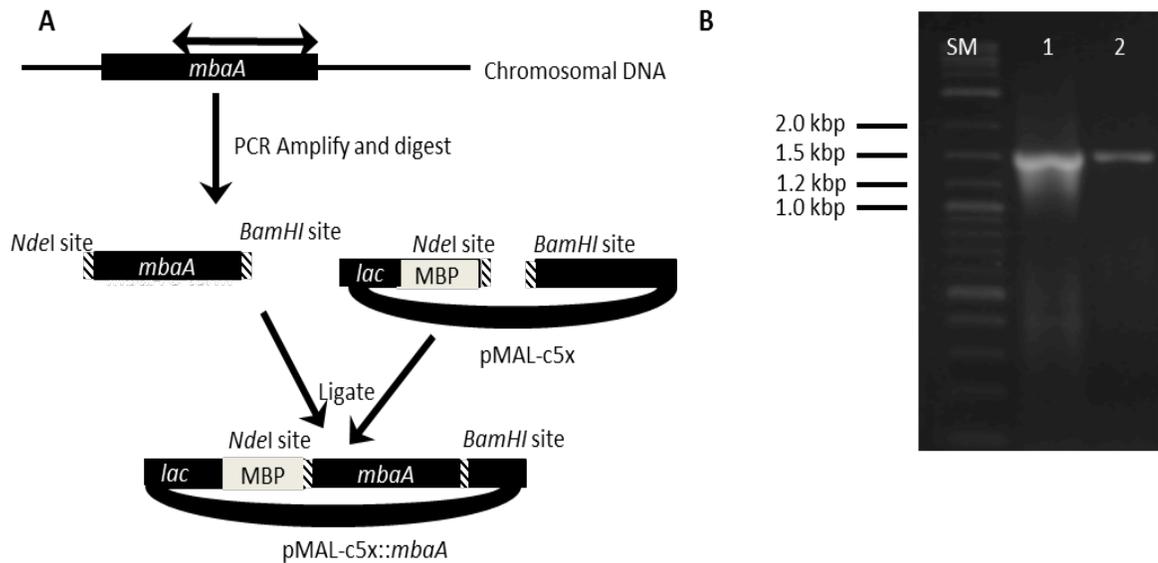


**Figure 7. Average shift in thermal stability of NspS 6His.** Bars show the average temperature (°C) increase in thermal stability of NspS 6His after addition of polyamines. Values are calculated using the average thermal stability of NspS without polyamine addition. Thermal shift was calculated from the average of three technical replicates.

Polyamines are a general class of molecules that have a net positive pH at physiological pH and all contain amine groups. Given their similar characteristics, a TSA was performed to determine NspS binding specificity. Putrescine and cadaverine were added to NspS in a TSA (Figure 6B). Addition of either putrescine or cadaverine increased the thermal stability of NspS by about 1°C, which is negligible (Figure 7). This indicates that there was no binding event and that NspS binding is specific to norspermidine and the structurally similar spermidine.

## Construction of *mbaA* plasmids

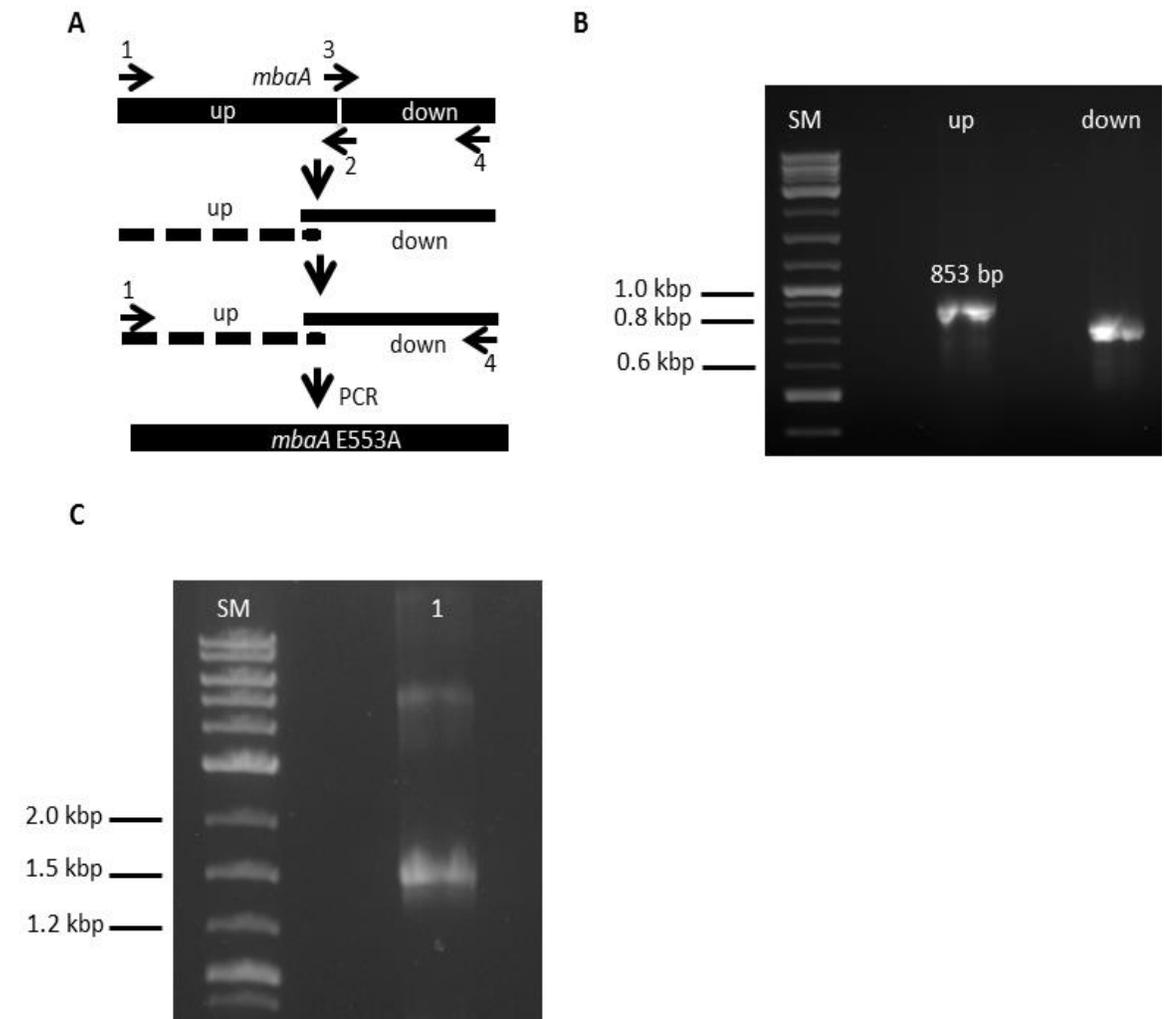
In order to determine the phosphodiesterase capacity of MbaA, wildtype and mutant *mbaA* sequences were cloned into an expression plasmid. To prevent complications involved in purifying integral membrane proteins, a 1.5 kbp fragment of *mbaA* was amplified from genomic DNA, which has the nucleotide sequence coding for the GGDEF and EAL domains C-terminal to the transmembrane domain.



**Figure 8. Construction of the *mbaA* expression plasmid.** (A) Diagram of the cloning procedure used to synthesize the *mbaA* and *mbaA E553A* plasmids in the pMAL-c5x system. (B) Colony PCR of *mbaA* gene inserted into pMAL-c5x, lane 2, with PCR amplified *mbaA* genomic DNA, lane 1.

A fusion protein system was chosen to produce the MbaA protein given the potential for increased amounts of soluble protein. The expression plasmid pMAL-c5x was used to express *mbaA* genes in transformed NEB Express *E. coli* in order to produce protein. Two *mbaA* constructs were produced: *mbaA WT* (Figure 8) and *mbaA E553A* (Figure 9). The *mbaA WT* insert was prepared from genomic *V. cholerae* DNA by PCR amplification followed by restriction digest. The *mbaA E553A* insert was constructed by assembling two fragments that were amplified from genomic *V. cholerae* DNA and

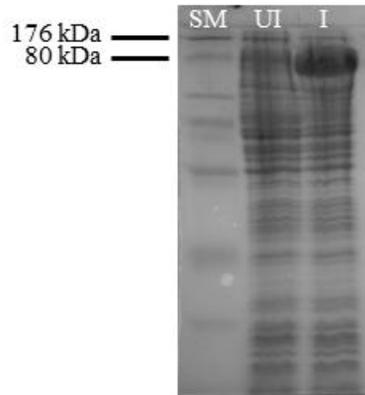
contained a single nucleotide substitution. After the construction of *mbaA E553A*, the fragment was inserted into pMAL-c5x and the resultant plasmid transformed into NEB express. Presence of the insert was verified by colony PCR amplification of the *mbaA* insert. After colony PCR was successful, plasmids were isolated and sent for sequencing to confirm that the nucleotide sequence was correct.



**Figure 9. Generation of MbaA E553A mutant.** (A) Diagram describing the mutagenesis by SOE PCR protocol for constructing the *mbaA E553A* fragment. (B) Agarose gel shows the “up” and “down” fragments amplified from genomic DNA. (C) The band at 1.5 kbp depicts successful splicing of “up” and “down” fragments into *mbaA E553A*.

## Production of MbaA WT and MbaA E553A

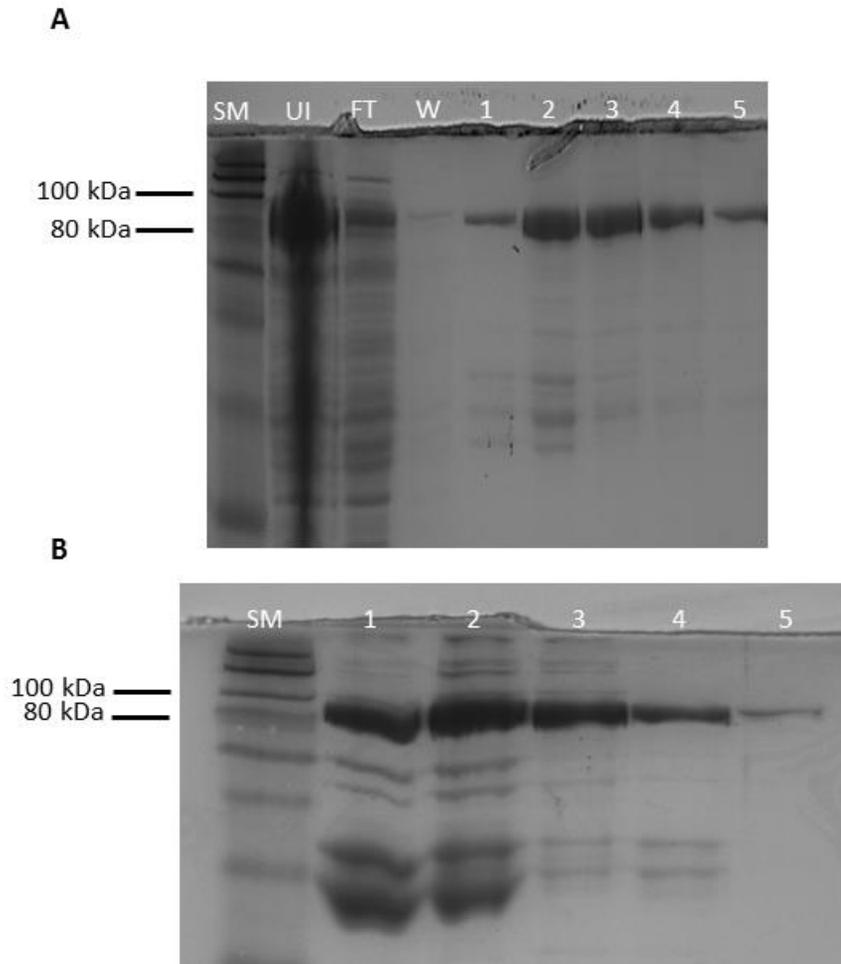
To analyze the enzymatic ability of MbaA, the *mbaA* gene cloned into pMAL-c5x was used to synthesize protein. Small scale experiments were used to determine the ideal expression conditions for MbaA synthesis. A 2 mL culture of NEB Express carrying *mbaA* was induced with IPTG overnight. After induction, the cells were lysed and analyzed by SDS-PAGE (Figure 10).



**Figure 10. Small scale optimization of MbaA expression.** A 2 mL overnight culture of NEB Express carrying the *mbaA* gene was induced overnight to produce protein. “UI” is the total cell protein from an uninduced culture and “I” is the total cell protein from the induced culture. The induced lane shows a strong band at 100 kDa that is absent from the uninduced and is consistent with the size of the MbaA/MBP fusion.

It was found that induction with 300 mM IPTG, overnight and at 30°C would produce the most protein. The procedure to produce MbaA protein was essentially identical for all MbaA constructs used in the study (Figure 4). Production of MbaA was most successful when induced during mid-log phase indicated by an  $OD_{655}$  of approximately 0.3. The purification of MbaA fusion protein was accomplished by using an amylose resin that has affinity for the MBP affinity partner. The protein could then be eluted using a solution of 10 mM maltose which would outcompete amylose for binding to MBP. Fractions were collected and analyzed by SDS-PAGE gel stained by Coomassie

Brilliant Blue (Figure 11). After gel analysis of the MbaA elution fractions, the protein was dialyzed against a 50 mM Tris solution at pH 8.5 overnight using a dialysis cassette.



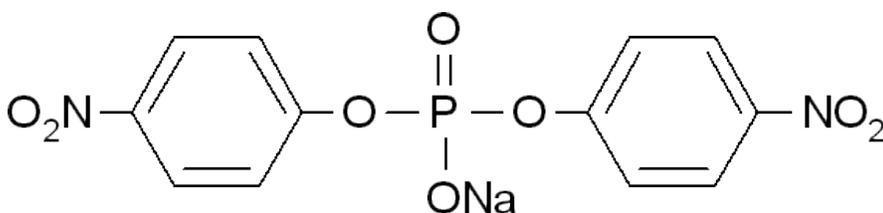
**Figure 11. Representative SDS-PAGE gels of MbaA production experiments.** (A) MbaA WT production from a 1 L culture. UI is the total cell protein from an uninduced culture, FT is the wash from the cobalt resin, W is the wash step in purification process and lanes 1-5 show samples from 1.5 mL elutions. (B) SDS-PAGE gel of MbaA E553A production experiment with lanes 1-5 showing the elution fraction from 5 samples. Both gels show MbaA protein at a molecular weight of 100 kDa.

### **MbaA is a Phosphodiesterase**

#### ***pNPP***

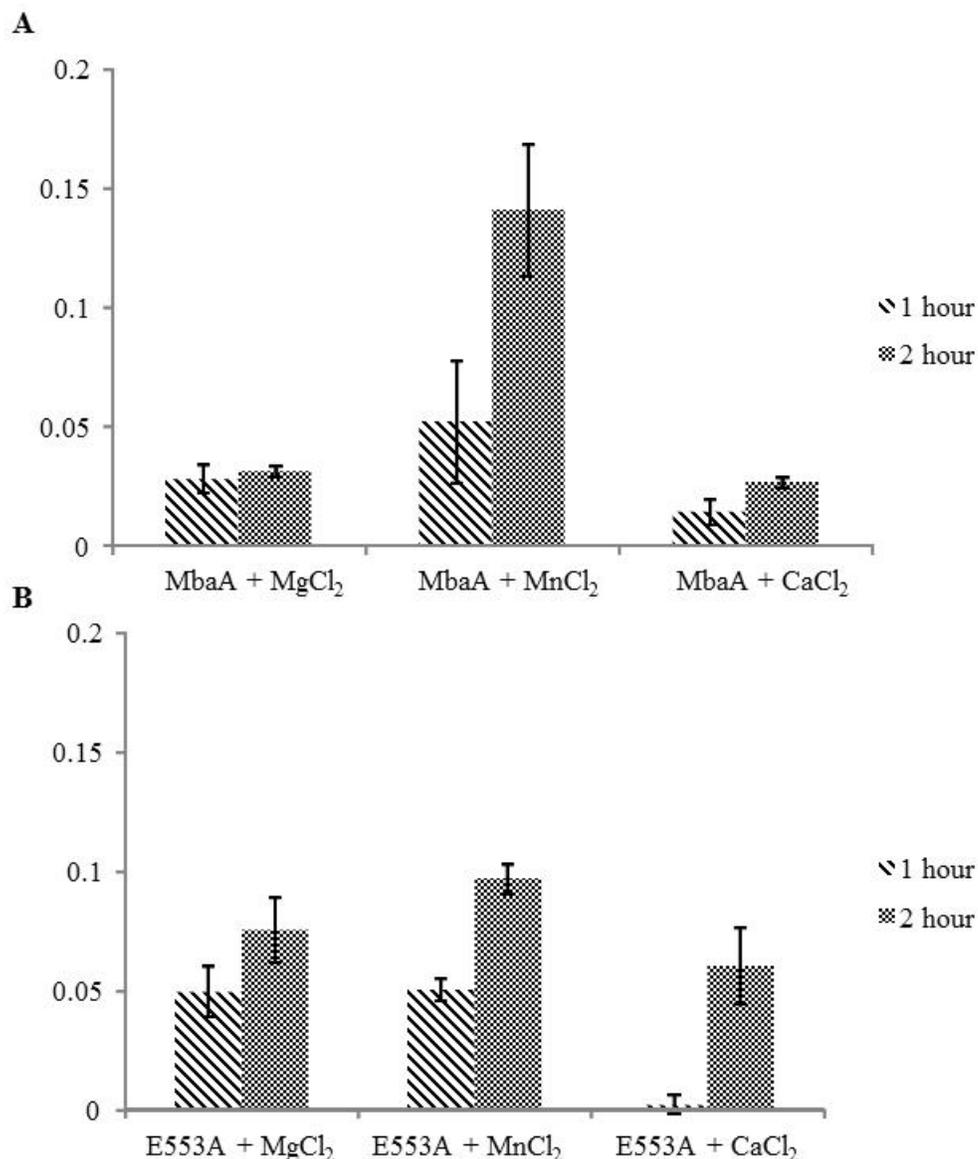
The first phosphodiesterase assay used in this study was performed using the reagent pNPP (Figure 12). This molecule has a phosphodiester bond available for cleavage by a phosphodiesterase and results in a color change detectable at 415 nm. This

assay has been used to successfully identify other PDE proteins. It was found that MbaA WT produced a yellow color in the pNPP assay which indicates that MbaA WT is a phosphodiesterase (Figure 13A). As part of the pNPP assay, 3 cationic cofactors were added to the assay one at a time, to determine their impact on activity.  $Mn^{2+}$ ,  $Mg^{2+}$  and  $Ca^{2+}$  were added to the reaction in the form of  $MnCl_2$ ,  $MgCl_2$  and  $CaCl_2$ . It was found that  $MnCl_2$  addition resulted in the strongest formation of yellow color and thus a higher absorption at 415 nm. The salts  $MgCl_2$  and  $CaCl_2$  did not produce a yellow color in the reaction after incubation indicating that they either inhibit the reaction or simply do not affect it.



**Figure 12. Bis(p-nitrophenyl) phosphate.** Molecule figure was sourced from Sigma Aldrich.

The pNPP assay was also used to analyze MbaA E553A using the same concentrations of the substrate and addition of the same cationic cofactors. When MbaA E553A was combined with  $MnCl_2$ , a yellow color developed to a similar degree as MbaA WT (Figure 13B). This was unexpected as the substitution of glutamic acid at position 553 with alanine should be a loss of function mutation as indicated in other phosphodiesterase studies. It is possible that the phosphodiesterase ability of MbaA is disrupted by the mutation but only in the degradation of a phosphodiester bond in c-di-GMP.

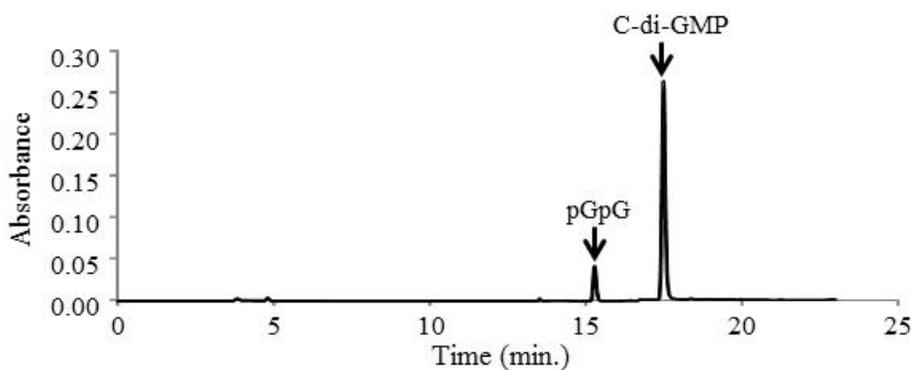


**Figure 13. pNPP assay of MbaA and MbaA E553A.** pNPP phosphodiesterase assay. Columns represent the absorbance of the samples at 415 nm. The samples develop a yellow color when the enzyme present is has the ability to cleave a phosphodiester bond in pNPP. (A) MbaA WT protein incubated with pNPP for 2.5 hours at 37°C. (B) MbaA E553A incubated with pNPP for 2.5 hours at 37 °C. Reactions were adjusted for background signal from pNPP + ion controls run along-side experimental groups.

### **HPLC**

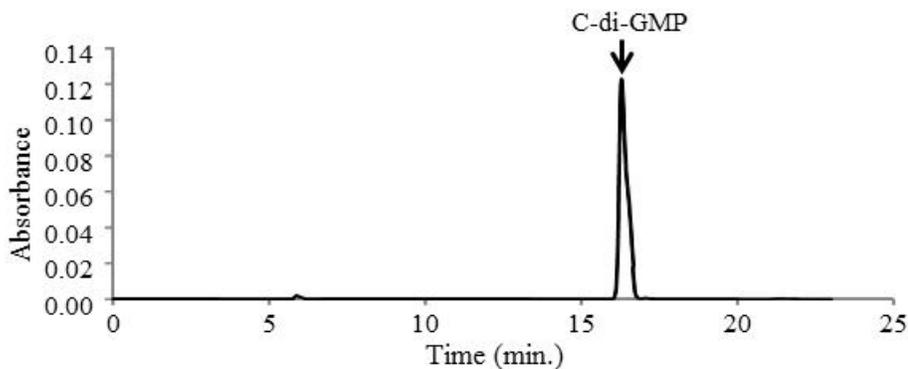
The pNPP assay uses a mimic for c-di-GMP; therefore, in an effort to provide more convincing results that MbaA is a phosphodiesterase, a second assay was performed. For the second MbaA phosphodiesterase assay, a pure sample of c-di-GMP

was used as the substrate. MbaA and c-di-GMP were combined with one of four cations and allowed to react for 2.5 hours at 37°C. The reaction products were then separated by HPLC, and the traces were compared with c-di-GMP and pGpG standards to identify peaks. The HPLC traces show that MbaA is a c-di-GMP phosphodiesterase (Figure 14). From the HPLC traces it was possible to calculate the amount of pGpG produced by MbaA during the incubation step. The area under the peaks in the HPLC trace can be used to quantify the amount of whatever has eluted. The area under the c-di-GMP standard peaks corresponding to 100  $\mu$ M or 4 nanomoles per reaction was compared to the area under the pGpG peaks. There was an average of 0.38 nanomoles of pGpG produced over the 2.5 hour period by 10  $\mu$ g of MbaA fusion protein.



**Figure 14. HPLC analysis of PDE assay.** HPLC trace of MbaA WT PDE assay with  $\text{MnCl}_2$  as the cationic cofactor. C-di-GMP and pGpG peaks were identified by running pure samples of each in a separate run. MbaA was added for a final total of 10  $\mu$ g per reaction and c-di-GMP at 100  $\mu$ M.

The same assay was used to evaluate the enzymatic capacity of the mutant MbaA E553A. In this assay,  $\text{MnCl}_2$  was used as the cationic cofactor since it was this cofactor that gave a positive result in the pNPP assay. It was found that MbaA E553A was incapable of degrading c-di-GMP to pGpG (Figure 15).



**Figure 15. Phosphodiesterase assay evaluating MbaA E553A.** 10  $\mu\text{g}$  MbaA E553A was incubated with  $\text{MnCl}_2$  and 100  $\mu\text{M}$  c-di-GMP and allowed to react for 2.5 hours at 37°C. The resultant peak was identified by running a purified sample of c-di-GMP in a different run.

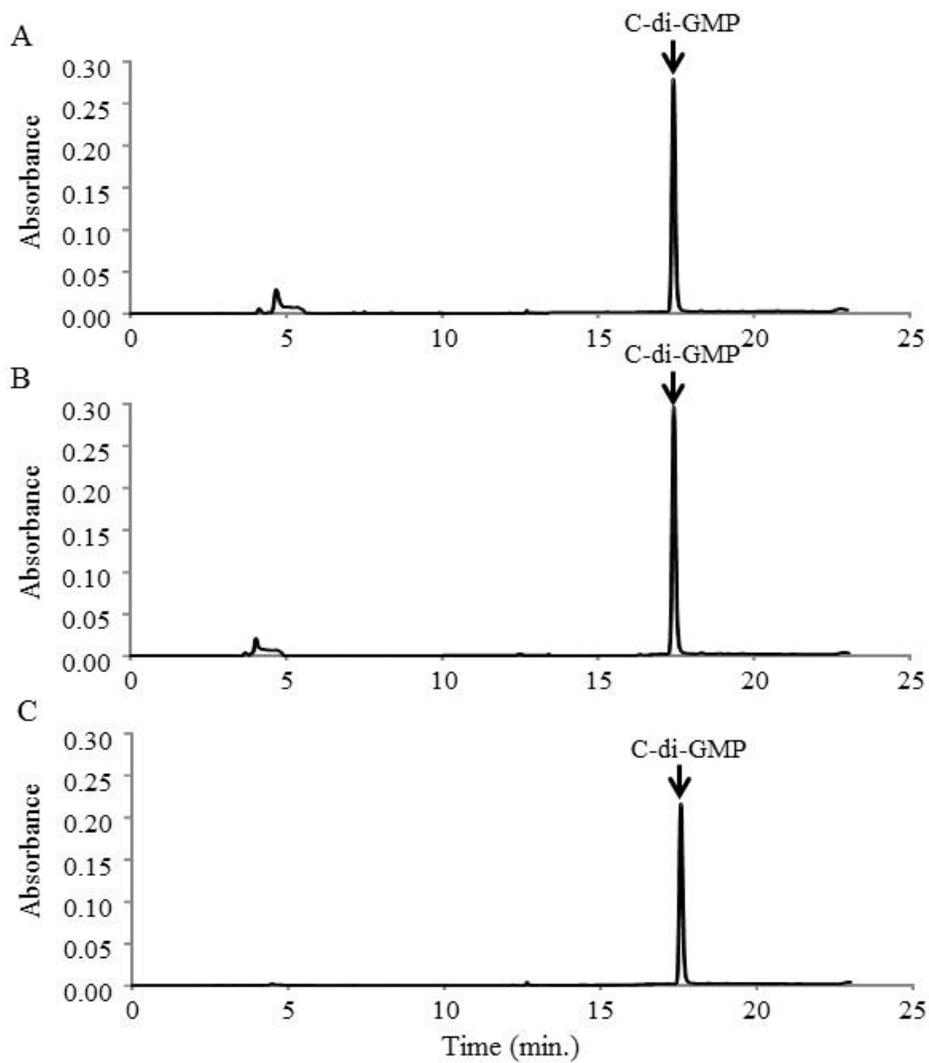
### **MbaA PDE activity requires cationic cofactors**

Phosphodiesterase proteins require a cationic cofactor for proper functioning and can be negatively impacted by other cations. Typically, either  $\text{Mg}^{2+}$  or  $\text{Mn}^{2+}$  are required for proper functioning of a PDE where  $\text{Zn}^{2+}$  and  $\text{Ca}^{2+}$  will inhibit PDE activity (37).

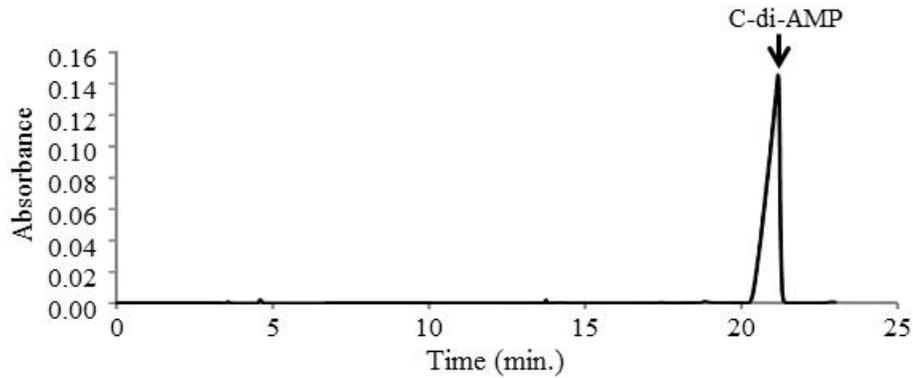
MbaA WT was placed in the same PDE assay described above only  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ , or  $\text{ZnCl}_2$  were added in place of  $\text{MnCl}_2$ . In each case, no c-di-GMP PDE activity was found (Figure 16). This shows that the PDE activity of MbaA requires  $\text{Mn}^{2+}$  and that none of the other cations added allow phosphodiesterase activity over a 2.5 hour period.

### **MbaA PDE activity is specific to c-di-GMP**

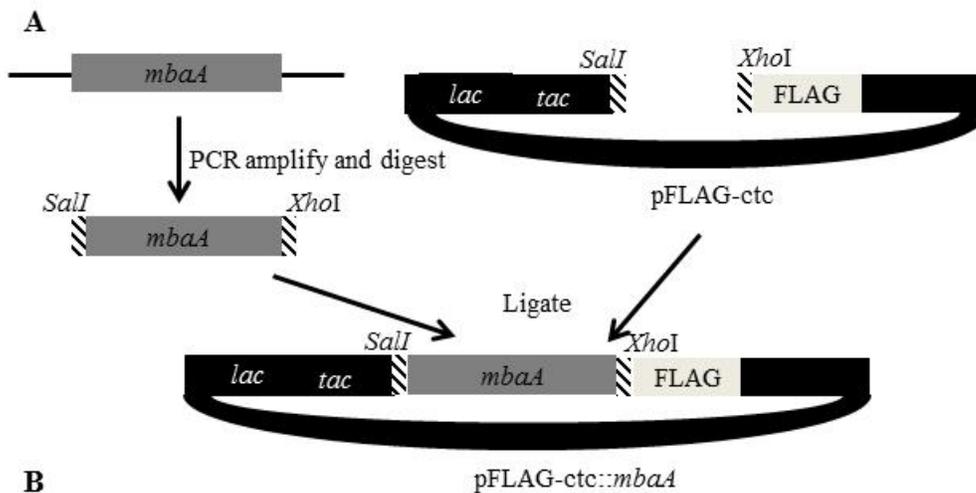
There are several cyclic nucleotides that are present in bacterial cells and so there is the possibility that MbaA will degrade several different molecules and not necessarily just c-di-GMP. To verify that MbaA PDE activity was specific for c-di-GMP, a phosphodiesterase assay was performed with c-di-AMP as a substrate and  $\text{MnCl}_2$  as the cationic cofactor. When the reaction products were separated by HPLC no peaks, aside from the c-di-AMP peak, were identified (Figure 17). This shows that MbaA is not capable of degrading c-di-AMP and is likely only active against c-di-GMP.



**Figure 16. HPLC traces of MbaA WT with varying cationic cofactors.** All PDE assays used MbaA at 10  $\mu\text{g}$  in the reaction and c-di-GMP at 100  $\mu\text{M}$ . C-di-GMP was identified by running a pure sample. (A) MbaA WT with  $\text{MgCl}_2$  as the cationic cofactor. (B) MbaA WT with  $\text{CaCl}_2$  as the cationic cofactor. (C) MbaA WT with  $\text{ZnCl}_2$  as the cationic cofactor.



**Figure 17. MbaA WT phosphodiesterase assay with c-di-AMP.** 10  $\mu$ g MbaA WT was incubated with 2 mM  $MnCl_2$  and 100  $\mu$ M c-di-AMP for 2.5 hours at 37°C. C-di-AMP was identified by running a standard during another run.



**Figure 18. pFLAG-ctc cloning.** (A) Proposed cloning strategy for pFLAG-ctc plasmids carrying *mbaA* (2 kbp) and *mbaA E553A* (2 kbp). (B) Agarose gel of *mbaA* amplification from genomic DNA. Fragment is 2 kbp and is comprised of the entire *mbaA* gene

### **Construction of a *V. cholerae*-compatible expression plasmid for *mbaA***

The phosphodiesterase MbaA has the ability to degrade c-di-GMP which should have an effect on biofilm development in *V. cholerae*. To determine the effect of MbaA and MbaA E553A on biofilm development, two plasmid constructs should be synthesized. The genes for *mbaA* and the relevant mutants were amplified and digested for insertion into pFLAG-ctc (Figure 18). The cloning of *mbaA* into pFLAG-ctc was not successful due to complications in achieving complete digestion. The pFLAG-ctc plasmid attaches a FLAG tag to the gene inserted into the multiple cloning site. The pFLAG::*mbaA* construct would be transformed into *V. cholerae* lacking a chromosomal copy of *mbaA*.

## Discussion

The periplasmic protein NspS was found to be capable of binding the polyamine norspermidine. Binding was assessed by a Thermal Shift Assay (TSA), which is based on the principle that ligand binding proteins should be more thermally stable when bound to their ligand. NspS alone was found to have a thermal stability of 45°C. The thermal stability reported is the temperature at which half of the protein has denatured or the rate of change in fluorescence is at its maximum. When norspermidine was added to a purified sample of NspS and subjected to the same gradual increase in temperature, the protein had a thermal stability of 55°C. This increase in thermal stability is indicative of a binding event between NspS and norspermidine, its predicted ligand. Addition of spermidine to purified samples of NspS also showed a 10°C shift in the thermal stability of NspS indicating spermidine binding. In an effort to show that binding to NspS was specific, the polyamines putrescine and cadaverine were added to purified samples of NspS. The thermal stability of NspS with added cadaverine or putrescine was approximately 46°C, which is a negligible difference compared to NspS alone indicating there was not a binding event. These data show that NspS is capable of binding norspermidine and spermidine, providing a mechanism for *V. cholerae* to detect those polyamines.

Previous experiments in our lab have shown that addition of spermidine to *V. cholerae* cultures inhibits biofilm formation in a NspS-dependent manner (24).

Spermidine is structurally very similar to norspermidine so it is not surprising that spermidine might bind NspS. What is unexpected is that there is a pronounced phenotypic difference in the effect of spermidine and norspermidine on biofilms. The TSA used in this study to show binding of protein to its ligand does not provide a  $K_d$  value so it is difficult to see if spermidine or norspermidine bind more tightly to NspS. It is evident, however, that they both stabilize NspS to a similar degree; therefore, the binding affinity to NspS for the two polyamines are likely to be comparable. This is the first step in the model for norspermidine regulation of biofilms in *V. cholerae*. The next goal of this study was to provide evidence that MbaA degrades the second messenger c-di-GMP.

The current study has shown that MbaA has the ability to act as a c-di-GMP specific phosphodiesterase. Two separate assays showed that not only is MbaA capable of degrading phosphodiester bonds but that it degrades the phosphodiester bonds in c-di-GMP specifically. The first assay was based on the degradation of the substrate pNPP. This assay showed that MbaA is an active phosphodiesterase and that  $Mn^{2+}$  was required as a cationic cofactor. This assay was also used to evaluate the E553A mutant that should not have phosphodiesterase activity. The pNPP assay showed that when MbaA E553A was combined with pNPP and  $MnCl_2$  at 37°C for 2.5 hours a yellow color developed. This was unexpected, as the E to A substitution in E553A should have resulted in a loss of function. It also raised a lot of questions since the publication that was the source of this method also assayed an E to A mutation protein and found a loss of activity (38). This, however, does not rule out that the glutamate at position 553 is required for the enzymatic degradation of c-di-GMP. It is possible that the mutation of the glutamate to

alanine removed phosphodiesterase activity against c-di-GMP but not phosphodiester bonds in other molecules.

The second phosphodiesterase assay involved incubating a purified sample of MbaA and c-di-GMP again with  $\text{MnCl}_2$  as the source of  $\text{Mn}^{2+}$ . This reaction was filtered and the reaction products were separated by HPLC. This assay showed again that MbaA was a functional phosphodiesterase indicated by the production of a pGpG peak after incubation of the protein with c-di-GMP. The second phosphodiesterase assay also showed that MbaA E553A is not an active phosphodiesterase of c-di-GMP. After a 2.5 hour incubation of MbaA E553A with c-di-GMP and  $\text{MnCl}_2$ , no pGpG was detected during HPLC separation. These data confirm that the glutamic acid residue at position 553 of MbaA is required for enzymatic degradation of c-di-GMP and that MbaA behaves similarly to other phosphodiesterases.

Cationic cofactors are necessary for the function phosphodiesterases. In the literature, four cations are mentioned and used in studies of phosphodiesterases:  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  (37, 39). The cations associated with catalyzing phosphodiesterase activity are  $\text{Mn}^{2+}$  and  $\text{Mg}^{+}$  while the cations that inhibit activity are  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ . The pNPP assay described was performed prior to the HPLC phosphodiesterase assay and was used to determine that  $\text{Mn}^{2+}$  was the preferred cofactor. When the cations  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  were added to phosphodiesterase assays there was no detectable production of pGpG indicating that their presence was not sufficient for MbaA activity. This is consistent with published literature on phosphodiesterases.

Since the protein used in the phosphodiesterase assays was a fusion protein of maltose binding protein and MbaA, the  $k_{\text{cat}}$  of the reaction was not calculated. However,

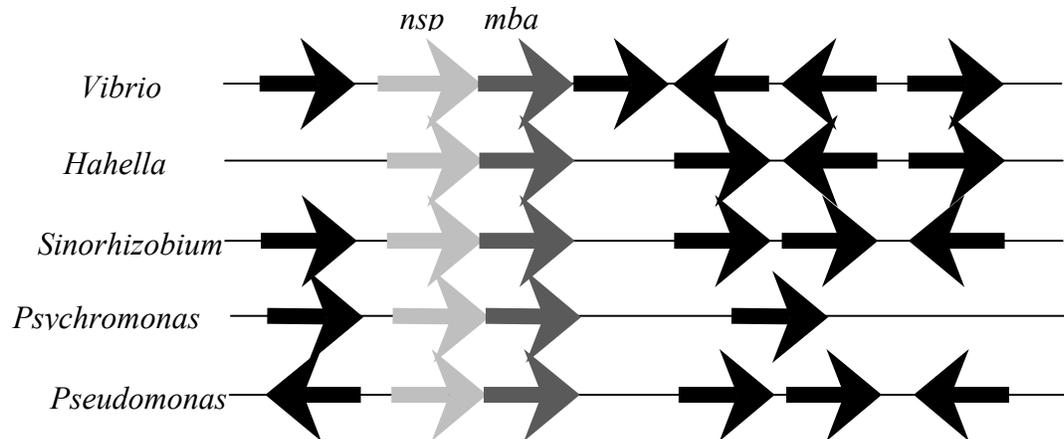
it is possible to compare the apparent activity of MbaA to other phosphodiesterases. The phosphodiesterase RocR from *Pseudomonas aeruginosa* has a  $k_{\text{cat}}$  of  $0.67 \text{ s}^{-1}$  (40). This value means that RocR produces one molecule of pGpG approximately every two seconds. We did not calculate the  $k_{\text{cat}}$  of MbaA but we do know that there was on average 0.38 nanomoles of pGpG produced by MbaA over 2.5 hours. This equates to an average of  $2.29 \times 10^{14}$  molecules of pGpG produced during the reaction by a total of  $4.82 \times 10^{14}$  molecules of MbaA. Over the course of 2.5 hours then, MbaA is producing on average, 0.47 molecules of pGpG per molecule of MbaA per second. The  $k_{\text{cat}}$  of RocR is slightly higher, but the apparent activity of MbaA seems to be comparable to that enzyme. The reported activity of MbaA has a large amount of variation that makes absolute quantification of its enzymatic capacity difficult. This discrepancy between replicates could be a result of using an MbaA/MBP fusion protein in the PDE assays. Since PDE proteins are predicted to act as homodimers, it is possible that the MBP fusion partner is interfering with that dimerization in an unpredictable fashion. Even though MbaA PDE activity may not be as robust as a fusion protein it was shown to have the ability to degrade c-di-GMP. This information then supports the norspermidine based biofilm signaling model proposed earlier.

It is clear that there is a signaling cascade in *V. cholerae* that responds to norspermidine and affects biofilm development. Further, it is clear that NspS and MbaA are both involved in that signaling at least in part by their interaction as well as the binding capacity of NspS and PDE activity of MbaA. This study did, however, reveal a point that must be addressed. The periplasmic binding protein NspS was found to bind spermidine in addition to norspermidine. As stated earlier, spermidine has the inverse

effect on biofilm formation as norspermidine so additional study is required to evaluate that interaction. I hypothesize that spermidine somehow blocks the inhibitory function of NspS on MbaA. This lack of attenuation could be through an inhibition of NspS/MbaA interaction with NspS is bound to spermidine or a change in the conformation that allows binding but no effect on MbaA activity.

A NspS/MbaA like signaling system could represent a new class of environmental signal transduction in bacteria. This new class of signaling system would be comprised of a periplasmic signaling protein and a membrane bound interacting partner that degrades c-di-GMP. Bioinformatic analysis shows that *nspS* and *mbaA* like genes are not found in all *Vibrio* species. For example, *nspS* and *mbaA* are absent from *Vibrio vulnificus* and *Vibrio fisheri*. Further bioinformatic analysis shows that *nspS* and *mbaA* like genes are found together in species distantly related to *V. cholerae* (Figure 19) (22, 23). When *nspS*-and *mbaA*-like genes are found in these other species they are not located in the same genomic context. This could suggest that *mbaA* and *nspS* like genes function together and could be found in disparate species due potentially to horizontal gene transfer. If the presence of *nspS* and *mbaA* in multiple species is the result of horizontal gene transfer then the question arises of how the organisms the genes are found are connected. An example of a complication of showing gene transfer is that *nspS* and *mbaA* genes are found in *V. cholerae* an aquatic pathogen and *Psychromonas ingrahamii* first isolated from polar ice. *nspS* and *mbaA* are found together in several species, suggesting that the two are acting together potentially in a similar manner to those in *V. cholerae*. We have shown that NspS and MbaA function together in *V. cholerae* and that homologs of the genes coding for NspS and MbaA are found in several disparate species. This

indicates that a NspS and MbaA like signaling system could be prevalent in bacteria but several questions remain.



**Figure 19. Genomic position of *nspS* and *mbaA* like genes in disparate species.** The figure depicts the genomic position based on bioinformatic analysis of *nspS* and *mbaA* homologs in several species distantly related to *V. cholerae*. The light and dark grey arrows show the position and orientation of *nspS*, *mbaA* and their homologs on the chromosome. Black arrows are genes found surrounding *nspS* and *mbaA* like sequences. Black arrows also indicate genes that do not have homologs in the other genome regions presented. There is no conserved synteny in any of the genomic regions in this figure indicating that the genomic region depicted is different for every species.

The protein NspS was shown to have the ability to bind norspermidine and spermidine. Further, previous research from our lab has shown that spermidine and norspermidine have inverse effects on biofilm formation. The binding data obtained in this study are not capable of contributing to the calculation of the binding affinity for NspS and its ligands. To better understand the interaction of NspS and its ligands then, analysis by Isothermal Titration Calorimetry is recommended (ITC). A better understanding of how tightly NspS binds either norspermidine or spermidine could indicate which ligand binds more strongly for example. Knowing the binding affinity of NspS could then indicate a more specific role for NspS in polyamine signaling. When considering the different phenotypes observed after addition of norspermidine or

spermidine, understanding the interaction of MbaA and NspS is necessary to make any conclusions. To better analyze NspS/MbaA binding, a series of co-immunoprecipitation experiments with varying concentrations of polyamines would determine the effect of interaction. Lastly, MbaA has been confirmed as a phosphodiesterase indicated by the production of a pGpG peak after incubation with c-di-GMP. Phosphodiesterase proteins are often characterized by their reaction rate or  $k_{cat}$ . This information allows phosphodiesterase proteins to be compared and could give insight into the polyamine signaling complex. With the wide range of pGpG production observed in the PDE assay experiments a pure sample of MbaA lacking the MBP fusion partner will be necessary. Once the MbaA construct is free of the fusion partner, the same PDE assay should be performed and analyzed at different time points with varying concentrations of c-di-GMP.

This study provides data for the first norspermidine-based signaling system in bacteria as well as the potential for a new model of environmental signal detection and response. In bacteria, especially in c-di-GMP based signal transduction, there is little knowledge of specific signal inputs. Therefore the information presented here is significant in providing evidence for a novel signaling system complete with identity of the environmental input and a clear phenotypic response.

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### **Biographical Sketch**

Steven Randolph Cockerell was born in Memphis, TN and raised in Mount Airy, NC. He graduated from the University of North Carolina Asheville with a Bachelor of Science in Cell and Molecular Biology in December of 2010. After receiving a Master of Science degree in Cell and Molecular Biology from Appalachian State University in August of 2013 he will pursue a career in Brewing and Fermentation Science.