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By

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Abstract

Cyclic-di-GMP, a common bacterial second messenger, has been thought to help develop virulence and biofilms in bacteria, most specifically in *Salmonella* Typhimurium. By being able to dysregulate cyclic-di-GMP production, virulence may be better combatted. STM1987, an L-arginine-responsive diguanylate cyclase with a periplasmic sensory domain, dimerizes and generates the bacterial second messenger cyclic-di-GMP in response to the amino acid L-arginine in a pathway that also requires the periplasmic L-arginine-binding protein ArtI. Their biochemical responses to L-arginine and when they dimerize could help clarify this pathway, so I sought to develop a periplasmic dimerization sensor to better monitor these biochemical interactions. Similar to STM1987, the ToxR transcriptional regulator from *Vibrio cholera* is also activated by dimerization. By switching out the periplasmic domain of ToxR for the periplasmic regions of interest, I can better evaluate the cyclic-di-GMP response to L-arginine. This research aims to find the specific responses in this pathway to be able to use this in combatting bacterial virulence. I was able to successfully show that the STM1987 periplasmic domain dimerizes in response to L-arginine, providing an important insight into this signaling pathway.

Acknowledgments

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Introduction

Salmonella Typhimurium

Salmonella Typhimurium is a Gram negative, facultative anaerobic bacillus (*Salmonella - Medical Microbiology - NCBI Bookshelf*, n.d.). Existing around the world, *S.* Typhimurium is a food borne pathogen that causes Salmonellosis and produces a range of symptoms from common gastroenteritis to enteric fevers. While most healthy people will recover without treatment, the infection can spread beyond the intestines, causing enteric fevers that are life-threatening (*Salmonella Infection - Symptoms and Causes - Mayo Clinic*, n.d.). In addition to problems caused by curable *Salmonella* infections, *S.* Typhimurium has increasingly become resistant to many antibiotics including amoxicillin and chloramphenicol, amongst others (Umair & Siddiqui, 2020). One of the mechanisms which assists *S.* Typhimurium in antibiotic resistance is by invading and surviving within the host's own cells.

To successfully invade and survive within host cells, *S.* Typhimurium must adhere to, invade, survive, and proliferate in these host cells. Macrophage infection by *S.* Typhimurium primarily is driven by the phagocytosis process that is employed to prevent systemic infection in most cases. In epithelial cells, this invasion process involves bacterial secretory system proteins. After either of these invasions, S. Typhimurium can either be killed by the cell, or proliferate within the cell (Gog et al., 2012). One mechanism that can assist with this survival mechanism and to promote virulence in mice is low levels of c-di-GMP concentrations after phagocytosis by macrophages (Petersen et al., 2019). To better understand if this is a possibility, a system to analyze these interactions may be useful.

Cyclic-di-GMP

Biofilms and Motility

Bacterial signaling and behavior is facilitated by first and second messengers, where extracellular first messengers regulate levels of cytoplasmic second messengers to carry a signal to effector proteins (Newton et al., 2016). Cyclic-di-GMP (c-di-GMP) is a common second messenger present in bacteria that coordinates many different aspects of bacterial growth, signaling, and behavior including motility, virulence, biofilm formation, and cell cycle progression. Usually, microorganisms are freely suspended, but biofilms cause these microorganisms to aggregate and be enclosed by a material primarily composed of polysaccharides (Donlan, 2002). C-di-GMP has also been shown to have an effect on bacterial motility, which is critical in bacterial virulence (Sun et al., 2018). Whereas high levels of c-di-GMP have been shown to increase the incidence of biofilm formation, low levels of c-di-GMP have been shown to increase motility.

Diguanylate Cyclases and Phosphodiesterases

Synthesis and degradation of c-di-GMP is controlled by diguanylate cyclases (DGCs) and c-di-GMP-specific phosphodiesterases (PDEs) respectively (Jenal et al., 2017). DCGs encode GGDEF domains to combine two molecules of GTP to produce one molecule of c-di-GMP, while PDEs encode either EAL or HD-GYP domains to hydrolyze c-di-GMP to a linear pGpG to reverse the process (Hengge, 2016). There are usually multiple GGDEF, EAL, and HD-GYP domains present in bacterial genomes, suggesting that the regulation of c-di-GMP is highly complex and dependent on many factors (Sisti et al., 2013). These DGCs and PDEs typically contain sensory domains that induce dimerization of the respective protein, activating its

enzymatic ability. This then links c-di-GMP to the sensing of specific environmental cues, affecting the appropriate alteration in bacterial physiology and gene expression.

Bacterial Sensing Through Periplasmic Domains

There are several ways that bacteria can sample the environment, communicate with each other, and then elicit the appropriate responses in order to survive. Periplasmic binding proteins are receptors in the periplasm of bacteria that detect changes and mediate various processes such as transport, chemotaxis, quorum sensing, biofilm formation, and motility. Because these periplasmic proteins can affect biofilm formation and motility, they can play a role in antibiotic resistance (Borrok et al., 2009). Two-component systems are another key system in bacterial signal transduction. Typically comprised of a histidine kinase for sensing external input signals and a response regulator to communicate the appropriate change in the physiology of the bacteria, several studies have shown that two-component systems are crucial for expression of virulence factors, viability, and growth (Tiwari et al., 2017). When the histidine kinase is activated by a signal, the kinase activity is then activated, and autophosphorylation of itself then occurs. This phosphorylated kinase can then transfer that phosphate to the response regulator, stimulating a down-stream response.

L-Arginine Sensing in a Cyclic-di-GMP Pathway

Many organisms contain several c-di-GMP modulating enzymes with periplasmic sensory domains, but there are few that have been linked to particular signals that modulate their activity. As shown by Mills et. al., some of these signals include L-arginine, glucose, salicylic acid, and N-acetyl-D-glucosamine. Of these, N-acetyl-D-glucosamine, glucose, and L-arginine

were shown to increase c-di-GMP, while salicylic acid was shown to decrease c-di-GMP. L-arginine was shown to produce the largest change in c-di-GMP and at the lowest concentration of all the compounds tested (Mills et al., 2015). This study also showed that this response is specific to L-arginine.

Salmonella Typhimurium's genome contains 17 cyclic-di-GMP metabolizing enzymes, including the diguanylate cyclase STM1987. This previous study identified an *S.* Typhimurium c-d-GMP pathway that responded to L-arginine by increasing c-di-GMP concentrations through activation of the DGC STM1987. This pathway also required an L-arginine sensing periplasmic binding protein, ArtI, but the exact mechanism of ArtI's interaction with STM1987 is unknown. ArtI may induce dimerization of STM1987 to increase the concentration of free c-di-GMP (Mills et al., 2015). Figuring out the exact mechanism of this process is crucial to better understanding this regulatory pathway, and hopefully to find a way to decrease incidence of bacterial virulence.

ToxR

Role in *Vibrio cholerae*

Vibrio cholerae, the bacteria that causes cholera, has multiple mechanisms for its pathogenesis. Both the *tcp* operon, which codes for the toxin coregulated pilus, and the *ctx* operon, which codes for cholera enterotoxin, must be expressed. This process is regulated by two membrane proteins, ToxR and ToxS (Bina et al., 2003). ToxR is a transmembrane DNA binding protein that positively controls transcription of genes for the cholera toxin and contributes to its pathogenicity (Parsot & Mekalanos, 1990). For this system to work, the transmembrane receptor ToxS must detect an appropriate signal within the periplasm, induce dimerization of ToxR, and

stimulate expression from ToxR-regulated promoters. The *ctx* promoter then activates to allow *Vibrio cholerae* to adapt appropriately to its environment.

Use as a Sensor

Because ToxR in *Vibrio cholerae* dimerizes to produce an appropriate response, similar to DGCs and PDEs, this mechanism is very adaptable with the proteins of interest. The ToxR system is well understood and can be used as a model for c-di-GMP enzyme dimerization. By replacing the periplasmic fragment of ToxR for the periplasmic regions of interest that are related to c-di-GMP production, we can detect dimerization-induced transcriptional expression of different reporters to measure mCherry for fluorescence, CamR for chloramphenicol resistance, and LacZ(Y) for orange and blue colorimetric. This system and figuring out the pathways is crucial because we can use *Salmonella* Typhimurium as a model organism, and once we figure this pathway out, any periplasmic signaling protein can be put in place and expanded to other bacteria and other signals.

Plans

After finding appropriate conditions for optimal growth, this system can then be translated for use in other bacteria and signaling proteins, which is the long-term goal. For this experiment specifically, once the optimal mCherry reporter conditions have been found, we can then transition to using chloramphenicol resistance and LacZ(Y) as reporters. But first, I sought to test my hypothesis that L-arginine results in dimerization of the STM1987 periplasmic domain, and that this dimerization could be detected through our ToxR Dimerization Reporter system.

Methods and Materials

Bacterial Culture Conditions

Standard growth of bacteria was conducted in LB broth at 37°C shaking at 250rpm. Bacteria for L-arginine testing was grown for about 24 hours at 37°C, shaking in 2mL of minimal media. This minimal media consists of .5X M63 salts (21 mM K_2HPO_4, 11mM KH_2PO_4, 3.8 mM $(NH_4)_2SO_4$, ~3.8 mM KOH) (Mills et al., 2015), 1X essential MEM amino acids, 1X nonessential MEM amino acids, 0.23% glycerol, 1mM $MgCl_2$, 10uM $FeSO_4$, 1 mM NaCl, and autoclaved MilliQ water. When necessary, 100µg/mL ampicillin, 50µg/ml kanamycin, or 34µg/ml chloramphenicol was included for strain selection. The various bacterial strains used are shown in Table 1.

Name	Strain	Source
Mach1	*E. coli* str. W Δ*recA1398 endA1 fhuA* Φ80Δ(*lac*)M15 Δ(*lac*)X74 *hsdR*(r_K–m_K^+)	Invitrogen
EM012	*Salmonella enterica* serovar Typhimurium 14028	ATCC
O395	*Vibrio cholerae*	ATCC
MG1655	*Escherichia coli*	ATCC
DMST001	14028, pEP392	This study
DMST002	14028, pEP421	This study
DMST003	14028, pEP413	This study
DMST004	14028, pEP404	This study
DMST005	14028, pEP414	This study
JHST001	14028, pEP455	This study
JHST002	14028, pEP451	This study
JHST003	14028, pEP449	This study
JHST004	14028, pEP450	This study
JHST005	14028, pEP416	This study

Table 1: Bacterial Strains Used.

Cloning

The method used to create these plasmids with our proteins of interest was a method called Gibson cloning; this method requires three enzymatic activities, including a 5’

exonuclease to generate long overhangs, a polymerase to fill in the gaps of the annealed single stranded regions, and a DNA ligase to seal the nicks of the annealed and gaps that were filled in (Gibson et al., 2009). The plasmids used are shown in Table 2.

Name	Plasmid	Ab resistance	Description
pUC19	pUC19	Amp	New England Biolabs
pMMBA	pMMB67EH	Amp	(Fürste et al., 1986)
pEP392	pUC-TDR4-emptymCherry	Amp	TDR4 construct containing the empty mCherry reporter
pEP421	pUC-TDR4-1987mCherry	Amp	TDR4 construct containing the 1987 periplasmic fragment and the mCherry reporter
pEP413	pUC-TDR4-3615mCherry	Amp	TDR4 construct containing the 3615 periplasmic fragment and the mCherry reporter
pEP404	pUC-TDR4-ArtImCherry	Amp	TDR4 construct containing the ArtI periplasmic gene and the mCherry reporter
pEP414	pUC-TDR4-PhoQmCherry	Amp	TDR4 construct containing the PhoQ periplasmic fragment and the mCherry reporter
pEP416	pMMBA-TDR4-empty-mC	Amp	pMMBA containing empty TDR4 construct with mC reporter
pEP455	pMMBA-TDR4-1987-mC	Amp	TDR4 construct in pMMBA with mC reporter and 1987 periplasmic domain
pEP451	pMMBA-TDR4-3615-mCherry	Amp	TDR4 construct in pMMBA with mCherry reporter and 3615 periplasmic domain
pEP449	pMMBA-TDR4-ArtI-mCherry	Amp	TDR4 construct in pMMBA with mCherry reporter and ArtI periplasmic domain
pEP450	pMMBA-TDR4-PhoQ-mCherry	Amp	TDR4 construct in pMMBA with mCherry reporter and PhoQ periplasmic domain
	mCherry donor		(Shaner et al., 2004)
CS944	pKD3	Cam/Amp	Template vector for frt-Cam-frt lambda red marker, in R6kγ backbone (Datsenko & Wanner, 2000)

Table 2: Plasmids Used in this Study

Primer Name	Sequence	Use
rrnB Term Gfwd	5’-GGCTGTTTTGGCGGATGAGAGAAG-3’	Gibson primer within rrnB terminator to clone into MCS with rrnB terminator
pMMB GRvs1	5’-GCCGACCTGTATGAACGC-3’	Primers in backbone to amplify pMMB fragments for Gibson cloning
pMMB GFwd1	5’-CATGCCGGAGTTCGTCG-3’	
pMMB GRvs2	5’-CATCCGCTGGCAGTCCTA-3’	
pMMB GFwd2	5’-GCAGCTCGGTACTGGTC-3’	
Ptac MCS GRvs	5’-CAATTTCACACAGGAAACAGAATTCG-3’	Primer to amplify through the Ptac promoter to clone into Ptac MCS
TDR4 Ptac-ToxR	5’-ATAACAATTTCACACAGGAAACAGAATTCG ATGTTCGGATTAGGACACAACTCAAAAGAG-3’	Primer to amplify TDR4 at ToxR end for insertion into Ptac MCS
TDR4 rrnBTerm-PctxA	5’-GAAAATCTTCTCTCATCCGCCAAAACAGCC TACCGTTACCGGATTTCAATCACTTTGTGG-3’	Primer to amplify TDR4 at PctxA end for insertion into rrnB MCS (Ptac)
Cam GFwd	5’ - TATTTTTCTGTTAAACAAAGGGAGCATTAT ATGGAGAAAAAAATCACTGGATATACCACC - 3’	Primers to insert Cam marker into reporter plasmid
Cam GRvs	5’- GGATGGCCTTTTTGCGTTTCTACAAACTCT TCATCGCAGTACTGTTGTATTCATTAAGCA - 3'	
mCherry GFwd	5’ - TATTTTTCTGTTAAACAAAGGGAGCATTAT ATGGTGAGCAAGGGCGAGGAGGATAACATG - 3’	Primers to insert mCherry into reporter plasmid
mCherry GRvs	5’ - GGATGGCCTTTTTGCGTTTCTACAAACTCT TTACTTGTACAGCTCGTCCATGCCGCCGGT - 3’	
LacZY GFwd	5’ - TATTTTTCTGTTAAACAAAGGGAGCATTAT ATGACCATGATTACGGATTCACTGGCCGTC - 3’	Primers to insert LacZY into reporter plasmid
LacZY GRvs	5’ - GGATGGCCTTTTTGCGTTTCTACAAACTCT TTAAGCGACTTCATTCACCTGACGACGCAG - 3’	
LacZ GRvs	5’ - GGATGGCCTTTTTGCGTTTCTACAAACTCT GTTATTATTATTTTTGACACCAGACCAACT - 3’	Reverse primer to insert LacZ into reporter plasmid
rrnB Term Gfwd (Gateway Fragment)	5’-GGCTGTTTTGGCGGATGAGAGAAG-3’	Gibson primer within rrnB terminator to clone into MCS with rrnB terminator
pMMB2GRvs1 (Gateway Fragment)	5’-CAACAGCGAGGCAGCATG-3’	Primers to improve efficiency within

pMMB2GFwd1 (Gateway Fragment)	5’-GCATGGAGCCGAAAA-3’	pMMB backbone for Gibson reactions
pMMB2GRvs2 (Gateway Fragment)	5’-CATCCGCTGGCAGTCCTA-3’	
pMMB2GFwd2 (Gateway Fragment)	5’-GCAGCTCGGTACTGGTC-3’	
Ptac MCS GRvs (Gateway Fragment)	5’-CAATTTCACACAGGAAACAGAATTCG-3’	Primer to amplify through the Ptac promoter to clone into Ptac MCS

Table 3: Primers Used in this Study

Gibson PCR cloning fragments were generated using the primers found in Table 3, Phusion DNA Polymerase (ThermoFisher), dNTPs, buffer, and DNA template. Coding sequence for the ToxR DNA binding/transmembrane domain and the *ctxA* promoter were cloned from the *V. cholerae* O395 genome. The transcriptional terminator was cloned from the pBAD24 vector, while the mCherry fragment was cloned from a lab fluorescent expression vector. We also generated a CamR reporter using the pKD3 (Datsenko & Wanner, 2000)plasmid as a template, and a LacZ(Y) reporter using the *E. coli* MG1655 genomic DNA as a template, but we were able to achieve our aims without using these reporters yet. These components were first Gibson cloned into the high copy pUC vector to generate a ToxR-empty control. Periplasmic regions of interest were then cloned from the *S.* Typhimurium 14028 genome into this vector to generate C-terminal fusions to the ToxR fragment. The periplasmic domain of STM1987 and the entire ArtI periplasmic binding protein without secretion signal peptide were cloned to test their dimerization in response to L-arginine. As controls, the periplasmic domains from another c-di-GMP metabolizing enzyme (STM3615) and the histidine kinase PhoQ (Dubey et al., 2016) were also cloned into the ToxR vector. Subsequent constructs were transferred to the low copy

pMMBA vector. Both pUC and pMMBA contain an ampicillin resistance gene to ensure that the plasmid was successfully inserted.

Conjugation and Phage Transduction

To get these plasmids into the *Salmonella*, two methods were used: conjugation/phage transduction and electroporation. The conjugation/phage transduction protocol began by growing up 2ml overnight cultures with ampicillin (pUC or pMMBA), an antibiotic-resistant *S.* Typhimurium strain (a previously constructed Δ*sseG*::Cam strain was used), and the pRK2013 conjugation helper strain. The next day, these were washed twice in LB without antibiotics by centrifuging and resuspending. Strains were then combined in the triparental mating mixture (helper/donor/recipient), centrifuged, the pellet was resuspended in 50µl, and it was plated to a dried LB plate to allow conjugation to proceed. After an overnight incubation at 37°C, these conjugation spots were struck to LB agar plates containing ampicillin and chloramphenicol to select for *S.* Typhimurium bacteria that had acquired the pUC or pMMBA vector.

To transfer the bacteria from the *ΔsseG::*Cam to wild type *S.* Typhimurium, a mutant variant of the P22 phage that indiscriminately packages random DNA was used to transduce the vector (Casjens et al., 1988). Phage transduction began with a 2mL LB overnight cultures with ampicillin of the donor Δ*sseG*::Cam with vector (pMMBA or pUC). The next day, 1mL of LB and 1.5ul of a bacteriophage (P22-HTInt) grown from the wild-type *Salmonella* was combined, and then 500ul of the overnight donor vector culture was added for a final phage concentration of 1:1000. This was then incubated at 37°C in the shaker overnight. At the same time, a 2mL LB culture of the recipient wild type *Salmonella* strain was started. The next day, 1mL of the phage culture was centrifuged at maximum speed to pellet the bacteria. 900ul of the supernatant was

removed and 100ul of chloroform was added to the supernatant, vortexed well, and allowed to sit for 5 minutes to kill any remaining bacteria. The sample was vortexed again and then centrifuged to separate the layers. The supernatant produced from this step is now the bacteriophage that was to be used.

To 1mL of LB, 2ul of the bacteriophage was added for a final phage concentration of 1:1000 and then mixed. 150ul each of the recipient *Salmonella* strain was added two separate tubes; to the negative control tube, 150ul of LB was added, and to the experimental tube, 150ul of the diluted bacteriophage was added. All tubes were incubated at 37°C and shaken for 1-2 hours, then each of these was plated to LB agar plates containing ampicillin. As long as the control plates did not have colonies present, colonies from the transduction plates were struck out onto green phage plates to cure the culture of phage (Schmieger, 1972). From these same plates, any single, white colonies were picked and struck out onto another phage plate to ensure no more phage is present the next day. If there are single, white colonies on these plates, they were picked the next day and grown in LB with ampicillin. This was grown overnight, and a PCR was performed on the overnight culture the next day to confirm presence of the vector. A glycerol stock was frozen down for future use.

Electroporation

As an alternative method to get the prepared plasmids into the wild-type *Salmonella*, overnight cultures of Mach1 *E. coli* containing the ToxR Dimerization Reporters were started in LB and ampicillin. A second culture of recipient wild type *Salmonella* was started in 2ml of LB. The next day, in new LB, a 1:1000 dilution of the recipient *Salmonella* strain was started, and this culture was grown for about 2 hours, or until 1 optical density (OD_{600}). ToxR Dimerization

Reporter plasmids were then purified from Mach1 *E. coli* using a miniprep kit (IBI Scientific). For every plasmid that needed to be transferred to the bacteria, 1.5 mL of the culture was centrifuged, the supernatant was decanted, and the pellet was washed and resuspended in 750ul of DNase free water. There were 3 total washes, and the supernatant was decanted again after the third wash. The bacteria were resuspended in at least 550ul of DNase free water, adding another 100ul for every sample above 5. To 97ul of electrically competent cells, 3ul of the purified plasmid was added. This was transferred to an electroporation cuvette, and an automated protocol on the electroporator was selected to shock the cells and get the plasmid in the cells. 500ul of SOC was added to the cuvette, mixed, transferred to a bigger tube, and the bacteria was grown for 1-2 hours. This was repeated for every plasmid needed. After their growth, these reactions were plated to LB agar plates containing ampicillin, single colonies were grown overnight, and these cultures were PCR tested for presence of the vector. As each sample was completed, they were frozen down in glycerol for later use.

mCherry ToxR Dimerization Reporter Assay

2mL overnight cultures of empty mCherry (mC), ArtI-mC, 1987-mC, 3615-mC, and PhoQ-mC were started and grown in M63 minimal media at 37°C. The next day, M63 without amino acids, which were replaced with water, was made, and 4 different testing conditions per strain were made. Every strain contains ampicillin, but the four different conditions were with IPTG and with L-arginine, with IPTG and without L-arginine, without IPTG and with L-arginine, and without IPTG and without L-arginine. The bacteria were added 1:100, and this was grown for 2 hours, shaking at 37°C. 200ul of each sample was added to a 96-well plate, and the mCherry fluorescence was read in the Synergy HTX plate reader (Biotix). Where noted, an

OD600 reading was also taken of the bacterial culture alongside the mCherry fluorescence readings so that we could normalize to bacterial density.

Statistical Analysis

To evaluate the response of the reporter system, first the arginine effect of each strain needed to be calculated; this was done by dividing the fluorescence amount of the strain with the arginine by the fluorescence of the same strain without the arginine. To get the relative effect, the arginine effect value for each strain was divided by the arginine effect value for the empty-mCherry. This was to normalize all the values to the negative control. Once enough data was gathered using this method, a one-way matched ANOVA test was run to test for statistical significance.

Results

ToxR Dimerization Reporter Construction

Due to the ToxR system in *Vibrio cholerae* dimerizing in a similar way to predicted c-di-GMP metabolizing enzymes, this system was adapted with my proteins of interest to test whether STM1987 dimerizes in response to L-arginine. The plasmids of interest, including empty-mCherry, 1987-mC, ArtI-mC, 3615-mC, and PhoQ-mC were generated by Gibson cloning (Figure 1). Then, electroporation, conjugation, and phage transduction were used to move these plasmids into the bacteria, which was wild-type *Salmonella* Typhimurium. To test the system itself, overnight cultures of the bacteria with different plasmids were grown overnight at 37°C while shaking in minimal media with essential amino acids. The next day, 1 mL of the overnight cultures was removed, centrifuged, and washed down twice with the same minimal media made

without amino acids. As the amino acid mixture contains L-arginine, this is required to generate a no-arginine condition. These were resuspended in 1 mL of the media, and the 2-hour cultures were started at 0.25 OD_{600} in the same minimal media without amino acids. Each plasmid had one sample with 20mM L-arginine and without L-arginine. These were again grown at 37°C while shaking, and then 200ul of each sample was added to a 96-well plate and the fluorescence was measured using the Synergy HTX plate reader (Biotix).

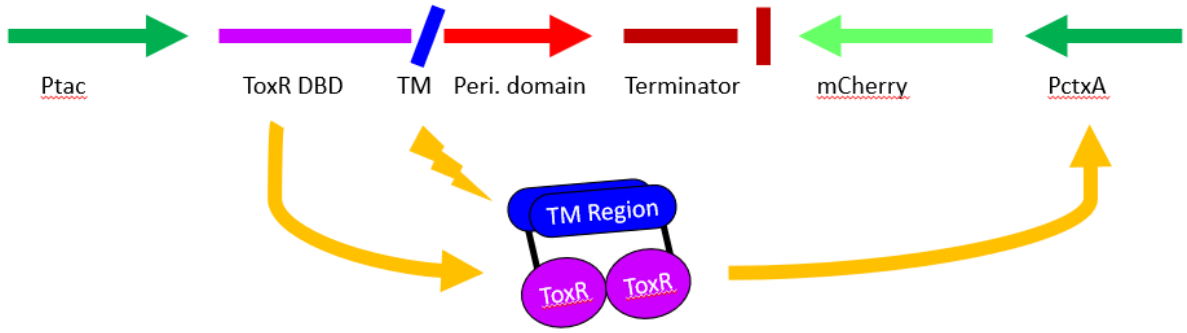

Figure 1: Plasmid map of the ToxR Dimerization Reporter. Ptac is the IPTG inducible promoter that controls expression of the ToxR fusion, ToxR DBD is the ToxR DNA binding domain, TM is the transmembrane region, the peri. domain is the periplasmic region, PctxA is the ToxR-inducible promoter, and mCherry is the fluorescence reporter.

Testing and Optimization

A large portion of this project was determining the appropriate conditions for growth. There were many adjustments and testing conditions to determine what would work best. The first set of tests used low copy pMMBA plasmids, had L-arginine at 100uM, and the IPTG was added at .5mM. We failed to see any significant results, most likely due to a mixture of insufficient conditions, so we began to optimize the assay. Among variables tested included different IPTG concentrations (0.1mM and 0.5mM), adding IPTG to the overnight cultures in addition to the two-hour cultures, giving strains 3 hours of growth the next day instead of 2

hours, or concentrating the bacteria via centrifugation and resuspension after a 2-hour growth. After comparing the fluorescence of these strains to a wild type *S.* Typhimurium strain without any vector, it was apparent that the low copy pMMBA reporter plasmids were not giving high enough fluorescence to see significant results.

We then decided to switch strategies and try the high copy pUC reporter vector. This seemed to work well, and the new protocol was to use pUC because it provides a higher copy number, and therefore higher fluorescence readings. After being grown overnight in M63 media with amino acids and ampicillin, the cultures were grown without amino acids for 2 hours with and without L-arginine the next day. The finalized protocol became to grow up the bacteria with the pUC plasmids overnight in M63 with amino acids and ampicillin, spin down 1 mL of the overnight culture the next day, wash twice with M63 media without amino acids, and resuspend in 1 mL of M63 without amino acids with ampicillin with or without L-arginine. The OD_{600} was measured, and all cultures were grown for 2 hours starting at 0.25 OD_{600}, with and without 20 mM of L-arginine, which became our standard protocol.

L-Arginine Induces Dimerization of the STM1987 Periplasmic Domain

To determine how L-arginine influences dimerization of the STM1987 and ArtI periplasmic components, we conducted the optimized protocol alongside the empty, STM3615, and PhoQ periplasmic reporters. Once the fluorescence numbers were obtained from the plate reader, various statistical analyses were conducted. First, the L-arginine effect was calculated by dividing the fluorescence of each strain with L-arginine by the fluorescence of the sample without L-arginine. This indicated how much fluorescence change there was in response to the addition of L-arginine. Then, because we considered there could be non-specific effects of L-

arginine, we normalized to the empty mCherry vector control to remove those effects from analysis. This gave us the relative arginine effect. (Figure 2). Only the STM1987 periplasmic domain exhibited a significant response to the addition of L-arginine.

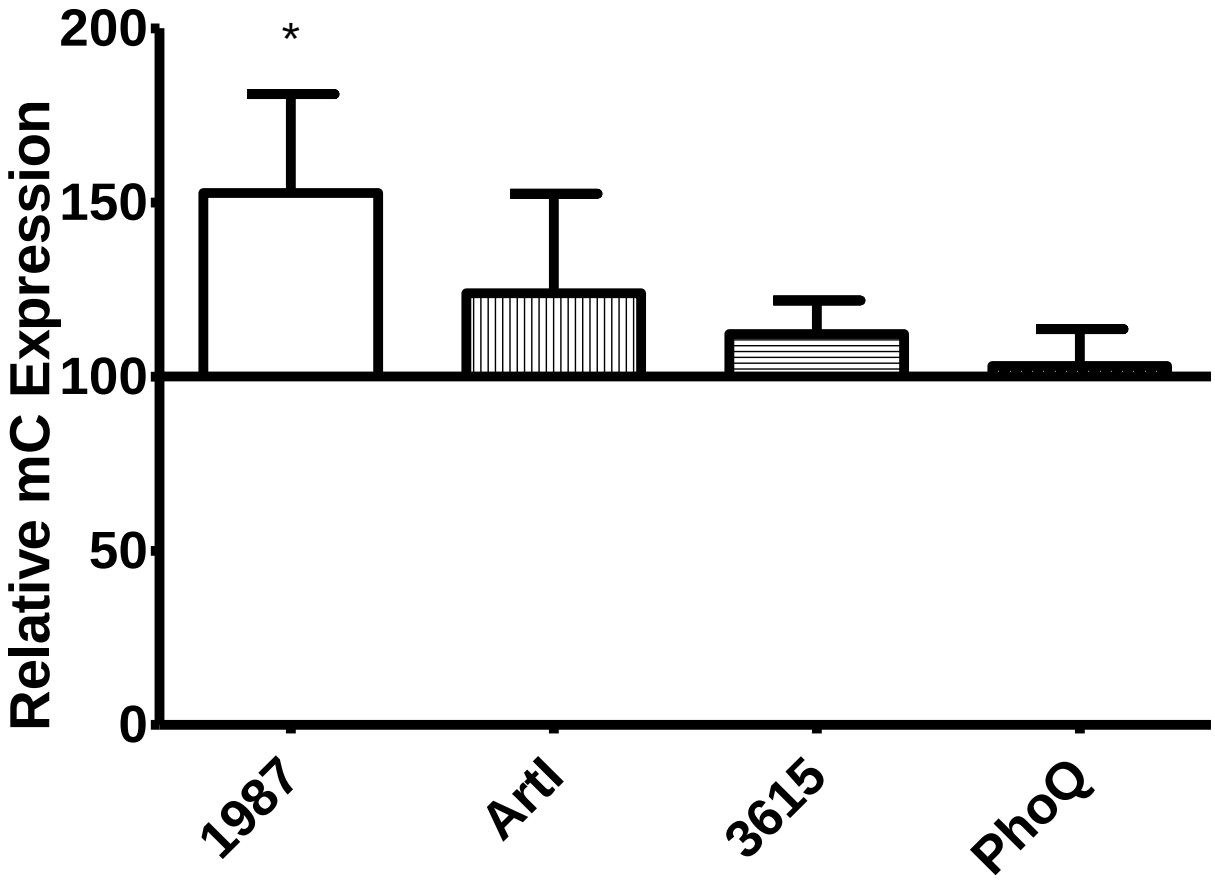

Figure 2: STM1987-ToxR fusion dimerizes in response to L-arginine. Relative mCherry expression in percentages. mCherry-empty is not included due to the relative expression being 100%, and all the other data is normalized to that. STM1987 shows statistical significance, with $p<0.05$. Statistical analysis completed using a One-Way Matched ANOVA test.

Discussion

The ToxR Dimerization Reporter was created to evaluate the biochemical interactions involved in the *Salmonella* and L-arginine system to evaluate if STM1987's sensory periplasmic

domain will dimerize in response to L-arginine. The ToxR system in *Vibrio cholerae* dimerizes in the same way that c-di-GMP metabolizing enzymes are predicted to do, so the ToxR system was adapted for use in the testing of STM1987 to determine if it does dimerize in response to L-arginine.

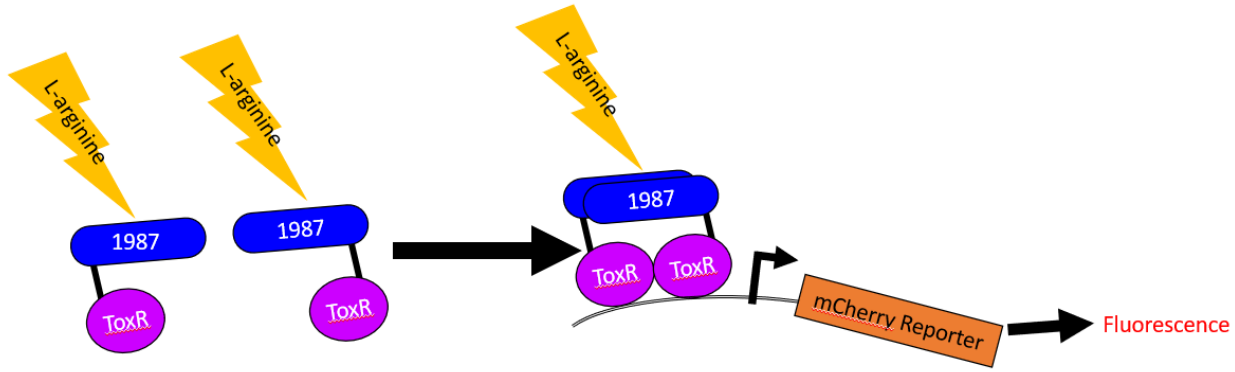

Figure 3: Model of STM1987-ToxR fusion dimerizing in response to L-arginine, causing the mCherry reporter to show fluorescence

Using my ToxR Dimerization Reporter, I was able to show that the addition of L-arginine resulted in dimerization of the ToxR fusion and expression of mCherry (Fig. 3).
This finding supports my hypothesis and opens this system up to future work discovering the mechanisms of this regulatory pathway. Interestingly, ArtI seems to show an increase in fluorescence, although results weren't statistically significant. While we anticipate that ArtI binds arginine, that does not necessarily mean that it dimerizes in response to it; based on these results, it is very possible that it does not dimerize in response. This slight upward change could be due to dimerization response but an instability in the ArtI-ToxR fusion, or could just be background. Repeating these experiments, or generating an ArtI protein deficient in L-arginine binding, may help to resolve this question. Both STM3615 and PhoQ periplasmic domains show very little change in response to L-arginine, consistent with them having no role in the L-arginine

c-di-GMP response. We believe that the fluorescence shown by STM3615 and PhoQ could be background fluorescence as these strains do not show much difference from the empty-ToxR mCherry reporter, but further testing is needed to confirm this. While the ToxR Dimerization Reporter still requires further testing, based on these results it would appear that this system does work and is selective enough that it will not cause dimerization of everything.

Future Plans

There are several avenues still to explore, both within the STM1987/ArtI/L-arginine system and expanding the ToxR Dimerization Reporter to further pathways. These experiments were mostly conducted with pUC plasmids, but pMMBA plasmids can be used as well, as this optimization proved to be difficult. Further testing can be conducted to differentiate what the original problem was, and how the fluorescence can reach the readable range. One option may be that we need to separate the ToxR fusion construct from the mCherry reporter. Placing them on separate vectors may allow us to better fine tune our results. To better understand the STM1987 dimerization, various concentrations of L-arginine can be tested to determine minimum values for this system to still work correctly. Different reporters can also be tested; here, only mCherry was tested, but LacZY, which shows blue and orange colorimetric changes, and CamR, which shows chloramphenicol resistance, can also be used as different reporters. In addition, we can also expand the STM1987 dimerization reporter into different mutant strains to determine what is necessary for this to work properly. For instance, ArtI knockout mutants can be created to see if ArtI is necessary to dimerize STM1987, as would be hypothesized due to the requirement for ArtI in L-arginine-induced activation of STM1987 c-di-GMP production. Finally, this system

can also be expanded to other periplasmic domains, as the main goal is for this system to be adaptable for anything that follows this same process.

Conclusion

Cyclic-di-GMP is a common bacterial second messenger that is thought to regulate virulence in *Salmonella* Typhimurium. By discovering the parts of these c-di-GMP pathways and their biochemical interactions, virulence may be better combatted with the disruption of these pathways. Additionally, if this pathway works, it can then be adapted to test any bacterial signal that causes dimerizes in the same way. The experimentation conducted so far has shown that STM1987, a diguanylate cyclase, does dimerize in response to L-arginine. This was shown with statistical significance, and because the negative controls used did not show statistical significance, it can be concluded that this system is selective, and that it does not cause dimerization of every plasmid that is put into this system; it only dimerizes those that should be dimerizing in response. As most c-di-GMP modulating enzymes and histidine kinase sensors of two component systems are thought to activate through dimerization, this provides a wealth of future opportunities to investigate using this ToxR Dimerization Reporter.

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