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Investigation on Bacterial Signaling through Generation of a ppGpp Biosensor

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Investigation on Bacterial Signaling through Generation of a ppGpp Biosensor

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Abstract

Guanosine tetraphosphate (ppGpp) is a bacterial signaling molecule involved in activating the stringent response, a cellular reaction to environmental stress that downregulates cell division and metabolism processes to conserve nutrients. The stringent response is implicated in some instances of antibiotic persistence, so broadening the current understanding of ppGpp signaling is useful. This thesis seeks to generate a ppGpp biosensor that will bind ppGpp and emit fluorescent light in its presence, which will allow for improved research into the pathways and functions of the signaling molecule. To generate a novel ppGpp biosensor, I converted a biosensor previously used to detect cyclic di-GMP (a different signaling molecule) to contain a binding site transformed to now bind specifically with ppGpp. The genetic sequence for the cyclic di-GMP binding site was replaced with the ppGpp hydrolase domain which has a specific affinity for ppGpp; however, hydrolase activity would provide unwanted breakdown of the ppGpp, so it is mutated further to neutralize hydrolase activity. The desired outcome of this thesis results in a biosensor with a binding site that has a specific and sufficient binding affinity for the ppGpp molecule. Using this, we can determine how ppGpp levels are regulated in bacteria under conditions of stress, and how this signaling molecule is related to the survival of bacteria in response to antibiotic treatment.

Introduction

Increasing incidences of bacterial infections that cannot be treated with antibiotics is a growing area of concern in modern medicine. Multi-drug resistant bacteria are becoming increasingly common, and these infections typically have high rates of morbidity and mortality (1). While some bacteria genetically code for resistance to antibiotics, bacteria may employ several different mechanisms for persisting in the presence of antibiotics. By monitoring bacterial sensing, these mechanisms can be better understood, and new targets may be found for treating persistent bacterial infections.

Second messenger molecules are a common sensory pathway that relay primary signals after cell surface receptors bind with a first messenger molecule. Both eukaryotes and bacteria utilize second messenger systems to maintain optimal intracellular conditions. In humans, for example, the adrenaline response elicits a production of the first messenger – epinephrine – which is circulated throughout the body and bound by cell surface receptors. In response, an increasing amount of the second messenger molecule, cyclic adenosine monophosphate (cAMP), is generated within triggered cells. Cyclic AMP targets protein kinase A to stimulate glucose production and prepare the body for intense activity (2). Bacteria use second messengers in a similar way, with first messengers relaying information about the nutritional environment and growth conditions, and second messengers influencing intracellular activity for the bacteria to grow optimally.

Roles and Regulation of ppGpp:

One important bacterial second messenger is guanosine tetraphosphate (ppGpp). In *E. coli* and many other bacterial organisms, a buildup of ppGpp activates stress survival genes and

genes that upregulate amino acid biosynthesis, while conversely inhibiting DNA primase, ribosomal RNA synthesis, and rapid growth genes (3). This means that, in the presence of high ppGpp, bacteria will slow or halt growth processes in favor of nutrient production and survival processes. These conditions are typically a response to amino acid starvation like that seen during the stationary phase of liquid culture growth, but can also arise due to other nutrient stress.

ppGpp production in *E. coli* is regulated by the RelA and SpoT enzymes, both of which have synthetase activity that produce ppGpp by transferring pyrophosphate from adenosine triphosphate (ATP) to a guanine diphosphate (GDP) when nutrient deprivation occurs. Upon return to a nutritional environment, ppGpp is degraded by the additional hydrolase domain of the SpoT protein, which removes a pyrophosphate and leaves GDP (4). Among the targets of ppGpp is RNA polymerase, which is responsible for triggering slowed growth and increased stress and survival functions.

This situation of increased survival and limited growth of bacteria is known as the stringent response. I hypothesized that the stringent response is potentially linked with antibiotic persistence, a state which allows bacteria or colonies to persist in the presence of antibiotics by shutting down division, growth, and the uptake of these environmental antibiotics (3). While persistence differs from outright antibiotic resistance – which refers to a genetic ability to evade specific antibiotics – antibiotic persistence may still be a key area of focus in overcoming growing challenges with antibiotic-persistent infections.

Detecting ppGpp

While research into ppGpp and its role in bacterial persistence is ongoing, we would like to create a biosensor to detect bacterial ppGpp levels for further investigation. In order to achieve this, a fluorescent resonance energy transfer (FRET) mechanism can be used to visualize and quantify ppGpp levels using fluorescent imaging or detection of fluorescent output in a plate reader. FRET-based biosensors are designed so that two fluorescent protein domains flank a binding domain to detect a desired molecule. When the fluorescent domains are close together, excitation of one will permit light to transfer between the biosensors that in turn excites the second fluorescent domain (5). If the distance between the two fluorescent domains increases, FRET fluorescence decreases while emission from the first fluorescent domain increases. When the target molecule is bound binds to the biosensor, it undergoes a conformational shift and the distance between the two fluorescent domains is altered. By quantifying the amount of FRET fluorescence versus emission from the first fluorescent domain, we can determine the relative number of biosensor molecules in either the bound or unbound state. This fluorescent output can be quantified with a fluorescent plate reader or visualized with fluorescent microscopy. As the binding of the target molecule to the biosensor is concentration dependent, we can then approximate the intracellular concentration of the target molecule.

Our lab has access to a FRET-based biosensor that detects the bacterial second messenger cyclic di-GMP, containing the cyclic di-GMP-binding domain YcgR flanked by teal and orange fluorescent domains (6). The construction of this biosensor would work for detection of ppGpp if the c-di-GMP binding site were swapped for a new one with affinity for ppGpp. Since the SpoT hydrolase domain has the needed affinity for ppGpp, we can incorporate that domain with cloning to result in a FRET-based ppGpp biosensor (**Figure 1**).

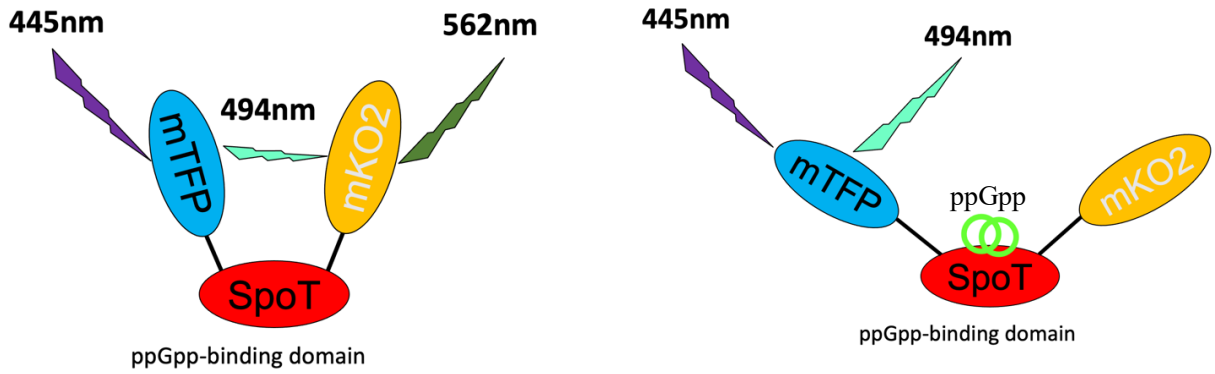

Figure 1. ppGpp Biosensor Design. In the unbound conformation, fluorescent light from the teal protein may transfer to the orange protein to produce a FRET wavelength of 562 nm. Upon binding ppGpp, a conformational change may increase the distance between fluorescent domains, decreasing FRET fluorescence and increasing emission of the teal wavelength at 494 nm.

Methods and Materials

Bacterial culture conditions

E. coli cultures used throughout were grown in lysogeny broth (LB) in 15mL tubes at 37ºC with shaking at 250 rpm. Mach 1 *E. coli* were used as electrocompetent cells for transformation, and DH5α *E. coli* were used for chemically competent transformation. Ampicillin at a concentration of 100 µg/mL was used to select for transformed *E. coli* colonies. Our colonies were plated on LB agar plates, which were typically incubated overnight at 37ºC.

Vector construction

Gibson cloning (7) was used for construction of our biosensor vectors. PCR reactions for Gibson cloning fragments included 5 µL of 5x Phusion HF buffer, 0.25 µL of Phusion DNA

polymerase (ThermoFisher), sufficient dNTPs, DNA template, and oligonucleotides were used per 25 µL reaction. Confirmation of vector construction was accomplished through 10µl PCR reactions using a 2X GoTaq DNA polymerase master mix (Promega). PCR conditions were set at 98ºC for denaturing, 55ºC for annealing, and 72ºC for extension. Initial denaturing at 98°C took place over 3 minutes. 40 PCR cycles were run with 30 seconds of denaturing, 30 seconds of annealing, and an extension time equal to 0.5 minutes per kilobase of product per cycle. Final extension ran 5-10 minutes before holding overnight at 12ºC. PCR fragments to be used in Gibson cloning reactions were treated with the restriction enzyme *DpnI* to degrade any remaining parent vector template to prevent carryover into the transformation. Gibson assembly facilitates the joining of our DNA fragments by adding complementary overlaps to the fragments, which are first electrophoresed in agarose gel. Desired sized fragments are cut from the gel and purified, then an incubation at 50°C for one hour with the Gibson assembly mix joins the complementary DNA fragments.

To transform our newly assembled DNA, competent cells are utilized (**Table 1)**. Competent cells have inducibly permeable membranes to facilitate transformation; chemically competent cells create pores with addition of $CaCl_2$, and electrocompetent cells are induced with electric pulse. Chemically competent cells were transformed by thawing on ice, mixing with the vector, and heat shocking at 42°C for 30 seconds. Electrocompetent cells were subcultured from an overnight culture, grown to an OD600 of about 1.0, washed three times in water, and mixed with the transformation vector. 100µl was added to a 0.1cm electroporation cuvette that was then shocked at 1.8kV. Both sets of transformants were resuspended in 500µl of SOC media, grown for an hour, and plated to antibiotic-containing agar plates. Plasmids were isolated from successful transformants using the IBI miniprep kit and protocol (9). These plasmids were

subjected to confirmation PCR using *Taq* DNA polymerase and the indicated oligonucleotides. PCR-confirmed vectors were submitted for sequencing and the culture was frozen down at -80°C in 20% glycerol for storage.

Table 1 – Bacterial strains used in this study		
Strain	Genotype	Source
MG1655	*E. coli*	ATCC
Mach1	*E. coli* str. W Δ*recA1398 endA1 fhuA Φ80*Δ*(lac)M15* Δ*(lac)X74 hsdR(rK–mK+)*	Invitrogen
DH5α	*E. coli fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Thermo Fisher
BL21 (DE3)	*E. coli fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS λ DE3 = λ sBamHIo* Δ*EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21* Δ*nin5*	Thermo Fisher

Table 2 – Plasmid vectors used in this study			
Name	Plasmid	Ab resistance	Description
pBAD-Tealspy	pBAD18-Teal-YcgR-mKO2	Ampicillin	Expression vector for cyclic di-GMP biosensor (6)
EM775	pET15b-Teal-BcsA-mKO2	Ampicillin	Biosensor purification vector for cyclic di-GMP biosensor (6)

pEP458	pBAD-Teal-SpoT-mKO2	Ampicillin	Expression vector for ppGpp biosensor with active hydrolase domain
pEP459	pBAD-Teal-SpoTSDM-mKO2	Ampicillin	Expression vector for ppGpp biosensor with mutated hydrolase domain
pEP430	pET15b-Teal-SpoTSDM-mKO2	Ampicillin	Biosensor purification vector for ppGpp biosensor with mutated hydrolase domain

Table 3 – Oligonucleotides used in this study		
Primer	Sequence	Purpose
SpoT Hyd GFwd	AAC TCC ACC GAC GGC ATG GAC GAG CTG TAC AAG ACT AGT AGC GGT GAA CCC TAT ATC ACG	Generate the SpoT hydrolase enzymatic domain PCR fragment for biosensor construction
SpoT Hyd GRvs	A CCT CAT CTT CAT CTC TGG TTT AAT CAC ACT GAC GGT ACC GTC CGG GCG AAG TGA G	
Teal-mKO2 YcgRSwap GFwd	GGT ACC GTC AGT GTG ATT AAA CC	Generate vector PCR fragments to swap YcgR domain with SpoT domain
Teal-mKO2 YcgRSwap GRvs	ACT AGT CTT GTA CAG CTC GTC C	
EM410	GGA TGG CAT GAC AGT AAG AGA ATT ATG CAG TGC	

EM411	GCA CTG CAT AAT TCT CTT ACT GTC ATG CCA TCC	
SpoT Hyd-SDM-GFwd	CG GCG CTG CTG CAT AAC GTG ATT GAA GAT AC	Site directed mutagenesis (SDM) primers to block hydrolase activity
SpoT Hyd-SDM-GRvs	GT ATC TTC AAT CAC GTT ATG CAG CAG CGC CG	
LacIq3' GFwd	CAC CAG TGA GAC GGG CAA C	Incorporate sensor into expression vector
LacIq3' GRvs	CAG GCG GTG AAG GGC	
TFP Fwd		
TFP Rvs		

Results and Discussion

ppGpp messaging is an important area to study in bacteria because of increasing multi-drug resistant infections. Antimicrobial resistance is considered a crisis, and the World Health Organization has identified resistant Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* as critical bacteria for new treatment studies (9). Furthermore, there is additional threat from antibiotic tolerant bacteria which produce persister cells that remain dormant in the presence of harmful antibiotics (9). These persistent cells are often implicated in diseases with great morbidity and mortality – like recurrent infection in cystic fibrosis patients. If ppGpp is implicated in triggering bacterial persister cells, understanding its roles in doing so could lead to essential new treatment options.

The creation of our ppGpp biosensor will allow for further studies on the effects of ppGpp as a bacterial second messenger. Most understanding of ppGpp comes from studies on *E.*

coli, but the ppGpp biosensor should be useful in investigating ppGpp levels in other species as well (9). It can also be used to monitor how ppGpp levels fluctuate with the cell cycle, and it can solidify ppGpp's role in the stringent response.

To create the biosensor, the ppGpp hydrolase domain of the SpoT protein was found to be suitable in its binding affinity to create a ppGpp binding site. Using Gibson cloning (7), the SpoT hydrolase domain was inserted in place of a cyclic di-GMP-binding domain (YcgR) from a previously published biosensor (6). The PCR reactions from **Table 4** will give us two PCR fragments from the biosensor vector and the SpoT hydrolase domain (Figure 2). These were then Gibson cloned together, transformed into DH5α *E. coli*, and plated onto LB agar plates containing ampicillin to detect successful transformants.

Table 4: Gibson PCR for Exchanging YcgR for SpoT in Biosensor Vector

Gibson PCR	**Primer 1**	**Primer 2**	**Template**	**Size**
SpoT Hydrolase	GFwd	GRvs	MG1655 genomic DNA	339bp
SpyV1	YcgRSwap Fwd	EM411	pBAD-Tealspy	1.55kb
SpyV2	EM410	YcgRSwap Rvs	pBAD-Tealspy	4.44kb

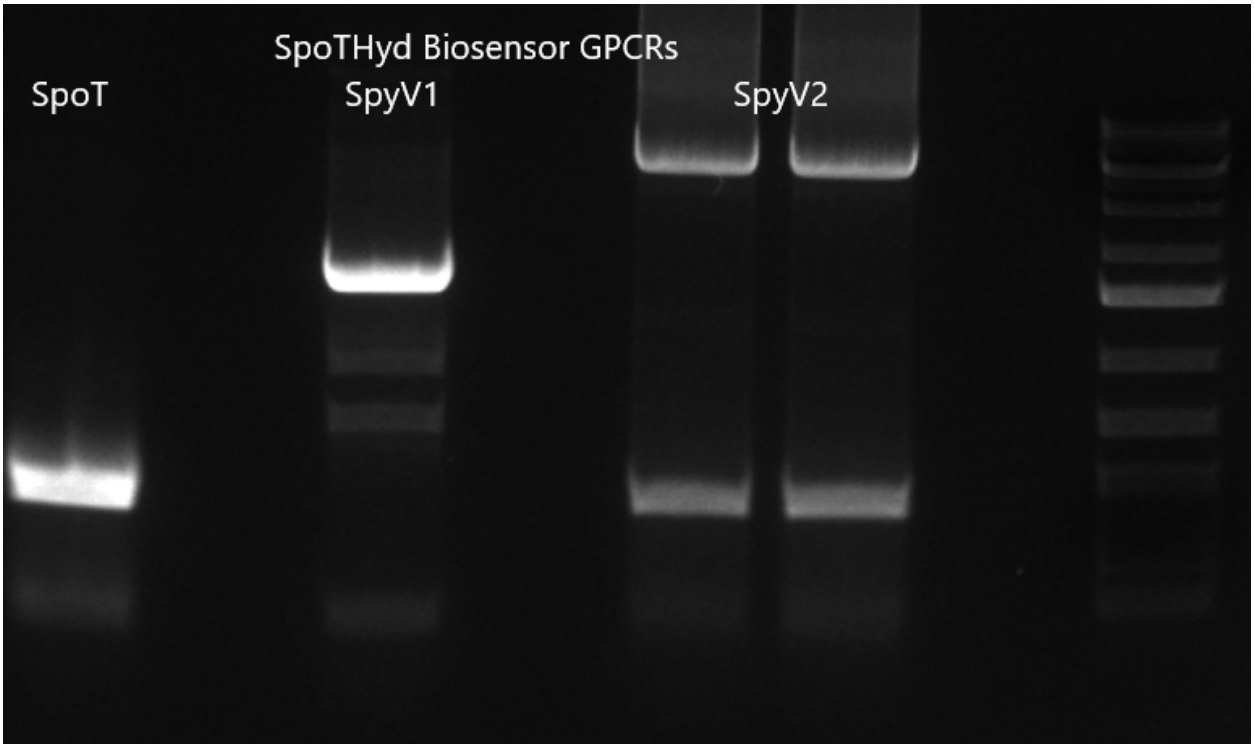

Figure 2. Gel electrophoresis of YcgR-SpoT exchange PCR fragments. The SpoT, SpyV1, and SpyV2 produce the expected sizes of fragments – 339bp, 1.55kb, and 4.44kb respectively – as compared to the GeneRuler 1kb plus (ThermoScientific) ladder (right).

Confirmation of Cloning:

Successful transformants were grown up and tested with PCR followed by gel electrophoresis to confirm the desired insert took place. Tests (**Table 5)** were run to confirm the desired presence of SpoT, and to ensure the YcgR was lost. Since the desired transformation took place, there was a 1.1 kB fragment in the SpoT tests, and despite some fragment in YcgR lanes, it is assumed they are only background because there is no presence of a 0.77kb fragment.

Table 5: Cloning Confirmation PCR			
PCR	**Primer 1**	**Primer 2**	**Size**

SpoT Test (S)	pFD TealSpy Fwd	SpoTHyd Biosensor GRvs	1.1 kB
YcgR Test (Y)	pFD TealSpy Fwd	pBAD18Spec YcgR GRvs	0.77 kB

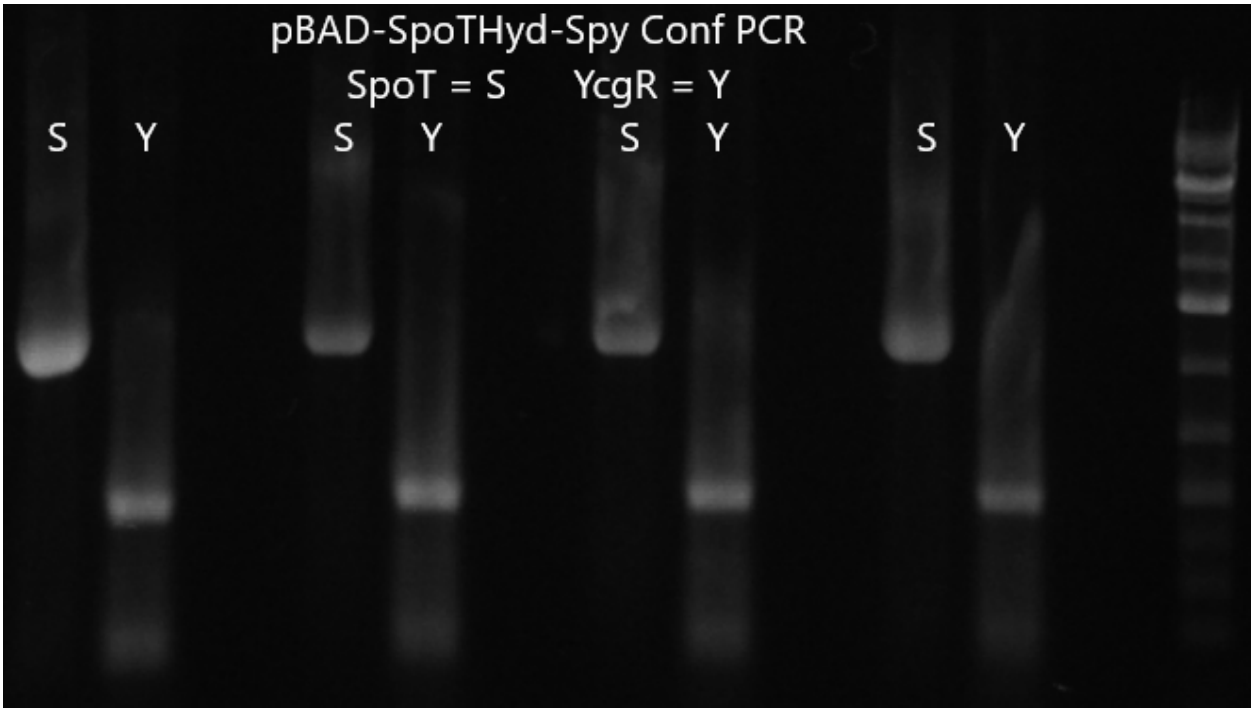

Figure 3: Gel electrophoresis to confirm desired exchange of SpoT for YcgR in the biosensor vector. This electrophoresis confirms the desired presence of the SpoT fragment and a lack of YcgR as compared to the GeneRuler 1kb plus (ThermoScientific) ladder (right).

Site Directed Mutagenesis:

The ppGpp hydrolase domain of SpoT had the specific binding affinity needed in our new biosensor, but it would facilitate unwanted breakdown of the messenger because the SpoT hydrolase domain is responsible for breaking down ppGpp. After swapping the domains into the biosensor, I incorporated a mutation into the SpoT hydrolase domain to halt digestion of ppGpp

but ideally maintain the binding affinity. By switching aspartic acid amino acid D78 to an asparagine (N), the domain should be useful as a ppGpp active site (**Table 6**) (10). This mutation is incorporated through the site directed mutagenesis (SDM) PCR reaction in **Table 7.**

Table 6: Point Mutation to Block Hydrolase Activity	
Wild Type Sequence	TG ATG GCG GCG CTG CTG CAT **GAC** GTG ATT GAA GAT ACT CCC
Desired Sequence after SDM	TG ATG GCG GCG CTG CTG CAT **AAC** GTG ATT GAA GAT ACT CCC

Table 7: Site-Directed Mutagenesis of SpoT Hydrolase			
Gibson PCR	**Primer 1**	**Primer 2**	**Size**
PCR #1	SpoTHyd-SDM-GFwd	EM410	1.8 kB
PCR #2	SpoTHyd-SDM-GRvs	EM411	4.6 kB

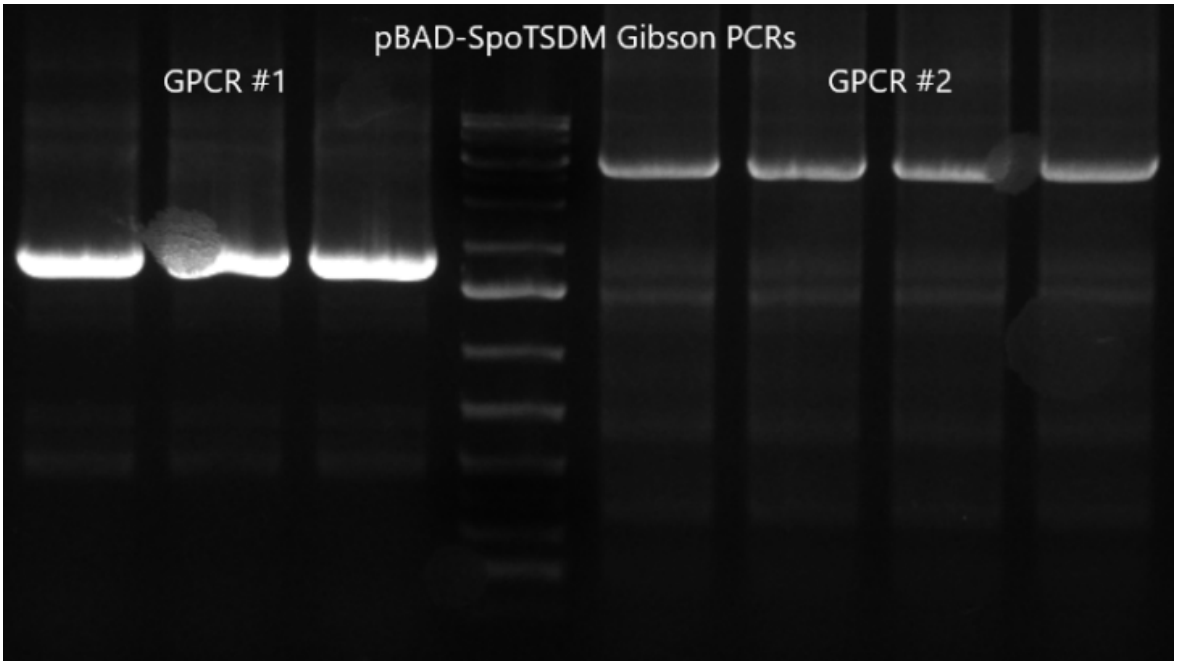

Figure 4. Gel electrophoresis of SDM products. The gel electrophoresis confirms the desired 1.8kb fragment from PCR #1, and 4.6kb fragment from PCR #2 as compared to the GeneRuler 1kb plus (ThermoScientific) ladder (middle).

These two PCR fragments were gel extracted, ligated together in a Gibson cloning reaction, and transformed into *E. coli*. Successful transformants were cultured and purified plasmids were sequenced to confirm the correct mutation and insertion took place (**Figure 5).**

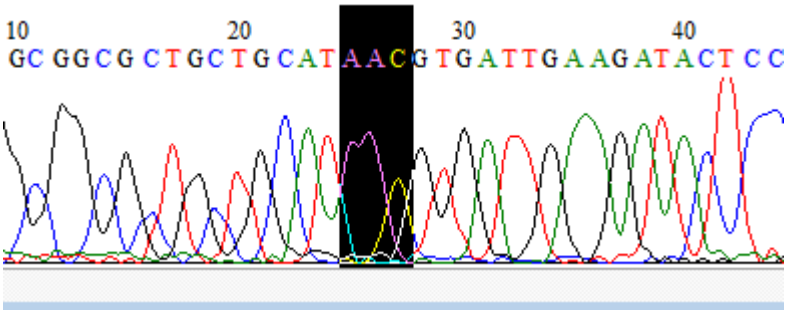

Figure 5. Genetic sequence of SDM product. The sequence of our mutated product confirms that the desired GAC->AAC mutation was incorporated.

Knowing the correct mutation was incorporated, we wanted to then transfer the ppGpp biosensor construct to a protein purification vector. This purification vector will allow for the biosensor to be His-tagged, expressed, and purified from BL21(DE3) *E. coli* cells. The ppGpp biosensor construct was incorporated into a pET15b expression plasmid via the PCR reaction in **Table 8.** Gel electrophoresis (**Figure 6**) confirmed the correct fragments, although the SpoT hydrolase fragment ran out of the gel and had to be rerun and directly purified.

Table 8: PCR for incorporation with expression vector

PCR	**Primer 1**	**Primer 2**	**Template**	**Size**
SpoTSDM Domain	GFwd	GRvs	pBAD-SpoT-SDM	0.4kb
pET15b #1	Teal-mKO2 YcgRSwap GFwd	EM411	pET15b-BcsATealspy	1.6kb
pET15b #2	EM410	LacIq3' GRvs	pET15b-BcsATealspy	3.4kb
pET15b #3	LacIq3' GFwd	Teal-mKO2 YcgRSwap GRvs	pET15b-BcsATealspy	2.2kb

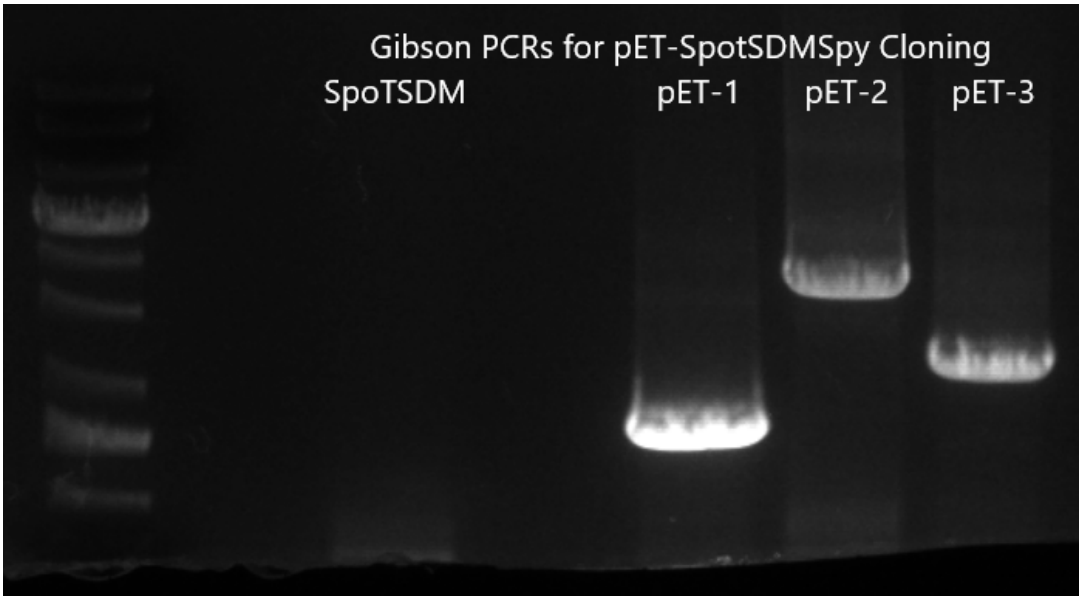

Figure 6. Gel Electrophoresis of Expression Plasmid Cloning. Despite the SpoT fragment running out of the gel, we confirmed SpoT fragment along with three correct vector bands as compared to the GeneRuler 1kb plus (ThermoScientific) ladder (left).

After confirming correct fragments were present for the vector insert, the fragments were purified, Gibson cloned, transformed into *E. coli*, and grown up on ampicillin. Plasmids from three colonies were isolated for a final PCR to confirm our biosensor was located in the pET15b vector (**Table 9**). According to our final gel electrophoresis (**Figure 7**), we had the desired bands to confirm our biosensor was successful. Finally, our biosensor is ready for protein expression via BL21(DE3) cells designed for easy uptake and expression of our vector.

Table 9: pET15b Insertion Confirmation PCR			
PCR	**Primer 1**	**Primer 2**	**Size**
Confirmation of insertion into pET15b	LacIq 3’ GFwd	SpoT biosensor GRvs	2.6 kB

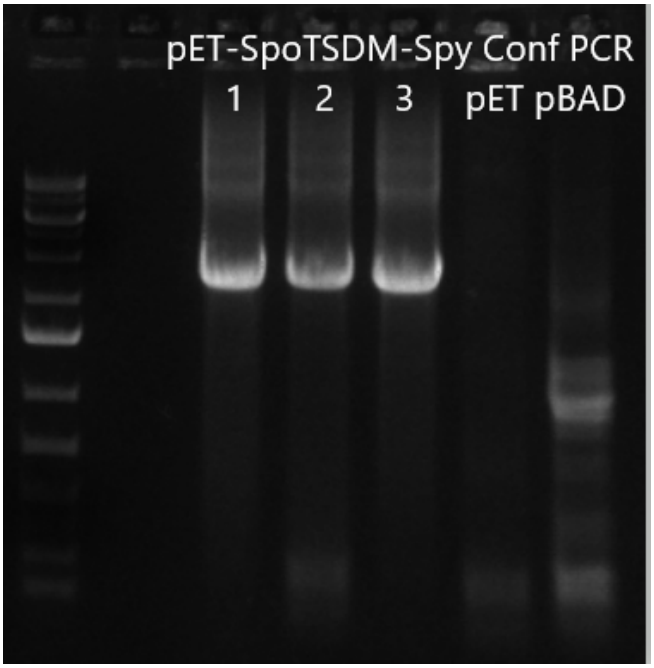

Figure 7. Gel Electrophoresis for confirmation of biosensor insert into vector. Our final gel electrophoresis confirms the presence of our biosensor in each of the colonies grown up tested as compared to the GeneRuler 1kb plus (ThermoScientific) ladder (left).

Now that our biosensor is ready for protein expression, we can test its efficacy as a ppGpp biosensor. This includes comparing the binding of wild type and SDM versions to ensure the SDM did not diminish affinity for ppGpp. It also includes confirming that the binding affinity is specific to ppGpp and that other molecules (GTP, GDP, etc) do not produce a signal. In vitro, hydrolase activity can be inhibited with the presence of certain ions, which is how we can accurately compare wild type and SDM versions. The biosensor can be used to monitor fluctuating ppGpp levels during the cell cycle and under different nutrient conditions to increase understanding of ppGpp's roles. It can also be used to test and compare ppGpp levels within different species of bacteria. With a successful ppGpp biosensor that can accomplish these

studies, it is possible that new understanding of bacterial persistence will be achieved, and that this knowledge could be used to address growing concerns with multi-drug resistance.

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