August 2017

An Inducible Fluorescent Reporter System to Measure Lux Operon Promoter Activity

Nicole Thunes
University of Wisconsin-Milwaukee

AN INDUCIBLE FLUORESCENT REPORTER SYSTEM TO MEASURE *LUX* OPERON PROMOTER ACTIVITY

by

Nicole Thunes

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Biological Sciences

at

The University of Wisconsin-Milwaukee

August 2017

ABSTRACT

AN INDUCIBLE FLUORESCENT REPORTER SYSTEM TO MEASURE *LUX* OPERON PROMOTER ACTIVITY

by

Nicole Thunes

The University of Wisconsin-Milwaukee, 2017
Under the Supervision of Dr. Charles Wimpee

Bioluminescence is the enzymatic production of light by a living organism. Many species of marine bacteria produce light with varying degrees of brightness. The *lux* operon is responsible for bioluminescence and is well studied, however it is currently unknown why different species of bacteria display different brightness levels. A dual-plasmid system designed to mimic the quorum-sensing induction of the *lux* operon was created and successfully implemented in *E. coli*. This was accomplished through the use of an arabinose-inducible plasmid containing a *luxR* gene from *Vibrio harveyi,* and then using the resulting LuxR protein to activate the *lux* promoter in a second plasmid. The second plasmid was created using a new vector containing a fluorescent reporter. An upstream region from a *Vibrio* species containing a promoter for the *lux* operon could then be inserted into the plasmid vector and induced using the previously made LuxR plasmid. The fluorescence and luminescence levels of different strains were compared in the hopes to better understand the impact of promoter activity on light production.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Introduction

Bioluminescence is the production of light enzymatically by a living organism. It is found in a variety of organisms such as bacteria, fish, squid, jellyfish, fungi, fireflies, and unicellular eukaryotes [5]. It is thought to have evolved independently up to 30 separate times, meaning that the ways these organisms produce light and the genes involved can vary substantially [8]. In the case of bacteria, luminescence is thought to have only evolved once, based on the similarity of the genes involved. These bacteria are also rather closely related, found in only three families of *Gammaproteobacteria*: *Enterobacteriaceae, Shewanellaceae,* and *Vibrionaceae* [6]. It is important to note that while only these families contain luminous bacteria, most members are actually non-luminous.

The genes responsible for light production in bacteria are contained in the *lux* operon. The basic *lux* operon has a common gene organization of *luxCDABE* [5, 6].

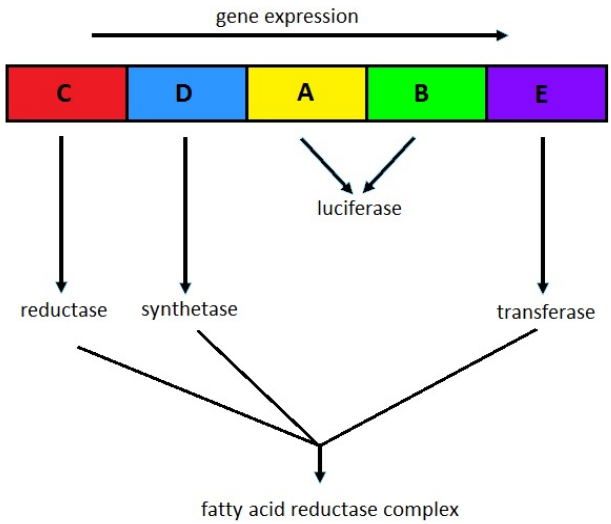


Figure 1. *Lux* Operon gene arrangement and function.

LuxAB encodes luciferase, a heterodimeric protein with alpha and beta subunits. *LuxCDE* encodes a fatty acid reductase complex, which synthesizes fatty aldehydes for the luminescence reaction. More specifically, LuxC acts as a reductase, LuxD is a synthetase, and LuxE is a transferase [5, 6]. *LuxG* is often present in the operon, and encodes a flavin reductase [5, 6, 12]. Other lux genes are sometimes present as well, but are not critical for the reaction to occur.

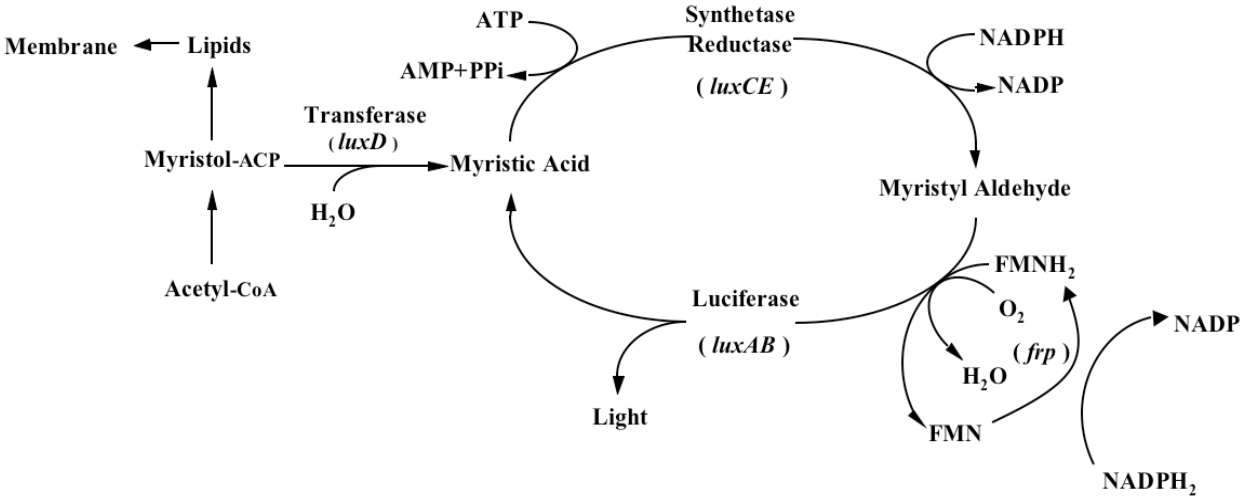


Figure 2. Bacterial luciferase reaction produced by the *lux* operon genes. Close et al. 2012

During the reaction, a long chain aldehyde is oxidized along with a reduced flavin mononucleotide ($FMNH_2$). This results in a long chain fatty acid, oxidized FMN, water, and emission of light (~490nm wavelength). Luciferase catalyzes the reaction [4, 5, 6, 7].

Many bacteria regulate bioluminescence through quorum sensing. There are differences in the way various species achieve this, but the quorum-sensing system of *Vibrio harveyi* is very well-studied and was used specifically as a model for the dual-plasmid induction system used in this

experiment. *V. harveyi* has a system of three parallel quorum sensing pathways. Autoinducer concentration (AI-1, AI-2, and CAI-1) indirectly regulates *luxR* mRNA translation [1, 3, 6, 14, 17]. *LuxR* is not located near the other *lux* genes, but encodes a transcription factor used to activate the *lux* operon [5, 6, 14, 15, 17]. LuxR is capable of binding to at least two sites upstream of the *lux* operon, although it has been shown that the binding site closest to the operon is the most critical [10]. A third binding site is thought to be located after the start of transcription [3]. At low cell densities, autoinducer concentration is also low, and *luxR* is not translated. At higher cell densities, autoinducer concentration is high, and *luxR* is translated. LuxR binds to a site (or multiple sites) in the promoter region of the *lux* operon, allowing for transcription of the *lux* genes and leading to light production [6, 17].

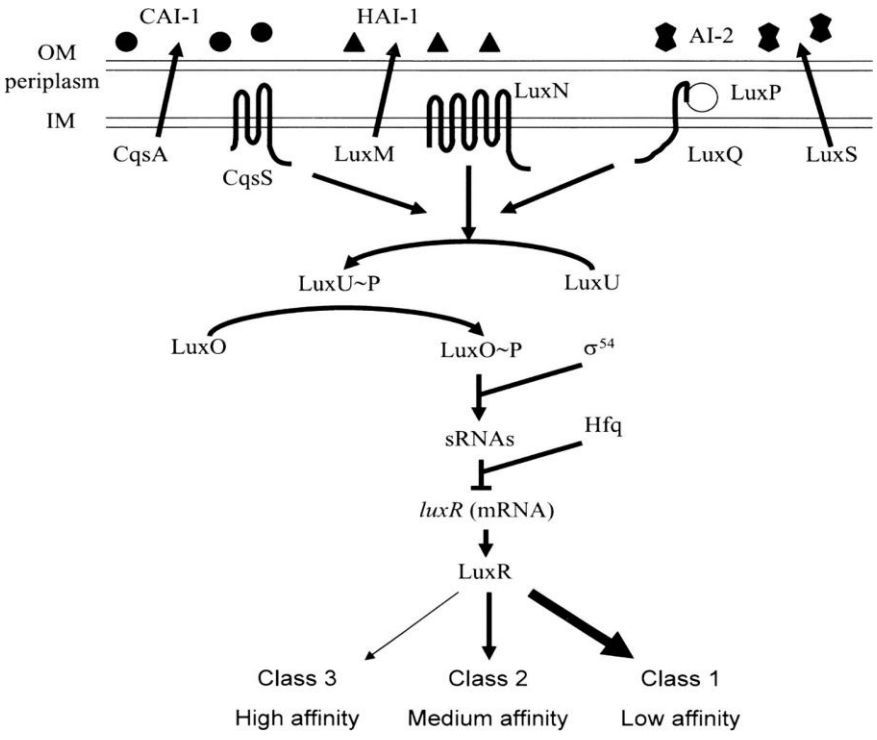


Figure 3. Quorum sensing in *V. harveyi*. Arrows show phosphate transfer during a low cell density state. Waters and Bassler 2006.

The dual-plasmid induction system for *lux* genes uses LuxR to activate the *lux* operon (Figure 4). One plasmid contains *luxR*, activated by arabinose, and the second plasmid contains a copy of a *Vibrio sp. lux* operon. Both plasmids have antibiotic resistance genes for selection purposes. The system was previously showed to induce light production in transformed *E. coli* cells using operons from *V. harveyi*, *V. vulnificus*, *V. chagasii*, *V. orientalis*, and *V. cholerae* [16].

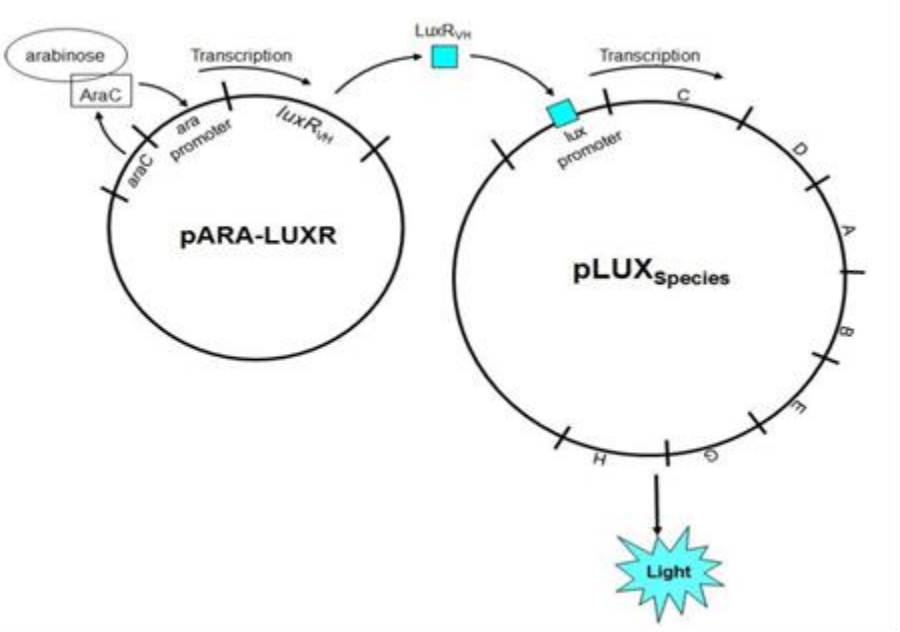


Figure 4. Diagram showing arabinose/LuxR induction of the previously used dual-plasmid system. Wannamaker 2013

The *lux* operon is well studied, but it is unknown why some species of bacteria produce greater amounts of light than others. The core *lux* operon is reasonably highly conserved [5, 6]. Because of this, it seems unlikely that differences in the core genes are causing different levels of luminescence. It does however seem possible that promoter activity may play a role, leading to

higher or lower levels of gene expression. To test this the dual-plasmid system was modified to study promoter activity by using only an upstream region from various *Vibrio* species instead of the full operon (Figure 5). A fluorescent reporter gene (either GFP or mCherry) was inserted directly after the upstream region to measure activity.

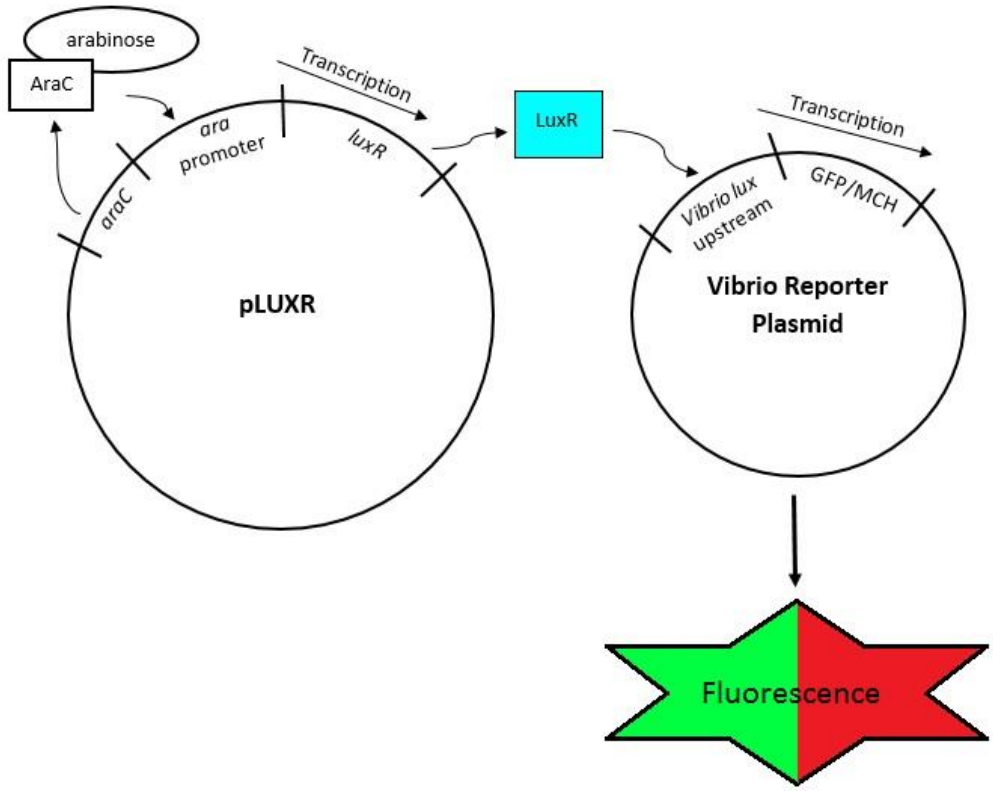


Figure 5. Modified dual-plasmid system containing a fluorescent reporter instead of the full *lux* operon as shown in Figure 4. Diagram shows arabinose/LuxR induction of the *Vibrio* reporter plasmid.

Hypothesis

If promoter activity of the *lux* operon does play a role in amount of light production, then there will be a correlation between the luminescence of various *Vibrio* species and the corresponding induced fluorescence of each. In short, promoter regions from brighter *Vibrio* species (specifically *Vibrio harveyi*) will show higher levels of fluorescence than promoter regions of dimmer species (*Vibrio chagasii)*.

Materials and Methods

Plasmids

Plasmid	Description
pGLO	Contains GFP gene. 5371bp, Bio-Rad
pMCH 2053	Contains mCherry, other properties unknown. McBride Lab
pLS6	Contains chloramphenicol resistance cassette. 5485bp. Wimpee Lab
pARA-LUXR (or pLUXR)	Modified pLS6. Contains *ara-luxR* insert cloned into SmaI site. 7350bp, Wannamaker, Wimpee Lab. Referred to as pLUXR in this paper.
pGEM-3Z	Contains ampicillin resistance cassette and *lacZ*. 2743bp, Promega.
pGFP	Modified pGEM-3Z with GFP inserted. 3431bp
pMCH	Modified pGEM-3Z with mCherry inserted. 3409bp
pVHGFP	pGFP with a *V. harveyi* upstream region inserted directly in front of GFP. 4073bp
pVCHGFP	pGFP with a short *V. chagasii* upstream region inserted directly in front of GFP. 3758bp
pVHMCH	pMCH with a *V. harveyi* upstream region inserted directly in front of MCH. 4051bp
pVCHMCH	pMCH with a partial *V. chagasii* upstream region inserted directly in front of MCH. 3736bp
pVCHLMCH	pMCH with a longer *V. chagasii* upstream region inserted directly in front of MCH. 3978bp

Table 1. List of plasmids used in this study. All plasmid maps were generated using RF-Cloning tool (Bond and Naus, 2012).

Plasmids pGLO and pGEM-3Z were acquired from the manufacturer and used unmodified. Plasmids pLS6 and pLUXR were previously used in another lab project (Wannamaker) and were also not modified. Plasmid pMCH-2053 was acquired from another lab and was used only as a source for mCherry. The remaining plasmids were constructed for this study.

Plasmid Construction pGFP

GFP was isolated from pGLO using PCR. Primers were designed to add a restriction site at each end of the reporter gene, EcoRI in front and HindIII at the end. Each restriction site was surrounded by 6 base pairs to allow the restriction endonucleases to properly digest the amplified fragment. A ribosome binding site was also included directly before the GFP start codon.

Primers were not needed for pGEM-3Z, which already included the required restriction sites. A dual restriction digest was performed on pGEM-3Z and the GFP PCR product, followed by ligation and transformation into JM109 competent *E. coli* cells. Blue/white screening using LB ampicillin plates coated with IPTG and X-Gal (50uL of each) was used to identify desirable transformants, which were then verified using colony PCR and eventually sequencing.

Plasmid Construction pMCH

MCH (mCherry) was isolated from pMCH 2053 using PCR. Primers added EcoRI and HindIII restriction sites as with pGFP, but no ribosome binding site was included. This ribosome binding site is not necessary, and was eliminated as a potential concern for undesirable reporter expression. The remainder of construction was identical to pGFP.

***Lux* Upstream Inserts**

All *lux* upstream regions were isolated from *Vibrio* genomic DNA using PCR. The full upstream region of *V. harveyi* was used (636bp), while in the case of *V. chagasii* a short upstream region (321bp) and full upstream region (563bp) were used.

Vibrio Reporter Plasmids

Both pGFP and pMCH were intended to be used as vectors for multiple *Vibrio lux* upstream regions. Primers were designed to add a BglII restriction site at the end of the vector and retain the EcoRI restriction site in front of the reporter. Similar primers were used to attach the correct restriction sites at the appropriate ends of the *lux* upstream region inserts. Dual-digests were performed on inserts and vectors, then the desired combinations were ligated and transformed into JM109 competent *E. coli* cells. LB ampicillin plates were used. Colony PCR was used to test for the *Vibrio* upstream insert combined with the fluorescent reporter. Successful colonies were then verified with sequencing and stored.

Transformation into Top10 *E. coli* cells

Plasmid DNA was isolated from verified colonies using Promega Wizard Mini-prep kit. Top10 cells were grown and made competent using $CaCl_2$. The Top10 cells were transformed with plasmid DNA to make the various combinations needed (Table 2). Antibiotic screening was used, followed by colony PCR for verification.

Dual-plasmid System

The dual plasmid system was initially constructed using JM109 cells. Plasmid DNA was isolated from earlier identified colonies using Promega kit. JM109 competent cells were transformed with both a pLS6/pLUXR plasmid and a *Vibrio* reporter plasmid in various combinations. Antibiotic screening was performed using ampicillin and chloramphenicol (Table 2). Colony PCR

was used to further verify that appropriate plasmids were present. This was successful, but data showed minimal fluorescence induction. Fluorescence results were more successful in Top10 *E. coli* cells, which were used afterward instead of JM109. The dual plasmid system was induced with arabinose added to the LB-ampicillin/chloramphenicol media. Top10 cells respond better to arabinose induction than JM109 because they have an *ara-14* genotype which blocks arabinose catabolism. Verified colonies were stored and grown in liquid media.

PCR and Sequencing

PCR reactions for plasmid construction were performed with Phusion to allow for accurate amplification of long fragments. This also created fragments with blunt ends. Colony PCR was performed using GoTaq Green Master Mix (Promega).

Restriction Digests

Restriction Digests were performed using enzymes from Promega. Dual-digests for EcoRI and HindIII used buffer B, while dual-digests for BglII and EcoRI used buffer D. Digests were incubated for 2 hours.

Fluorescence Measurements

Plasmids were grown in the appropriate liquid media (Table 2) in a 37°C shaker overnight. Liquid media used was LB with antibiotics and/or arabinose added. Ampicillin was used at a concentration of 100μg/mL, chloramphenicol at 25μg/mL, and arabinose at 2g/L. Time ranges between 20 and 24 hours were found to produce statistically significant levels of GFP and MCH,

although shorter growth times produced similar data. Fluorescence intensity and optical density were measured using a Tecan infinite 200Pro plate reader for 8 replicates of each plasmid combination type. Average fluorescence intensity with respect to OD was then calculated. Measurements were performed in the following order: OD at 600nm after an 8s shake, fluorescence (GFP) emission at 509nm using an excitation wavelength of 395nm, and fluorescence (MCH) emission at 601nm using an excitation wavelength of 575nm.

Media	**Plasmids**
LB Ampicillin	pGLO, pGEM, pGFP, pMCH, pVHGFP, pVCHGFP, pVHMCH, pVCHMCH, pVCHLMCH
LB Ampicillin + Arabinose	pGLO, pGEM, pGFP, pMCH, pVHGFP, pVCHGFP, pVHMCH, pVCHMCH, pVCHLMCH
LB Chloramphenicol	pLS6, pLUXR
LB Chloramphenicol +Arabinose	pLS6, pLUXR
LB Ampicillin + Chloramphenicol	pGEM+pLS6, pGEM+pLUXR, pGFP+pLS6, pGFP+pLUXR, pMCH+pLS6, pMCH+pLUXR, pVHGFP+pLS6, pVHGFP+pLUXR, pVCHGFP+pLS6, pVCHGFP +pLUXR, pVHMCH+pLS6, pVHMCH+pLUXR, pVCHMCH+pLS6, pVCHMCH+pLUXR, pVCHLMCH+pLS6, pVCHLMCH +pLUXR
LB Ampicillin + Chloramphenicol + Arabinose	pGEM+pLS6, pGEM+pLUXR, pGFP+pLS6, pGFP+pLUXR, pMCH+pLS6, pMCH+pLUXR, pVHGFP+pLS6, pVHGFP+pLUXR, pVCHGFP+pLS6, pVCHGFP +pLUXR, pVHMCH+pLS6, pVHMCH+pLUXR, pVCHMCH+pLS6, pVCHMCH+pLUXR, pVCHLMCH+pLS6, pVCHLMCH +pLUXR

Table 2. List of media used for plasmid combinations.

Luminescence Measurements

Luminescence was measured using a second plate reader. Both *V. harveyi* and *V. chagasii* were grown overnight in liquid SWC in a room temperature shaker. The following day, 1 mL of overnight culture was added to 50mL of new SWC and allowed to incubate in the shaker again at room temperature for two hours to achieve log phase. A 5 second shake was applied in the plate reader before measuring light output at 490nm.

Luminescence Imaging

Liquid cultures of *V. harveyi* and *V. chagasii* were streaked with a sterile q-tip onto LBSG 3% plates and allowed to incubate at room temperature overnight. The plates were uncovered and photographs were taken with long exposure times to capture light produced. Pictures were not edited or modified except to remove noise.

Results

Plasmid Construction

GFP from pGLO was inserted into pGEM-3Z create pGFP (Figure 6). Plasmid pGFP was initially constructed in JM109 cells, then verified with colony PCR (Figure 7) and sequencing.

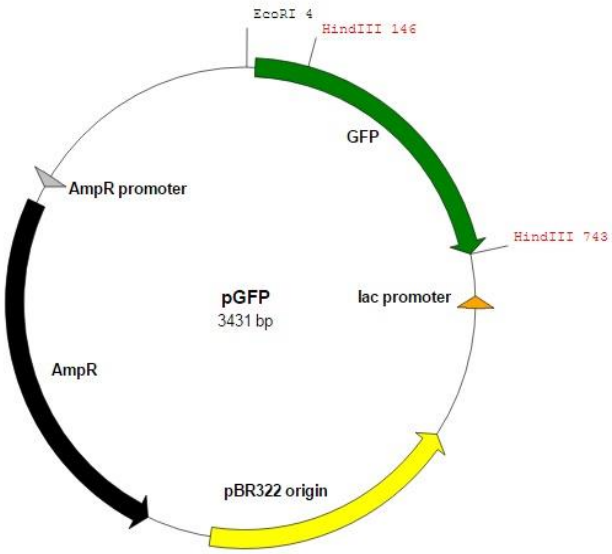


Figure 6. Map of pGFP.

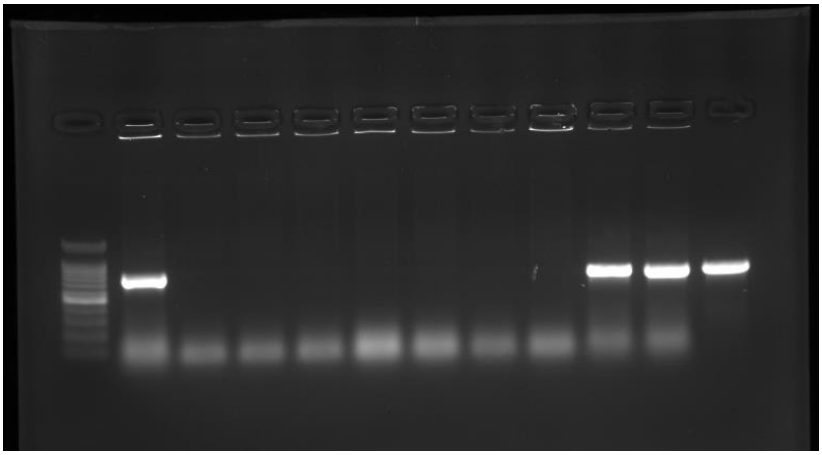


Figure 7. Colony PCR to check for GFP insert in pGFP transformed colonies. 100bp ladder shown. Last lane is a positive control. Three out of ten colonies were successful.

Alternate reporter gene mCherry was inserted into pGEM-3Z to create pMCH (Figure 8). Colony PCR was used for verification (Figure 9).

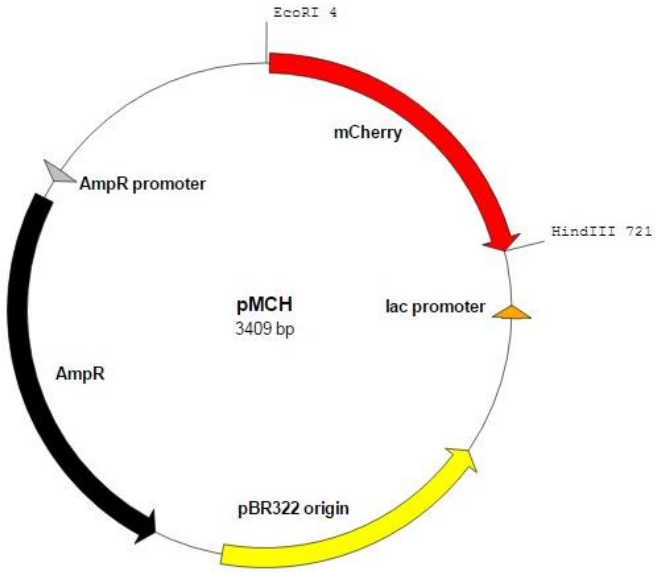


Figure 8. Map of pMCH.

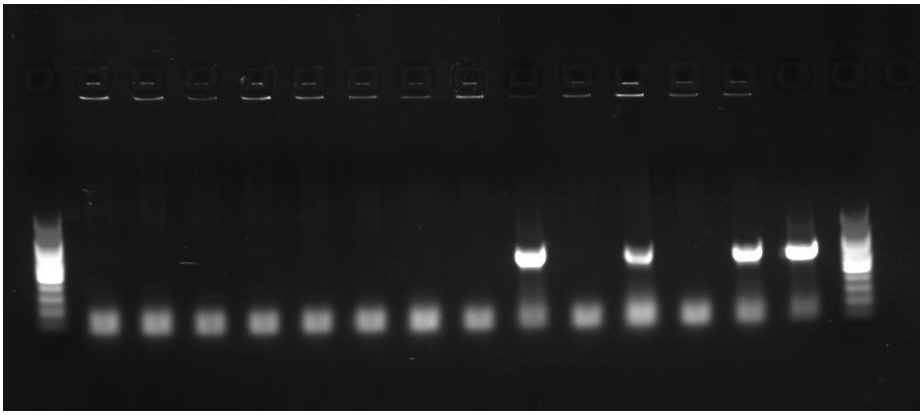


Figure 9. Colony PCR to check for MCH insert in pMCH transformed colonies. 100bp ladder shown. Lane 15 is a positive control. Three out of thirteen colonies were successful.

The *lux* operon upstream regions from *V. harveyi* (Figures 10, 11) and *V. chagasii* (Figures 15, 16) were inserted directly before the fluorescent reporter using both pGFP and pMCH vectors. In the case of *V. chagasii*, a longer upstream region was also included in pMCH only (Figure 20). All were verified with colony PCR (Figures 12, 13, 14, 17, 18, 19, 21).

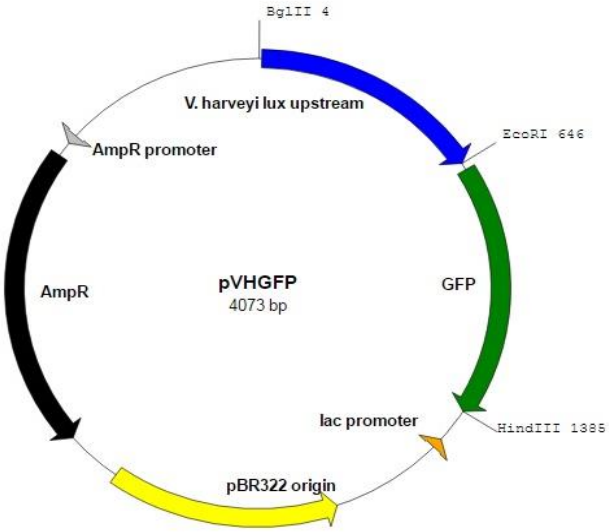


Figure 10. Map of pVHGFP.

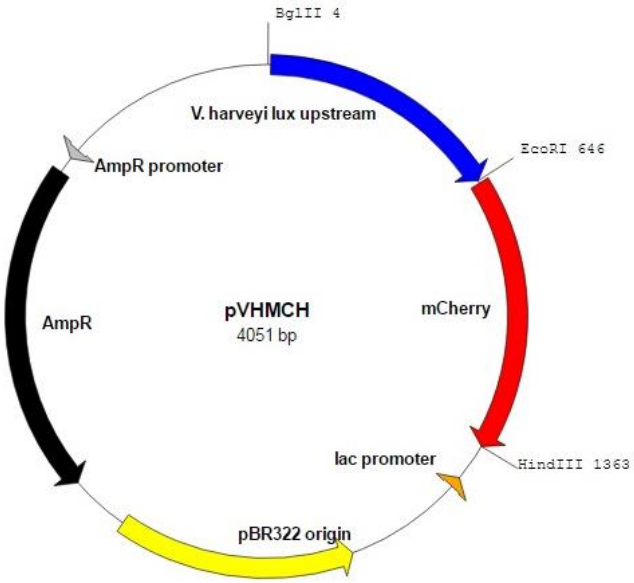


Figure 11. Map of VHMCH.

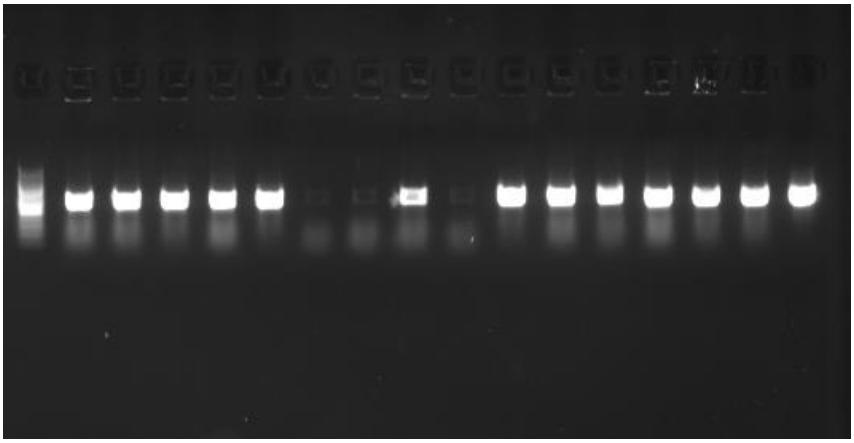


Figure 12. Colony PCR checking for *V. harveyi lux* upstream insert in pVHGFP transformed colonies. Efficiency was higher than anticipated, with thirty out of thirty-nine colonies tested showing success. Sixteen of the colonies tested are shown, using a 100bp ladder.

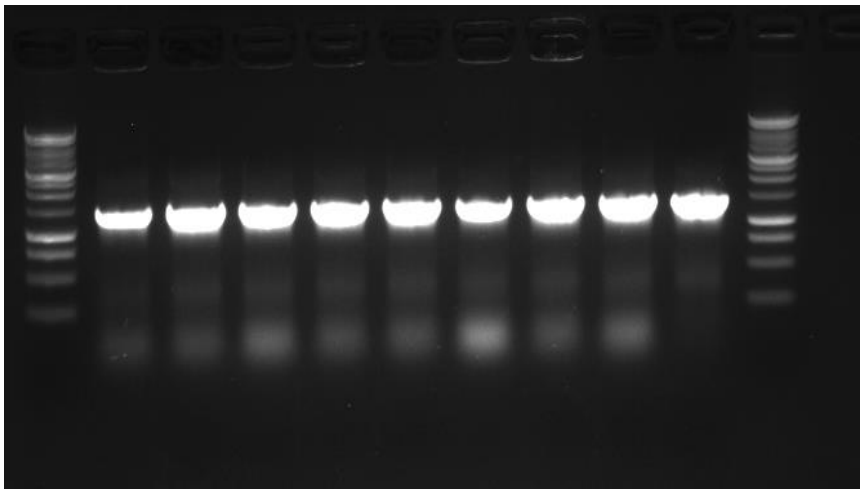


Figure 13. Colony PCR checking for *V. harveyi lux* upstream insert combined with GFP in pVHGFP transformed colonies. Eight colonies were chosen, which had been previously shown to have the *V. harveyi* upstream region (Figure 12). All colonies showed GFP as well as the *V. harveyi* insert (~1.4kb total length). A 1kb ladder is shown.

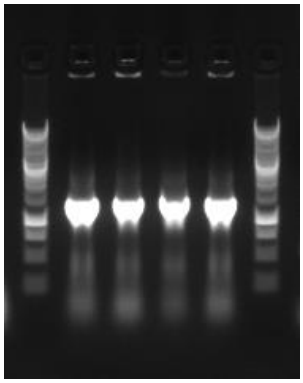


Figure 14. Colony PCR checking for *V. harveyi lux* upstream insert combined with MCH in pVHMCH transformed colonies. All colonies showed MCH as well as the *V. harveyi* insert (~1.4kb total length). A 1kb ladder is shown.

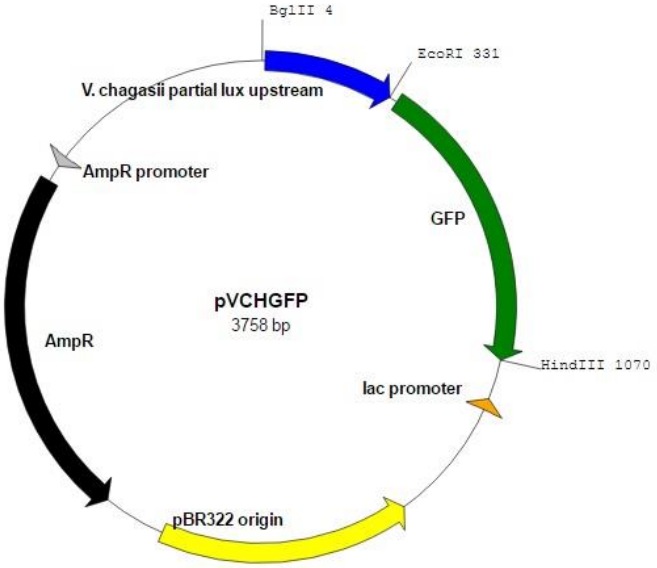


Figure 15. Map of VCHGFP.

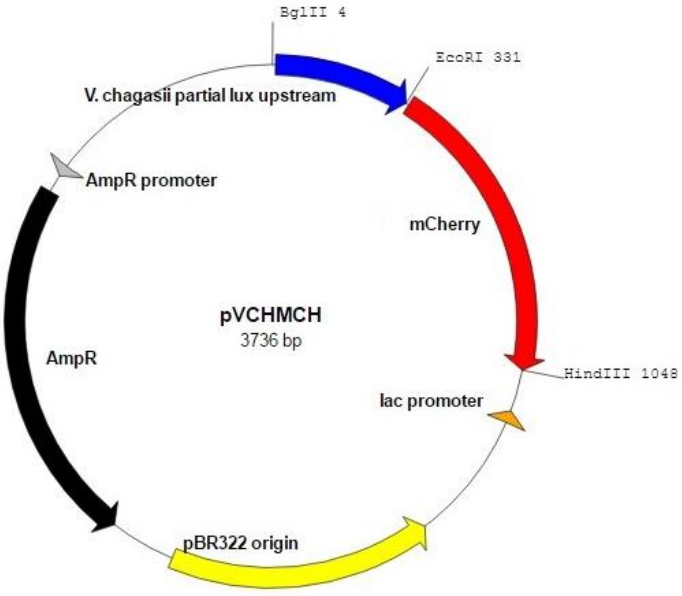


Figure 16. Map of VCHMCH.

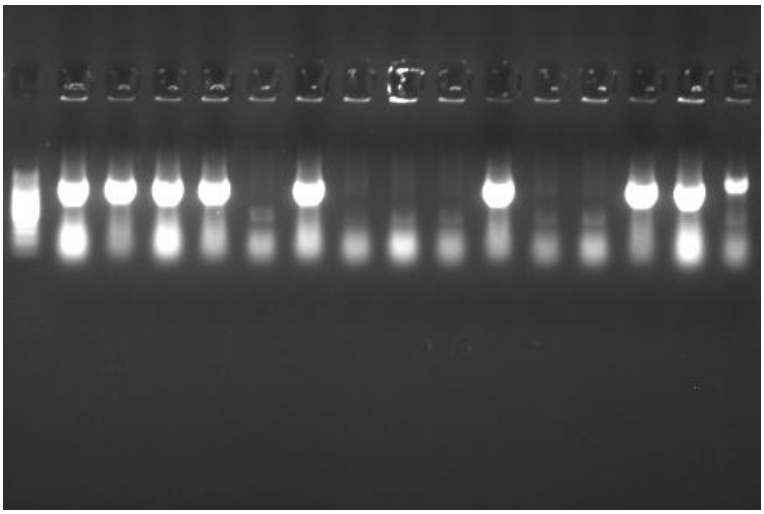


Figure 17. Colony PCR checking for *V. chagasii* partial *lux* upstream insert combined with GFP in pVCHGFP transformed colonies. Fifteen colonies were chosen, and eight show successful inserts. A 100bp ladder is shown.

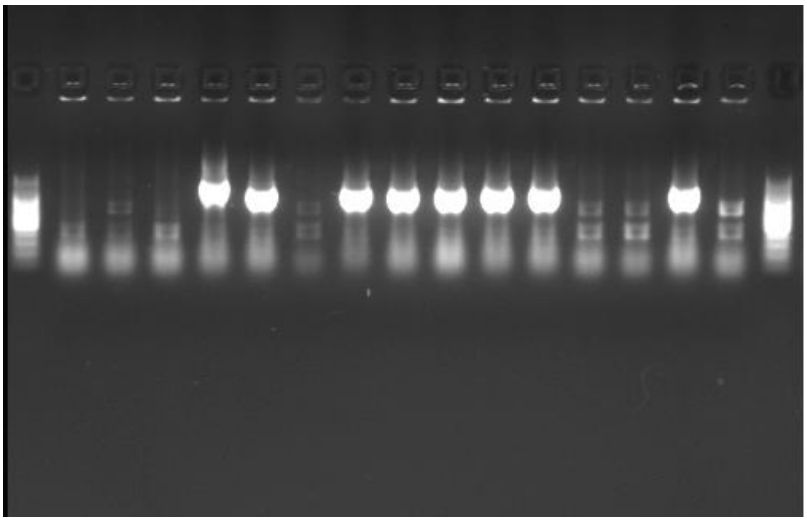


Figure 18. Colony PCR checking for *V. chagasii* partial *lux* upstream insert combined with MCH in pVCHMCH transformed colonies. Fifteen colonies were chosen, and seven show successful inserts. A 100bp ladder is shown.

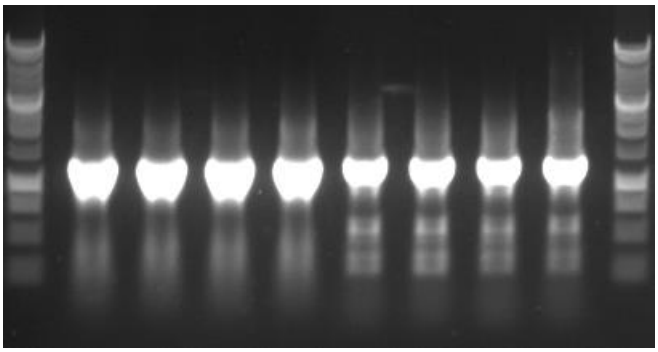


Figure 19. PCR from plasmid DNA isolated from colonies with pVCHGFP (lanes 2-5) and pVCHMCH (lanes 6-9). This was performed to better verify size of the inserts (~1.1kb) A 1kb ladder is shown.

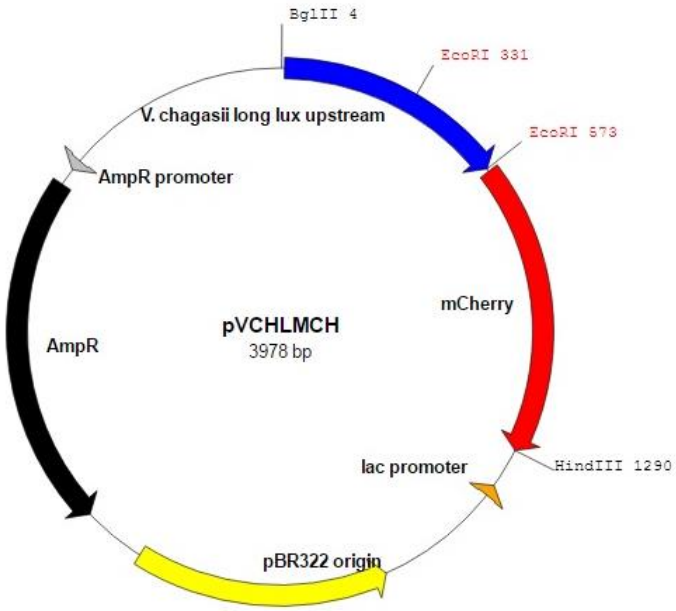


Figure 20. Map of pVCHLMCH.

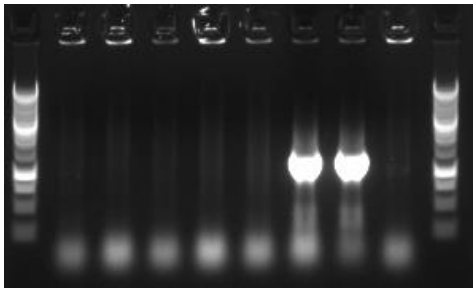


Figure 21. Colony PCR checking for *V. chagasii* full *lux* upstream insert combined with MCH in pVCHLMCH transformed colonies. Eight colonies were chosen, and two show successful inserts (~1.3kb). A 1kb ladder is shown.

After all plasmids were constructed and verified, Top10 *E. coli* cells were transformed with plasmids as described in Table 2 to create desired combinations. This was verified by colony PCR (Figure 22).

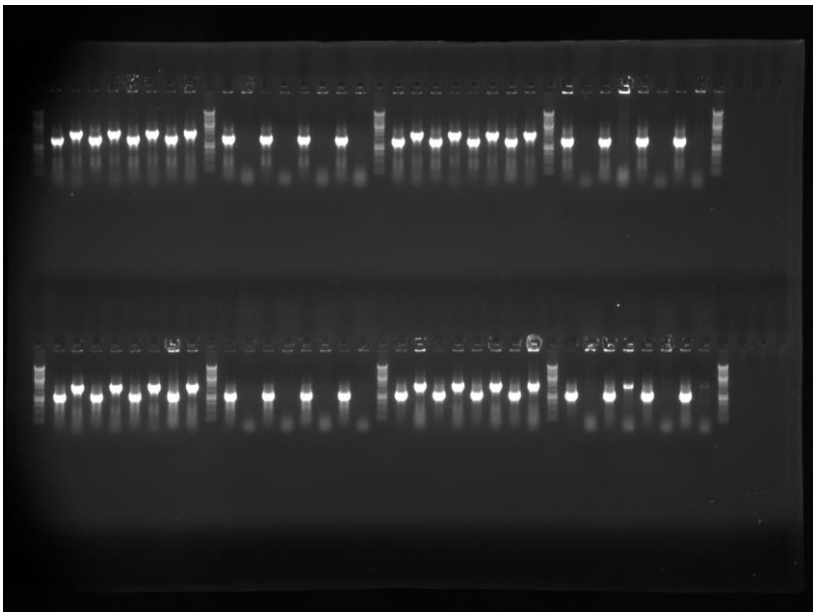


Figure 22. Example of colony PCR verifying dual-plasmid system in transformed Top10 cells. Each colony was tested for a *Vibrio* upstream region with fluorescent reporter, and then separately for ara-LuxR.

Fluorescence Measurements

All fluorescence measurements were performed using a Tecan 200pro plate reader. Fluorescence was divided by OD for each sample and the average fluorescence intensity was then calculated. Graphs show error bars representing standard deviation.

Initial testing of the dual-plasmid system was performed in JM109 *E. coli* cells. Cultures were incubated in a 37°C shaker for 18 hours. As seen in Figure 23, the system successfully induced fluorescence. However, fluorescence for strains without arabinose was higher than strains with

arabinose (Figure 24). This was discovered to be a problem with JM109 cells, and for this reason further testing was performed exclusively in Top10 *E. coli* cells.

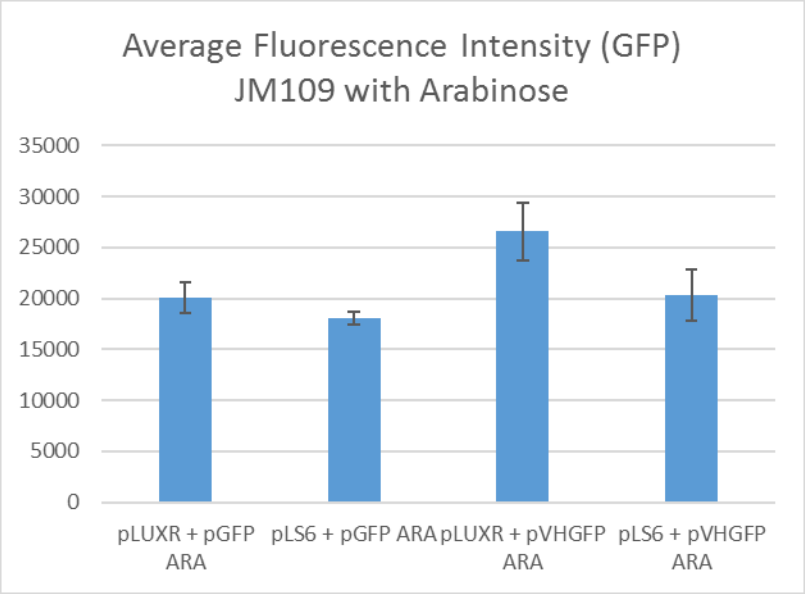


Figure 23. Average Fluorescence Intensity results for dual-plasmid system (pGFP and pVHGFP) in JM109 cells. All have been induced with 0.2% arabinose, and the system appears to be functioning properly.

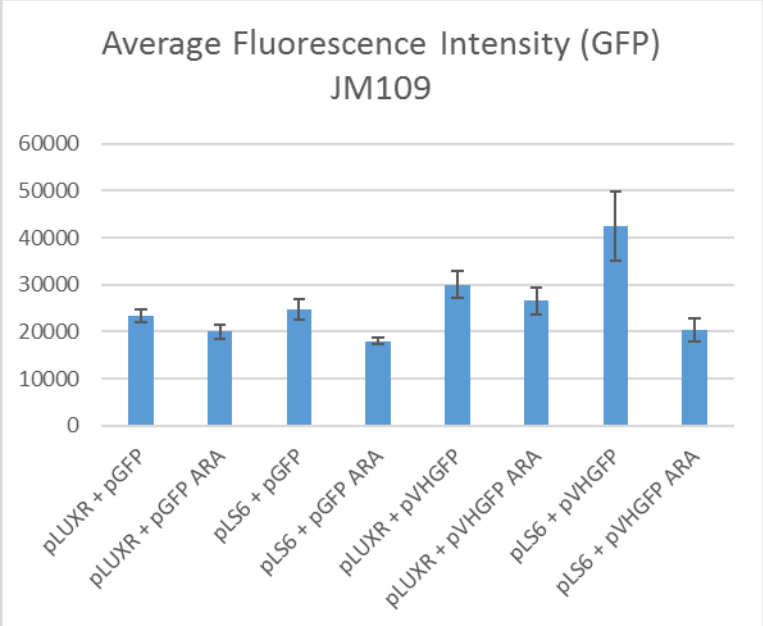


Figure 24. Average Fluorescence Intensity results for dual-plasmid system (pGFP and pVHGFP) in JM109 cells. Shows that strains without arabinose added actually have higher fluorescence than the arabinose-induced strains. This was later resolved in the Top10 cells.

Transformation of the dual plasmid system into Top10 cells resulted in appropriate fluorescent levels for induced and uninduced strains (Figure 25). Cultures were incubated in a 37°C shaker for 18 hours. A positive control containing pGLO was used for comparison and to be sure that GFP fluorescence was being detected properly. The combination of pVHGFP + pLUXR and arabinose shows a significantly higher level of fluorescence of almost 40000 units, meaning that it was inducing correctly. The uninduced version (pVHGFP + pLUXR without arabinose) shows a much lower value of 26000 units. This level is comparable to the other controls predicted to be incapable of fluorescence, both induced and uninduced. All controls are below the value reached for uninduced pGLO, which showed 29000 units. The only exception to this is pGFP + pLUXR with arabinose, which appears to be inducing despite the lack of an upstream region and therefore promoter. This fluorescence is lower than pVHGFP + pLUXR ARA, but not significantly.

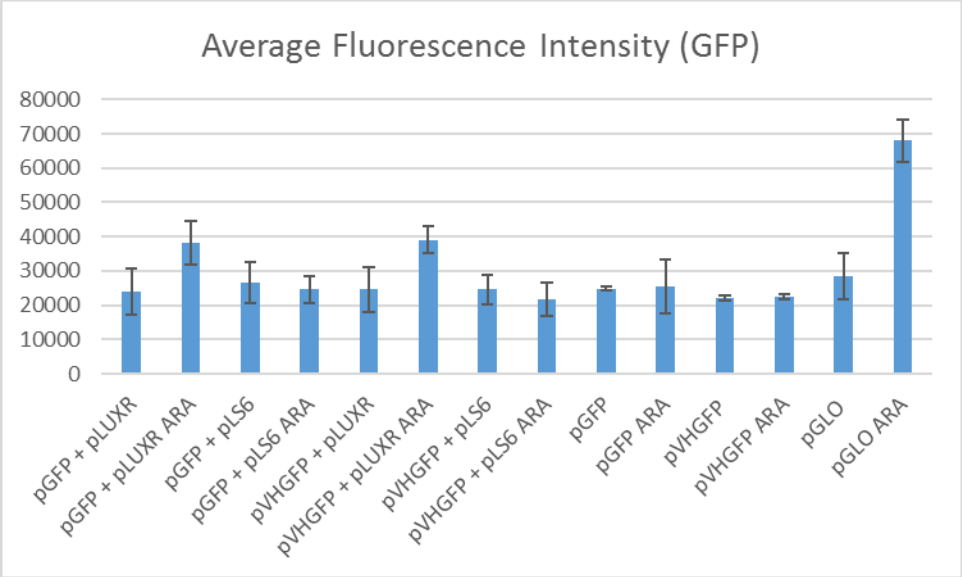


Figure 25. Average Fluorescence Intensity for dual plasmid system in Top10 cells. Plasmid combinations appear to be inducing properly compared to the JM109 results. Plasmid combination pVHGFP + pLUXR with arabinose is fluorescing at significant levels compared to controls, as expected. However, pGFP with no upstream region is somehow inducing as well.

After the dual-plasmid system was shown to be working correctly, plasmids containing the *V. chagasii* upstream regions were made and tested. A second fluorescent reporter (mCherry) was used in all *Vibrio* reporter plasmids to test if the induction of pGFP + pLUXR with arabinose was unique to GFP. Top10 cells were used exclusively, and cultures were incubated for 22 hours in a 37°C shaker.

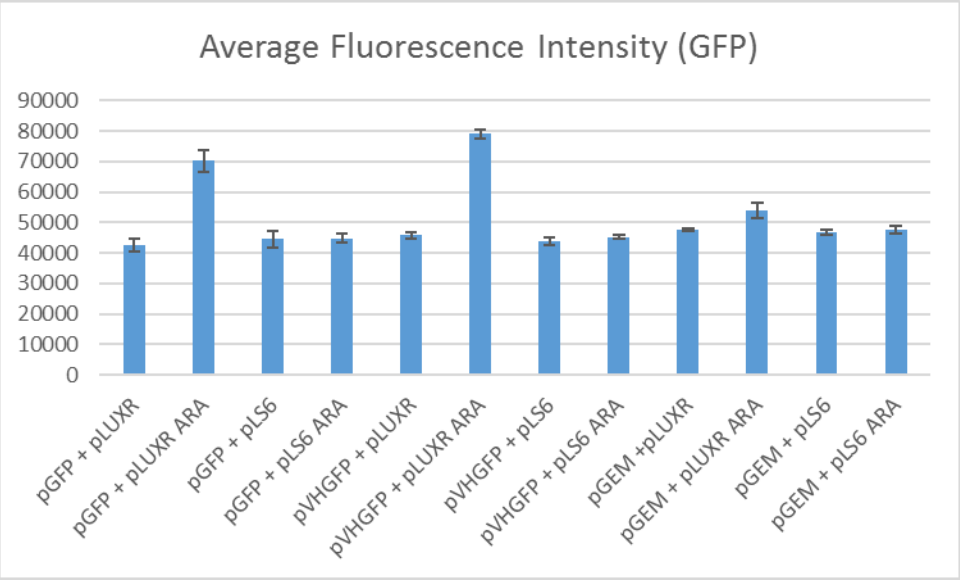


Figure 26. Average fluorescence intensity for dual-plasmid system in Top10 cells. The plasmid combination of pVHGFP + pLUXR induced with arabinose is fluorescing at high levels. Combination pGFP + pLUXR with arabinose also shows relatively high fluorescence, but is significantly lower than that of the induced pVHGFP combination. The longer growth time (22 hours) seems to have helped make this difference visible.

As shown in Figure 26, fluorescence results for newer readings were similar to previous values (Figure 25), but were able to reach higher levels due to the longer growth time of 22 hours. The control plasmid pGFP still showed fluorescence when induced with pLUXR and arabinose, but levels were not as high as induced pVHGFP. Another control combination of pGEM, induced and uninduced, was included for comparison. When induced with pLUXR and arabinose, pGEM does not fluoresce at high levels, indicating that this result is unique to pGFP.

The comparison using mCherry shows similar, but not identical results (Figure 27). As expected, pVHMCH induced with pLUXR and arabinose fluoresces at high levels. Induced pMCH fluoresces as well, but at lower levels. This is similar to the results of pGFP in Figure 26, however it is important to note that pMCH does not appear to be able to fluoresce as well as pGFP when compared to the appropriate VH plasmid.

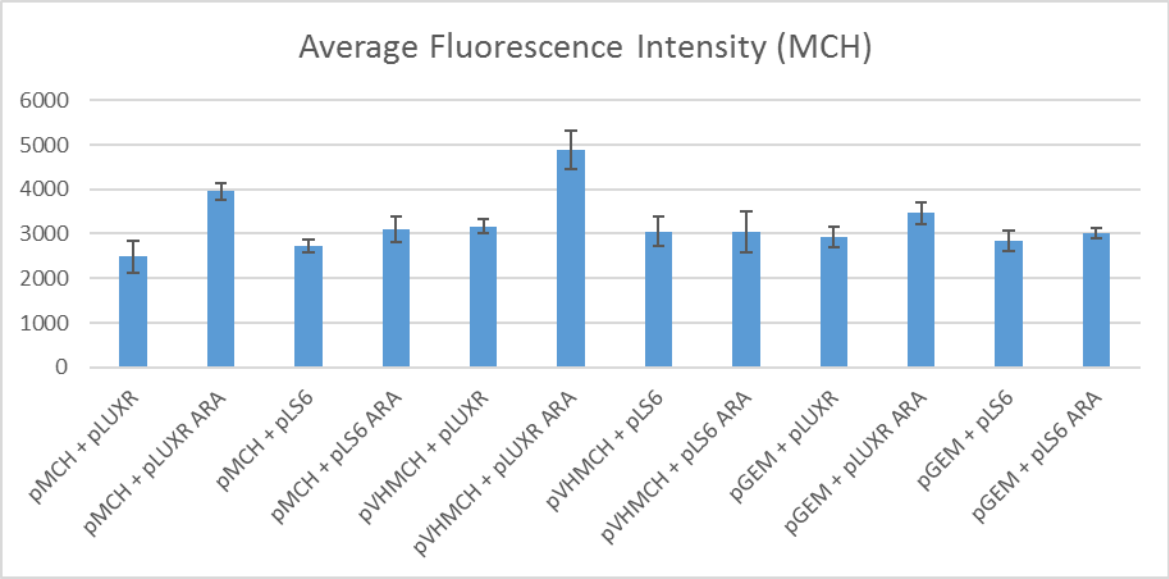


Figure 27. Average fluorescence intensity for dual-plasmid system in Top10 cells. The plasmid combination of pVHMCH + pLUXR induced with arabinose is fluorescing at high levels. Combination pMCH + pLUXR with arabinose also shows some fluorescence, but is significantly lower than that of the induced pVHGFP combination. Induced pMCH seems to produce less fluorescence compared to pVHMCH than induced pGFP produced compared to pVHGFP (Figure 26).

The remaining graphs (Figures 28, 29, 30, 31) use data collected at the same time as Figures 26 and 27. In Figure 28, induced and uninduced pVHGFP and pVCHGFP are shown. As expected, pVHGFP induced with pLUXR and arabinose fluoresced at the highest levels, nearly 80000 units. Plasmid pVCHGFP induced in the same way fluoresced at only 55000 units. The control plasmid

pGFP (with no promoter) showed fluorescence of 70000 units when induced. All three plasmids showed similar fluorescence levels when uninduced.

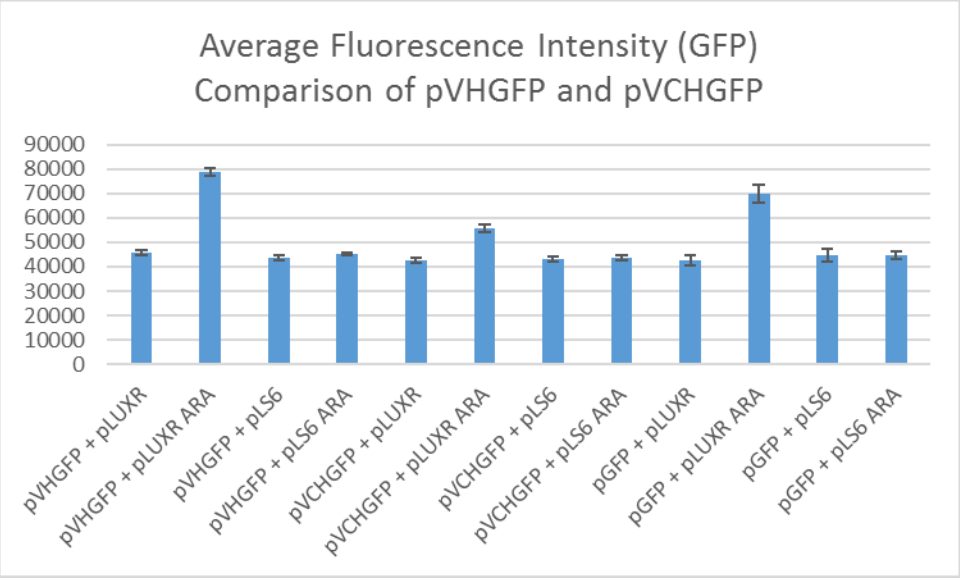


Figure 28. Average fluorescence intensity for dual-plasmid system in Top10 cells comparing fluorescence of induced pVHGFP and induced pVCHGFP. As expected, the much brighter *V. harveyi* plasmid (pVHGFP) shows higher fluorescence compared to the V. *chagasii* partial upstream plasmid (pVCHGFP). Uninduced levels of both are similar.

Results were similar using the mCherry reporter (Figure 29). When induced, pVHMCH showed the highest fluorescence levels, followed in order by pMCH, pVCHLMCH, and pVCHMCH. Levels of all four were similar when uninduced.

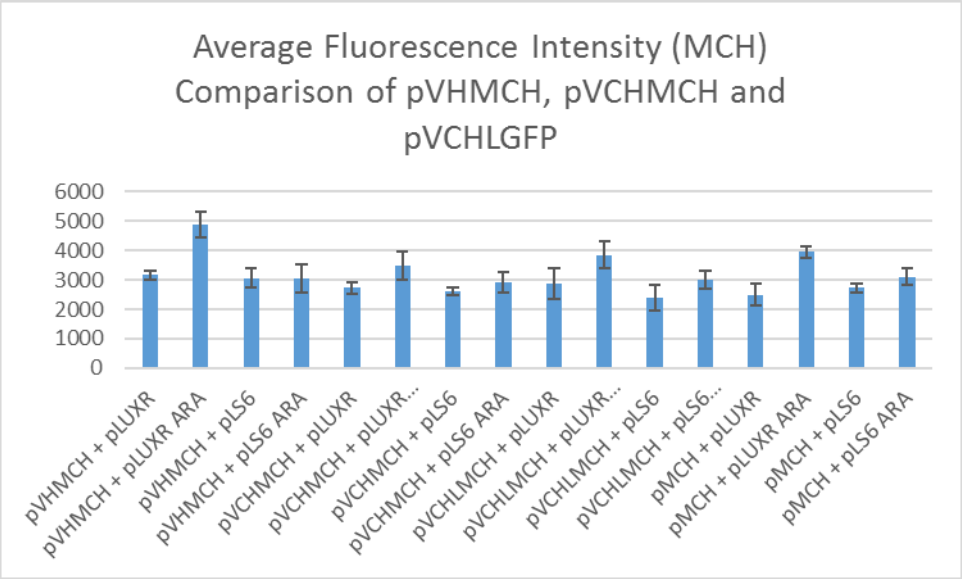


Figure 29.Average fluorescence intensity for dual-plasmid system in Top10 cells comparing fluorescence of induced pVHMCH, pVCHMCH, and pVCHLMCH. As expected, the much brighter *V. harveyi* plasmid (pVHMCH) shows higher fluorescence compared to the *V. chagasii* partial upstream plasmid (pVCHGFP) and the *V. chagasii* full upstream plasmid (pVCHLMCH). Although induced pVCHLMCH shows higher levels of fluorescence than pVCHMCH. Uninduced levels of all three are similar.

Various control plasmids were checked (Figures 30, 31). All seemed to fluoresce at similar levels, with the exception of pLUXR induced with arabinose, which showed slightly higher levels. Since this plasmid combination was used in all induced plasmids, this does not represent a serious concern. Interestingly, this result seems more extreme when using the mCherry reporter. It is uncertain what exactly pLUXR is causing to fluoresce, but since this study is about comparison and pLUXR is being used consistently, this is not a concern for this experiment. This may also be a partial reason for some of the fluorescence of the reporters without an upstream region (induced pGFP and pMCH).

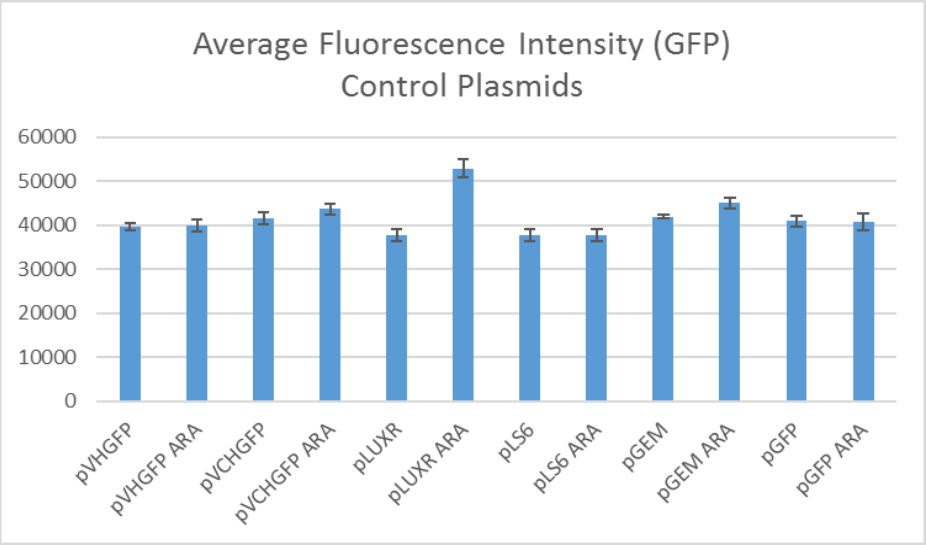


Figure 30. Average fluorescence intensity of control plasmids in Top10 cells. All show similar levels of fluorescence with the exception of induced pLUXR.

Fluorescence levels seem to differ more in general for the mCherry reporter plasmids, but it may also appear more extreme partially because of the smaller scale (Figure 31). Overall, the control plasmids show similar levels with or without arabinose, though they may differ from other plasmids. The exceptions are pLUXR, mentioned already, and pGEM which shows only slight variation. Plasmid pMCH, which was constructed using pGEM, shows no significant differences.

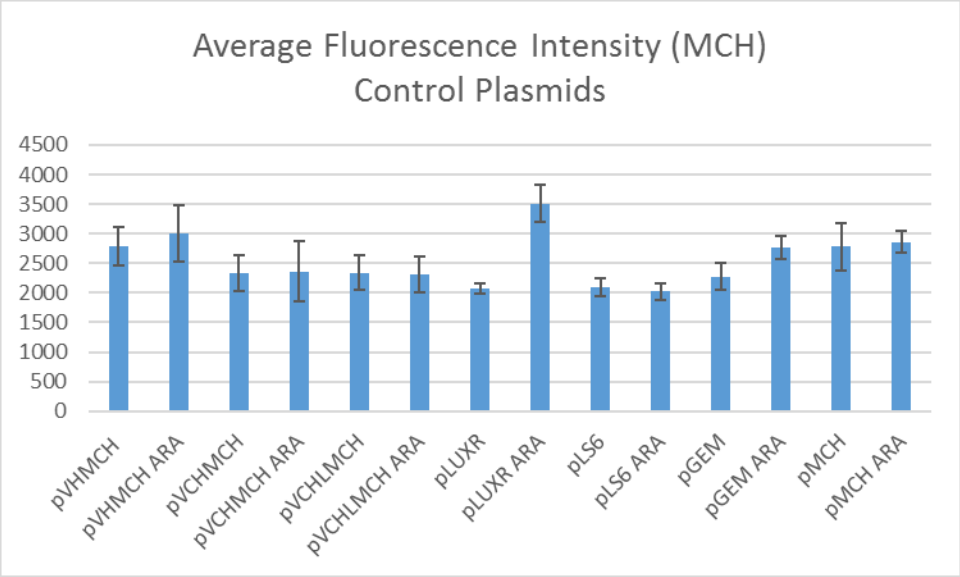


Figure 31. Average fluorescence intensity of control plasmids in Top10 cells. Induced pLUXR shows notably high fluorescence. There is some other variation between plasmids, but with the exception of pLUXR no other plasmid seems to be greatly affected by arabinose. Plasmid pGEM does show an increase with arabinose, but pMCH which is modified pGEM does not.

Luminescence Measurements

Growth Time	*V. harveyi*	*V. chagasii*	Empty SWC liquid media
2 hours	2473.74	72.51	69.72
3 hours	1708.37	75.79	63.34

Table 3. Luminescence measurements of *V. harveyi* and *V. chagasii* liquid cultures in SWC media. Shown in relative light units (RLUs).

Luminescence measurements using a luminometer plate reader function, show that *V. harveyi* is much brighter than *V. chagasii* (Table 3).

Luminescence Photographs

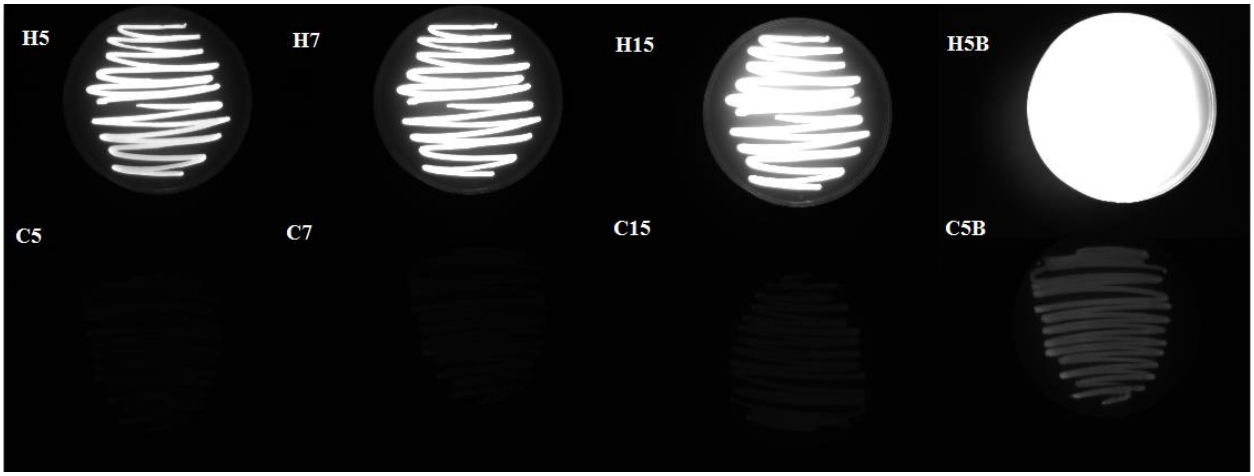


Figure 32. Long-exposure photographs showing luminescence of *V. harveyi* (top) and *V. chagasii* (bottom). Exposure times were 5 minutes (H5, C5), 7 minutes (H7, C7) and 15 minutes (H15, C15). H5B and C5B used a higher bin value of 4x4 with an exposure time of 5 minutes. Cultures were plated on LBSG 3% and incubated at room temperature overnight.

Long exposure photographs were also used to compare the light production of *V. harveyi* and *V. chagasii* (Figure 32). Using standard exposures up to 15 minutes, it is difficult to see any light for *V. chagasii,* while the *V. harveyi* plate is almost too bright at the 15-minute mark. The camera settings were changed to a higher bin value (4x4) to allow the light produced by *V. chagasii* to be more easily visible, although this shows excessive light for the *V. harveyi* plate. The comparison is nonetheless clear, with *V. harveyi* much brighter than *V. chagasii.*

Discussion

JM109 cells vs Top10

JM109 competent *E. coli* cells were originally used for convenience and efficient transformations. It was only after initial results were obtained (Figures 23, 24) that the flaws of the dual-plasmid arabinose induced system in JM109 cells became apparent. The results when looking at only the arabinose induced plasmids seemed to be reasonable, showing higher fluorescence levels for the *V. harveyi* plasmid combined with pLUXR (Figure 23). But when comparing the arabinose induced plasmids with the uninduced plasmids, all combinations exposed to arabinose showed lower fluorescence (Figure 24). The highest fluorescence was actually seen in the combination of pLS6 with pVHGFP without arabinose, which was not logical.

The dual-plasmid system was designed to use the same arabinose induction system as pGLO, which was already known to work well. Also, the dual-plasmid system had been induced with arabinose in the Wannamaker experiment (2013) previously. After investigation, it was discovered that the previous experiment functioned in Top10 *E. coli* cells. Top10 cells have an *ara-14* genotype, which blocks arabinose catabolism. This makes them much more suitable for arabinose induction systems than JM109 cells. This feature was tested using pGLO, which appears to create substantially more GFP in Top10 cells than when in JM109 cells. For this experiment, as mentioned in the Methods section, it was decided to perform initial transformations in JM109 cells, but then to convert everything to Top10 cells using plasmid DNA.

Inclusion of a Second Fluorescent Reporter

The decision to include mCherry as a second reporter was made after discovering the high fluorescence results of induced pGFP (Figure 25). It seemed possible that LuxR was binding to a site in GFP and somehow triggering higher than expected fluorescence levels. When uninduced, pGFP produced lower fluorescence comparable to other plasmids. The other possibility was that LuxR was binding to a site in pGEM-3Z (used to construct pGFP). As a way to avoid or at least mitigate some of these undesirable effects, mCherry was chosen as a second fluorescent reporter. The sequence of mCherry has a higher GC content compared to GFP (63% compared to 41%). Vibrio upstream regions typically have a lower GC content like GFP. If LuxR was finding ways to bind to GFP because of similarities to Vibrio upstream regions, then a reporter with a heavier GC content might show less LuxR binding.

While it is still unknown if LuxR binding is the cause of pGFP induction, it is quite obvious that induced pMCH shows less induction relative to other plasmid combinations than pGFP (Figures 26, 27). The use of both reporters was ultimately useful in comparing data, especially given the differences in scale. Later results using some of the control plasmids (Figures 30, 31) show that pLUXR with arabinose shows noticeable fluorescence compared to other controls, especially for the mCherry plasmids. This may also be part of the explanation for the fluorescence of induced pGFP and pMCH.

Fluorescence Measurements

Fluorescence measurements using both fluorescent reporters show highly similar results. Measurements from control plasmids were used to set a baseline and test for any problematic constructs (Figures 30, 31). The only notable plasmid seems to be pLUXR, which shows higher than expected fluorescence when induced with arabinose. As mentioned, this may be contributing to the induction of control plasmids pGFP and pMCH, but is not of concern when comparing induced values, as pLUXR and arabinose are both necessary for induction.

More importantly, the system shows proper induction for appropriate plasmids with GFP (Figure 28) and mCherry (Figure 29). The induced plasmids pVHGFP and pVCHGFP both show significantly higher fluorescence levels than uninduced plasmids. Plasmid pVHGFP, containing an upstream region from the brighter *V. harveyi,* shows the highest fluorescence as expected. Plasmid pVCHGFP actually shows slightly lower fluorescence than pGFP. This is not unreasonable given the relative dimness of *V. chagasii,* especially since pVCHGFP only contains a partial upstream region.

The induced plasmids pVHMCH, pVCHMCH, and pVCHLMCH also show significantly higher fluorescence than uninduced plasmids. Again, the *V. harveyi* plasmid pVHMCH shows the highest fluorescence. Plasmid pVCHLMCH shows higher fluorescence than pVCHMCH, which is logical given that pVCHLMCH contains the full upstream region. Induced pMCH is slightly higher but similar to pVCHLMCH.

Luminescence Results

As mentioned, bioluminescent bacteria display different levels of brightness. The species used for this experiment were chosen for their extreme differences in light production. *Vibrio harveyi* produced much more light than *Vibrio chagasii*, as can be seen in both the luminescence measurements (Table 3) and photographs (Figure 32). While it is possible that the time points used for the luminescence measurements may not have caught peak light production, the differences between the two species are still clear, with *V. harveyi* being inarguably brighter than *V. chagasii*.

Conclusion

During this study, it has been verified that *Vibrio harveyi* produces substantially more light than *Vibrio chagasii.* A dual-plasmid system using a LuxR/arabinose induction system was shown to function properly in Top10 *E. coli* cells and two fluorescent reporters were used to collect data. Based on these data, using both reporters, it was shown that plasmids containing *V. harveyi* promoters produced the highest amounts of fluorescence, compared to lower fluorescence levels of the *V. chagasii* full and partial upstream plasmids. While it is impossible to state conclusively if there is a correlation between promoter activity and brightness with such a small data set, this experiment provides compelling evidence about the existence of such a possible link.

References

[1] Bassler, B L. *How bacteria talk to each other: regulation of gene expression by quorum sensing*. Current Opinion in Microbiology. 1999. 2:582-587.

[2] Bond, S R, and C C Naus. *RF-Cloning.org: an online tool for the design of restriction-free cloning projects* Nucleic Acids Research 2012; doi: 10.1093/nar/gks396

[3] Chaparian, R R, S G Olney, C M Hustmyer, D A Rowe-Magnus, and J C van Kessel. *Integration host factor and LuxR synergistically bind DNA to coactivate quorum-sensing genes in Vibrio harveyi*. 2016. Molecular Microbiology. 101(5), 823-840.

[4] Close, D, T Xu, A Smartt, A Rogers, R Crossley, S Price, S Ripp, and G Sayler. *The Evolution of the Bacterial Luciferase Gene Cassette (lux) as a Real-Time Bioreporter.* Sensors. 2012. 12:732-752.

[5] Dunlap, P V. *Bioluminescence, Microbial.* Encyclopedia of Microbiology. 2009. pp. 45-61. Oxford: Elsevier.

[6] Dunlap, P V, and H Urbanczyk. *Luminous Bacteria*. The Prokaryotes. 2013. pp. 495-528. Springer Berlin Heidelberg.

[7] Engebrecht, J, and M Silverman. *Identification of genes and gene products necessary for bacterial bioluminescence.* Proc. Natl. Acad. Sci. USA. 1984. 81:4154-4158

[8] Hastings, J W. *Biological Diversity, Chemical Mechanisms, and the Evolutionary Origins of Bioluminescent Systems.* Journal of Molecular Evolution. 1983. 19(5): 309–321.

[9] Lyell, N L, A K Dunn, J L Bose, and E V Stabb. *Bright Mutants of Vibrio fischeri ES114 Reveal Conditions and Regulators that Control Bioluminescence and Expression of the lux Operon*. Journal of Bacteriology. 2010. pp 5103-5114.

[10] Miyamoto, C M, E E Smith, E Swartzman, J Cao, A F Graham, and E A Meighen. *Proximal and distal sites bind LuxR independently and activate expression of the Vibrio harveyi lux operon.* Molecular Microbiology. 1994. 14(2):255-262

[11] National Center for Biotechnology Information (NCBI). Basic Local Alignment Search Tool (BLAST).

[12] Nijvipakul, S, J Wongratana, C Suadee, B Entsch, D P Ballou, and P Chaiyen. *LuxG is a Functioning Flavin Reductase for Bacterial Luminescence*. Journal of Bacteriology. 2008. pp 1531-1538.

[13] Packiavathy, I A, P Sasikumar, S K Pandian, and A V Ravi. *Prevention of quorum-sensing-mediated biofilm development and virulence factors production in Vibrio spp. by curcumin.* Appl Microbiol Biotechnol. 2013. 97:10177-10187.

[14] Pompeani, A J, J J Irgon, M F Berger, M L Bulyk, N S Wingreen, and B L Bassler. *The Vibrio harveyi master quorum-sensing regulator, LuxR, a TetR-type protein is both and activator and a repressor: DNA recognition and binding specificity at target promoters.* Molecular Microbiology. 2008. 70(1):76-88.

[15] Swartzman, E, M Silverman, and E A Meighen. *The luxR gene product of Vibrio harveyi is a transcriptional activator of the lux promoter*. Journal of Bacteriology. 1992. pp 7490-7493.

[16] Wannamaker, A M. *Quorum Sensing in Vibrios and Cross-Species Activation of Bioluminescence Lux Genes by Vibrio Harveyi LuxR in an Arabinose-Inducible Escherichia Coli Expression System*. 2013. UWM

[17] Waters, C L, and B L Bassler. *The Vibrio harveyi quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers*. Genes and Dev. 2006. 20:2754-2767.

APPENDIX A: Primers

GFP

GFP Forward: ACCTGCGAATTCAGGAGATATACATATGGCTAGCAAAGG

GFP Reverse: CACGAGAAGCTTTTATTTGTAGAGCTCATCCATGCC

pGFP Forward: Same as GFP Forward

pGFP Reverse: GATGTCACCGGTGCCCTATAGTGAGTCGTATTAC

Sequencing Primers:

GFP Start: CCGCATCAGGCGCCATTCGCC

GFP End: GGATCCGTTCAACTAGCAGACC

T7 and SP6 Primers contained in pGEM-3Z were also used for sequencing.

mCherry

mCherry Forward: ACTTGCGAATTCATGGTGAGCAAGGGCGAGGAGG

mCherry Reverse: GTCGAGAAGCTTTTACTTGTACAGCTCGTCCATGC

Sequencing Primer MCH542: AGGTCAAGACCACCTACAAGGC

pMCH Forward: Same as mCherry Forward

pMCH Reverse: Same as pGFP Reverse

***Vibrio harveyi* Upstream**

VH Forward**:** TAGCTAAGATCTAATTCGCCCTCTCATTGGTTCGTG

VH Reverse: CCGTGCGAATTCATCAAGAGCTTCTCTTTTAAATTTTGG

Sequencing primer VH402: GATTCCGCTAGTGTTTAATAGCGC

***Vibrio chagasii* Upstream**

VCH or VCHL Forward: TAGCTAAGATCTGGAACCGTATAGCTTCTAACAACTGACC

VCHL Reverse: CCGTGCGAATTCATTAAAGGTAACTCCTCTTTATCTAG

Sequencing primer VCH331: AATCCTTGCAGACCTCTCAACGGC

*Note: VCH was created by digestion at the EcoRI site in the *V. chagasii* upstream region and not the result of primers. The primers were designed to produce VCHL, therefore there is no reverse primer for VCH.

Appendix B: *Vibrio lux* upstream sequences

Vibrio harveyi lux operon upstream sequence. Used in pVHGFP and pVHMCH.

AATTCGCCCTCTCATTGGTTCGTGATATATGCAATGAAAGGAATGTGCCAACTGATAATGCCCTCAATATCAATGCTTTAGATGT
AATTTTTTTATTTTTTTTAATATGAATCTCAAAATGACAATCGCTTTAAGCGGGTAAATCCGAGTTCAAAACCTGCGCTTTGCAA
AGAGCTCCGCACGTACACTGTCACACATCTAACTCGTACACTCGCCAATAGGAAGTACCCCATTAGTGGTTTCTCATGAAGTTC
ATACTTTTTCACCGAAAGTACTACTTTGGCTGAGAGGGTATTATTGAGGAATTACAGTTAAATCTAGTAGTAACAACTACTTAA
ACATCATTACTTATTTATAGTGTTACAAATAACATTAATAATTTATGCAATATTTATGAGTATGATTCCGCTAGTGTTTAATAGCG
CTGATAAAATCAAAAACTAAAGTGTTTGAAGTTTAGTTATTTGTTAAGTGTTACGACTAATTATAGATAAGAAGAACTATAATT
AAATTAAGTGATAATAGTTCTCGTTACTTTGAACTGTTTAATGTATTTGGTTAAAAGTTTTTAATTAACTTTAAAAAAATGATCCA
AGGAATTAATGTTTTCCAAAATTTAAAAGAGAAGCTCTTGAT

Vibrio chagasii partial *lux* operon upstream sequence. Used in pVCHGFP and pVCHMCH.

ATGAATCCTTGCAGACCTCTCAACGGCATTGAAAATACCCGTTTGACTATAAAAACCGTCGTAATCACTATGATTAATAATTATT
TAAAGTGGGAACTATTTATAATTATAATCAATTATTGTGAGTGGGAATGTTTTGATATGATTAACGCTTTTGAATATAAGTGGT
ATTTTCTCTTTCAAATAAATTAACTAACAGATAGGCTTTTTCTAGATAAAGAGGAGTTACCTTTAAT

Vibrio chagasii full *lux* operon upstream sequence. Used in pVCHLMCH.

GGAACCGTATAGCTTCTAACAACTGACCGCTAGATTGTGAGGATGGCTGCGTTCCCAGAAGGCAGATCCTTCCCACGGGAGGA
TTAGTGGGACTGATCGTTTGATACGAATAGCTTCATAGCATGACCTTGTTTCGTAAGCTCCATCGCCAGATATCTCATTGTTTCT
TTGGTGGGTCTTTTTGAGTAAGTTAGGTAGCACCTCACCGTCGCTCACATTCGATAGAGTCGCCGCTTCCCGTCAGTGCCATGC
TTTTTTGCTCGCTTACTAATGTATGGATAATGAGGGTATAACAATGGCAGTTGAGCAAGCTTGAAAACAGAATTCATGAATCCT
TGCAGACCTCTCAACGGCATTGAAAATACCCGTTTGACTATAAAAACCGTCGTAATCACTATGATTAATAATTATTTAAAGTGG
GAACTATTTATAATTATAATCAATTATTGTGAGTGGGAATGTTTTGATATGATTAACGCTTTTGAATATAAGTGGTATTTTCTCTT
TCAAATAAATTAACTAACAGATAGGCTTTTTCTAGATAAAGAGGAGTTACCTTTAAT

Appendix C: Fluorescent reporter sequences

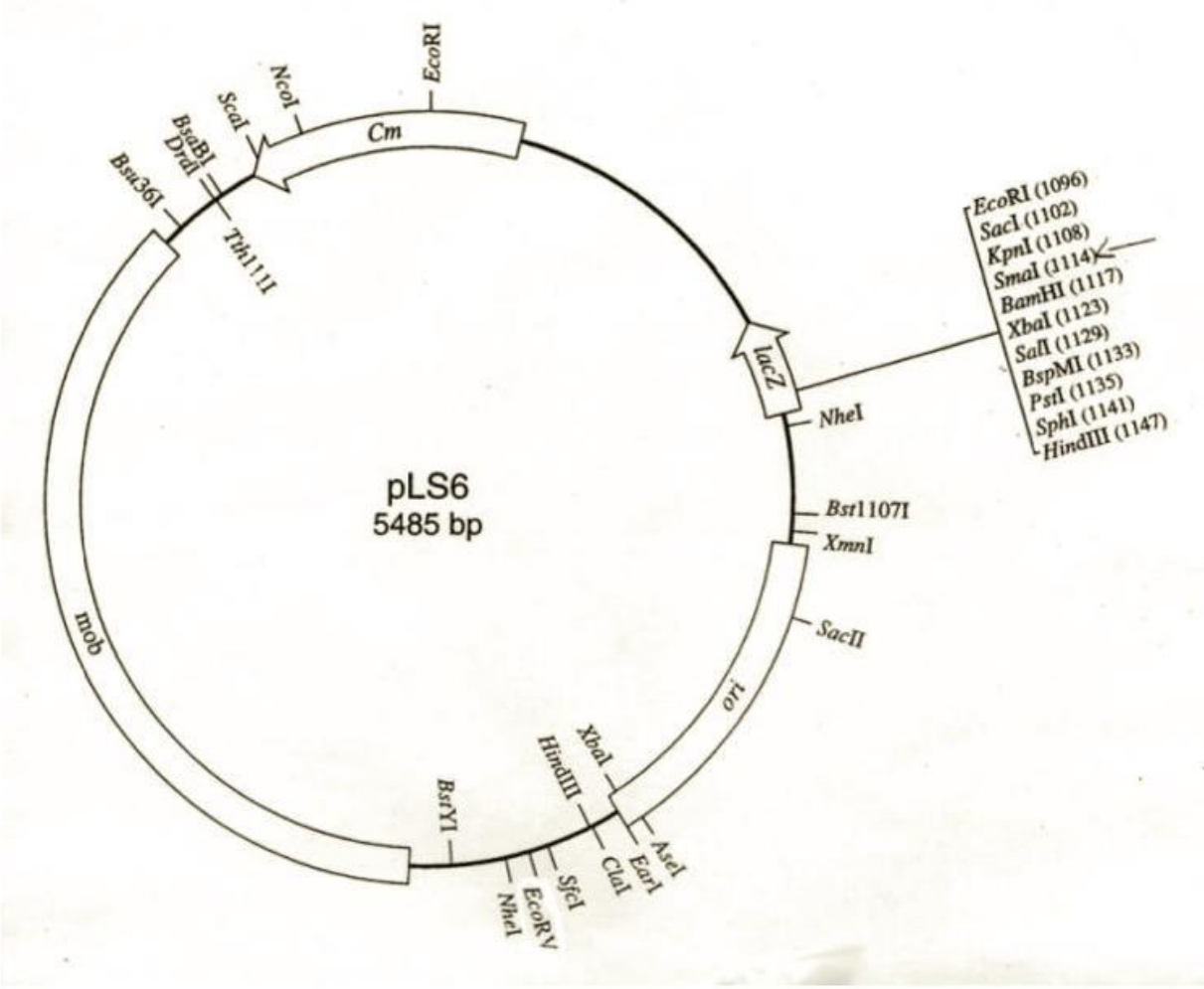
GFP (Green Fluorescent Protein) as obtained from pGLO (Bio-Rad).

ATGGCTAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTT
TCTGTCAGTGGAGAGGGTGAAGGTGATGCTACATACGGAAAGCTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTT
CCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTT
CAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTACAAGACGCGTGCTGAA
GTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTCGGACAC
AAACTCGAGTACAACTATAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAAT
TCGCCACAACATTGAAGATGGATCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTT
ACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTT
TGTAACTGCTGCTGGGATTACACATGGCATGGATGAGCTCTACAAATAA

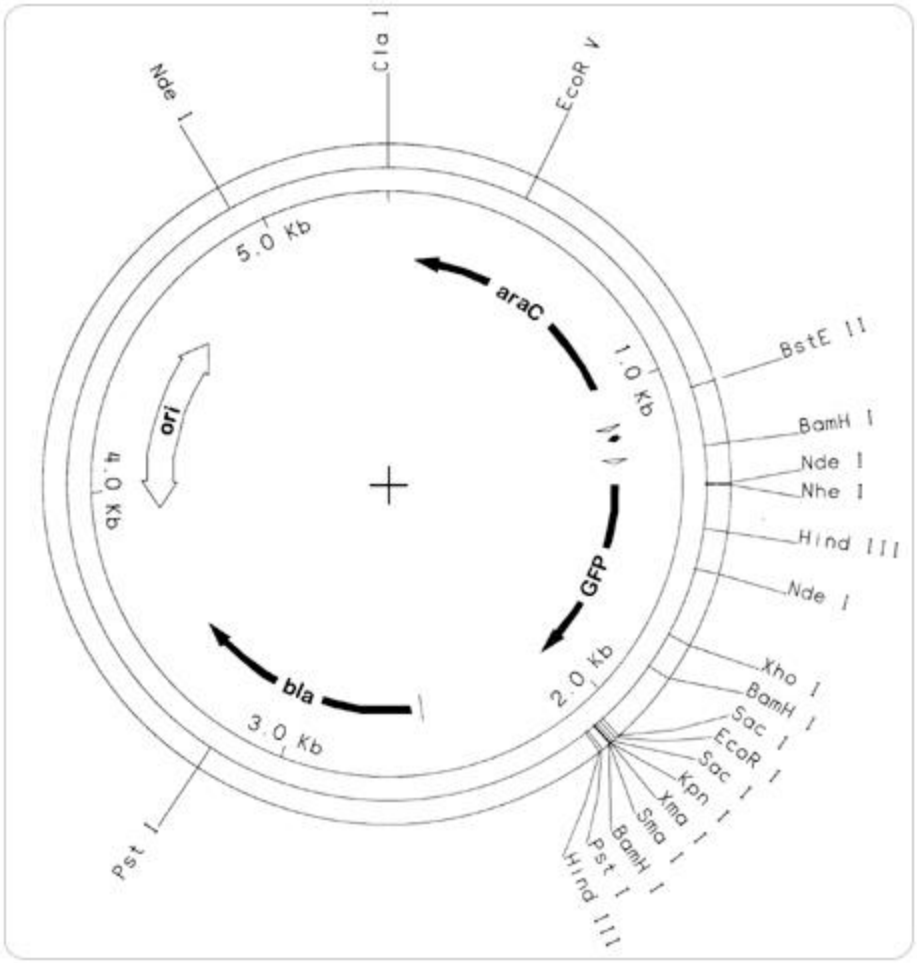
MCH (mCherry) as obtained from pMCH 2053 (McBride Lab).

ATGGTGAGCAAGGGCGAGGAGGACAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTG
AACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACC
AAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCC
GACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGG
TGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGAC
GGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGC
GAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCCGAGGTCAAGACCACCTACAAGGCCAAGAAGCCC
GTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGCTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTA
CGAGCGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAA

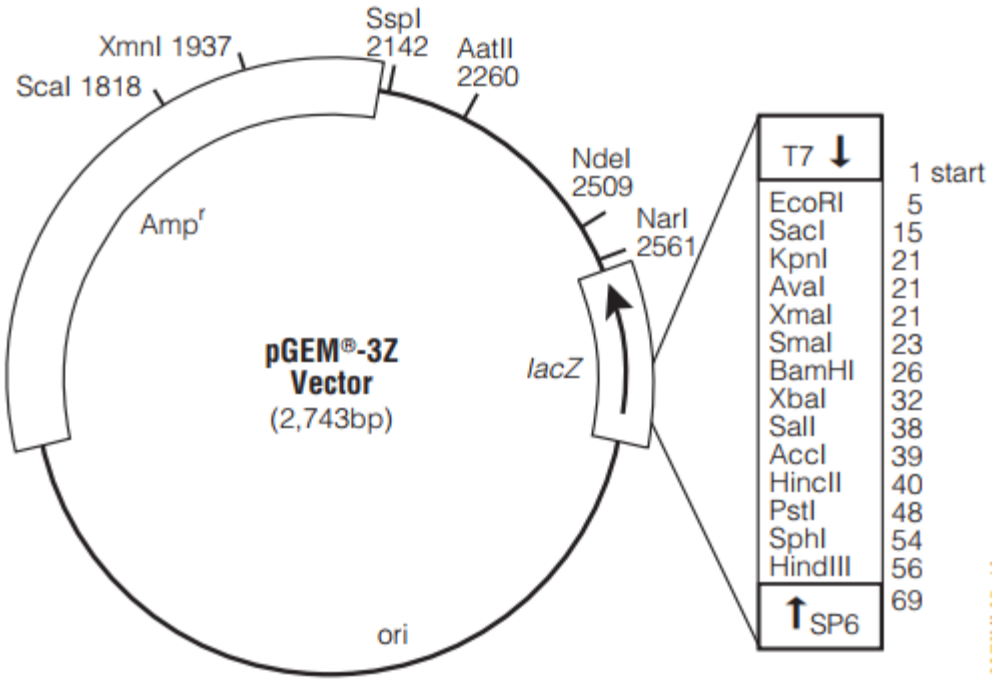
Appendix D: Plasmid Maps



Map of pLS6. Plasmid pLUXR was constructed by inserting *ara-luxR* at the SmaI site



Map of pGLO (Bio-Rad). Used to obtain GFP for construction of plasmids pGFP, pVHGFP, and pVCHGFP.



Map of pGEM-3Z (Promega), used as a basic cloning vector for plasmid construction during this study.

Appendix E: Media

LB (Lysogeny Broth)

1 Liter:

10g Tryptone

5g Yeast Extract

10g NaCl

For plates add 15g agar.

LB Modifications:

LB Ampicillin: LB with 100µg/mL ampicillin added.

LB Chloramphenicol: LB with 25µg/mL chloramphenicol added.

LB Ampicillin + Chloramphenicol: LB with 100µg/mL ampicillin and 25µg/mL chloramphenicol added.

For arabinose induction, 2g/L arabinose was added to LB.

SWC (Seawater complete)

1 Liter:

375mL 2x ASW

5g Peptone (or Tryptone)

3g Yeast Extract

3mL glycerol

2x ASW (Artificial Seawater)

1 Liter:

58.44 NaCl

10.15 $MgCl_2$

6g $MgSO_4$ (anhydrous; 12.3g if $MgSO_4.7H_2O$)

1.49g KCl

LBSG (LB with 2x salt with glycerol)

1 Liter:

10g Tryptone

5g Yeast Extract

20g NaCl

3mL glycerol

For 1.5% plates add 15g agar. For 3% plates add 30g agar.