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Knockout of the trans-acting riboswitches in Listeria monocytogenes to disrupt downstream virulence factors

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KNOCKOUT OF THE TRANS-ACTING RIBOSWITCHES IN *LISTERIA MONOCYTOGENES* TO DISRUPT DOWNSTREAM VIRULENCE FACTORS

BY

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DEDICATION

This thesis is dedicated to my loving wife and daughters for putting up with the long hours I spent in both the classroom and the lab.

ABSTRACT

Listeria Monocytogenes (*L. monocytogenes*) is a ubiquitous bacterium that can be found in both soil and water. This opportunistic intercellular pathogen is often food-borne and can lead to serious illness or death of those that are immunocompromised. Current medical research into the practical use of this pathogen has revealed that the intercellular mechanisms can be exploited to produce a cancer vaccine. Currently *L. monocytogenes* is being studied as a viable candidate for various other cancer vaccines and therapies. The purpose of this study is to reduce the downstream virulence factors that heavily rely on upregulation of the *prfA* gene. In this paper the pIMC plasmid is used in an attempt to knock-out the untranslated region (UTR) of the *prfA* gene located on the *LIPI*-1 pathogenicity island of the *L. monocytogenes*.

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CHAPTER 1

Introduction

Listeria monocytogenes is an intracellular pathogen that causes listeriosis, a disease that can range from mild to life-threatening. Several virulence factors enhance its ability to infect the human body. These virulence factors can be upregulated by the 5' riboswitch that becomes activated when the environmental temperature surrounding the pathogen is raised above 30 degrees C. These virulence factors facilitate the pathogenic response that not only accounts for the morbidity of the disease, but generates a host immune response. This characteristic of *L. monocytogenes* infection has generated interest in using this pathogen in order to develop new immunotherapies. These therapies include vaccines to prevent disease or to suppress tumors.

L. monocytogenes can recruit the body's immune system in a targeted response. This targeted response has allowed for the success of cancer vaccines using *L. monocytogenes*. There is, however, concern that this pathogen could revert to its wildtype or pathogenic form. This reversion of *L. monocytogenes* in an immunocompromised patient could be dangerous, resulting in active listeriosis.

The primary goal of this research project was to investigate the knockout of the untranslated region (UTR) of the *prfA* gene. Knockout of the *prfA* would, presumably, result in a decreased expression of the virulence factors found on the bacterial pathogenicity island as well as other genes controlled by PrfA protein. The pIMC plasmid

was used to knock out the UTR of the *prfA* gene, thereby reducing expression of the virulence factors of *L. monocytogenes*.

CHAPTER 2

Literature Review

Characteristics of *Listeria monocytogenes*:

The prokaryote *L. monocytogenes* is a Gram-positive, low guanine and cytosine (GC) ratio, non-spore-forming motile rod in the Firmicutes division of the Bacteria Kingdom (Liu, 2008). The Listeriaceae family contains the genus *Listeria*; a total of 17 species are described in literature. The 17 species include: *L. monocytogenes* (Pirie, 1940), *L. grayi* (Larsen & Seeliger, 1966), *L. innocua* (Seeliger, 1981), *L. welshimeri, L. seeligeri* (Rocourt & Grimont, 1983), *L. ivanovii* (Seeliger et al.,1984), *L. marthii* (Graves et al. ,2010), *L. rocourtiae* (Leclercq et al. , 2010), *L. fleischmannii* (Bertsch et al. , 2013), *L. weihenstephanensis* (Lang Halter et al. , 2013), *L. floridensis, L. aquatica, L. cornellensis, L. riparia and L. grandensis* (Bakker, et al., 2014) *L. newyorkensis*, and *L. booriae* (Weller, et al. 2015). Both *L. ivanovii* and *L. monocytogenes* cause human disease, but *L. ivanovii* more commonly causes disease in ruminant animals such as sheep (Guillet, et al. 2010).

L. monocytogenes causes listeriosis, a potentially fatal disease in humans (Goldfine & Shen, 2007). *L. monocytogenes* can either be found as a single cell or formed into short chains. It is a facultative anaerobe and can be motile at certain temperatures within the environment. It produces flagella at temperatures less than 30^{o}C, but is down regulated at temperatures above 30^{o}C (Toledo-Arana et al., 2009). Although it is a non-endosporeforming species, it can survive well in harsh

environmental conditions (Labbé & García, 2013), including high salt concentrations, acidic conditions, low pH, extreme temperatures and even short periods of desiccation (Takahashi, Kuramoto, Miya, & Kimura, 2011).

L. monocytogenes can thrive in a variety of environments. This bacterium is well adapted to life in the soil; it can also infect arthropods and animals. *L. monocytogenes* has been recovered from many environmental reservoirs, including soil, silage, groundwater and vegetation. This pathogen can live on decaying organic matter as a saprobe (Toledo-Arana et al., 2009). This species evolved through horizontal gene transfer; it can shift from a free-living cell in the environment to that of an intracellular pathogen in host cells (Cossart & Lebreton, 2014). *L. monocytogenes* does not require many nutrients for growth. The main nutrient requirements are biotin, riboflavin, thiamine, thioctic acid and various amino acids. *L. monocytogenes* does not grow well on salt media that only uses glucose as a sole carbon source (Goldfine & Shen, 2007).

Several clinical methods have been developed to detect the different species of *Listeria*. The basic Gram stain is used to verify the Gram-positive structure. Tumbling motility can be detected using a wet mount slide. Basic tests can confirm *Listeria*; more specific assays are designed to determine the actual species. BioMérieux produces a *Listeria* assay that uses a ten-well test strip for species identification. This test strip uses biochemical reactions to determine DIM (Differentiation /Innocua /Monocytogenes) positives and negatives based on the colorimetric test for naphthylamidase. The reaction is a result of the bacterium's response to esculin, α-mannosidase, the

acidification of D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucoside, D-ribose, glucose-1-phosphate and D-tagatose to detect the differences of the pathogenic strain *L. monocytogenes* and distinguish it from the more benign *L. innocua* (Falkow, Rosenberg, Schleifer, & Stackebrandt, 2006). Additional laboratory tests include the determination of hemolytic activity (*L. monocytogenes* is weakly β-hemolytic), the CAMP test reaction, hippurate hydrolysis, reduction of nitrates, and serotyping using antibody detection and more recently PCR (Leber, 2016).

L. monocytogenes is an opportunistic foodborne pathogen that is transmitted through ingestion. It is ubiquitous in the environment and of concern in the food industry, because of its ability to grow well in temperatures that range from 4 to 10^{o}C. This range allows it to grow at cooler temperatures and thus the species can propagate when stored under refrigeration temperatures. It can then be transmitted through ready-to-eat foods that do not undergo cooking. Normal food preservation tactics such as acidic conditions or high salt concentrations do not prevent the growth of the bacterium. *L. monocytogenes* has various methods to overcome these hostile environmental stressors. The move from free-living to intracellular pathogen requires virulence factors such as listeriolysin O (LLO), two phospholipase C enzymes (plcA/plcB) amongst others (Labbé & García, 2013). Many of these factors are regulated by the *prfA* gene on the pathogenicity island LPI-1 (Schmidt & Hensel, 2004).

Epidemiology of *Listeria monocytogenes*:

In 2001, listeriosis was added to the Centers for Disease Control and Prevention's (CDC) list of notifiable diseases to improve surveillance. The CDC recommends that all *L. monocytogenes* isolates be sent to the state public health laboratories for subtyping ("*Listeria* (listeriosis) | *Listeria* | CDC," n.d.). PulseNet (http://www.cdc.gov/pulsenet/index.html), the national molecular subtyping network for foodborne disease surveillance unit, is designed to find links between cases of outbreaks in foodborne disease. This facility uses DNA fingerprinting techniques or patterns of illness to identify and elucidate local and multistate outbreaks ("*Listeria* (listeriosis) | *Listeria* | CDC," n.d.).

The CDC estimates that in the United States of America there are approximately 1600 cases of listeriosis that occur annually, and of those, 260 cases are fatal. The CDC stated that in 2013, the average annual incidence of listeriosis cases in the United States was 0.26 cases per 100,000 individuals. Of the 831 foodborne outbreaks to occur in the United States in 2012, there were 4 confirmed listeriosis outbreaks and an additional suspected outbreak ("*Listeria* (listeriosis) | *Listeria* | CDC," n.d.).

Once ingested, the *L. monocytogenes* bacterium enters the host body, where environmental factors induce the upregulation of virulence factors that allow it to grow inside the host cell. There are many virulence factors that are upregulated by the shift to the host temperature of 37^{o}C (Chakraborty et al., 1992). Although most infections that occur in normal healthy adults are asymptomatic, there are occasionally illnesses

resulting in symptoms characterized by muscle aches, nausea, vomiting, fever, and fatigue. *L. monocytogenes* is generally considered a self-limiting pathogen. However, it can, on rare occasions, spread by passing through the blood-brain barrier and causing meningitis (Goldfine & Shen, 2007). Its ability to cross the blood brain barrier, the gastrointestinal barrier, and the placental barrier make *L. monocytogenes* a multi-systemic pathogen that can colonize many different host tissues. Symptomatic listeriosis occurs most often in those that are immunocompromised, making *L. monocytogenes* an opportunistic pathogen (Leber, 2016).

Listeriosis can present as a systemic or a localized infection. When listeriosis manifests as a systemic infection, it can present as septicemia. Listeriosis can cause an adult onset form as well as the neonatal/fetomaternal form. This form is thought to originate as a bloodborne infection in the mother that then colonizes the placenta, resulting in spontaneous abortion, a stillborn fetus, or septicemic syndrome (granulomatosis infantiseptica) that occurs in babies that survive to birth. The infection can remain asymptomatic in the mother, although it can present as listerial miscarriage. Women who experience this often complain of flu-like symptoms prior to miscarriage (Liu, 2008). Higher morbidity and mortality rates are associated with early-onset listeriosis. Early-onset listeriosis presents in newborns less than seven days after birth. ("*Listeria* (listeriosis) | *Listeria* | CDC," n.d.).

When listeriosis is diagnosed in otherwise healthy adults, it usually occurs in the central nervous system (CNS) (50 to 70% of patients), resulting in meningitis or

meningoencephalitis. The rate of mortality of brain infections associated with listeriosis ranges from 20-60%, depending on other underlying factors associated with the patient such as age or immune function ("*Listeria* (listeriosis) | *Listeria* | CDC," n.d.).

A variety of foods can become contaminated with *L. monocytogenes*. Dairy products, meats, seafood and vegetables are all potential sources for listeriosis transmission (Leber, 2016). The minimum infective dose is not known, and is very likely patient-dependent. It is estimated that 100 colony-forming units (CFUs) is within the acceptable risk parameter (Affairs, n.d.).

L. monocytogenes is ubiquitous in the soil and can contaminate produce before or during harvest; this is especially true of produce in contact with the soil. Cantaloupe contaminated with *L. monocytogenes,* originating at the Jensen farm located in Colorado in 2011, caused a multistate outbreak of listeriosis ("Multistate Outbreak of listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado | *Listeria* | CDC," n.d.). This outbreak involved 147 individuals in 28 states. The five subtypes of *L. monocytogenes* associated with this outbreak claimed the lives of 34 people, including a miscarried fetus ("Multistate Outbreak of listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado | Listeria | CDC," n.d.). The 147 individuals in this case ranged in age from less than a year old to 96 years of age. The reported median age was 76 years old, with most infected individuals over the age of 60. Seven of the reported cases were related to pregnancy. Four were diagnosed prior to birth; the remaining three were

diagnosed in the neonates ("Multistate Outbreak of listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado | *Listeria* | CDC," n.d.).

Pathophysiology of *Listeria monocytogenes*:

L. monocytogenes gains entry to epithelial cells of the gastrointestinal tract using surface proteins known as internalin InlA and InlB. InlA binds to the E-cadherin cell adhesion molecule, and InlB binds to the hepatocyte growth factor (HGF) receptor of the host (Goldfine & Shen, 2007). As shown in Figure 1, the binding of inlA and inlB forms a membrane bound vacuole around the *L. monocytogenes* cell. The *Listeria* then secretes listerolysin O (LLO), phospholipase C (PlcA, and PlcB). LLO is the pore forming cytolysin and PlcA and PlcB are two phospholipases that together break down the vacuole (Fig. 1) (Liu, 2008).

L. monocytogenes then replicates inside the cytosol using nutrients obtained from the host cell. At this point, it recruits the host cell actin via the actin assembly-inducing protein (ActA) (Freitag, Port, & Miner, 2009). The polymerization of actin forms a "comet" like tail, pushing the bacteria through the host cell's cytoplasm. After reaching the outer edge of the host cell, it pushes against the cellular membrane, causing the membrane to extrude into a nearby cell. This extrusion creates an invagination into the nearby host cell. A double vacuole forms around the *L. monocytogenes* cell, and the infection process starts over (Goldfine & Shen, 2007). It then replicates inside the cytosol of the new host cell. The ability of *L. monocytogenes* to

freely move inside the infected cell, as well as its ability to spread cell-to-cell, are two key factors in the pathogenicity of the species (Liu, 2008).

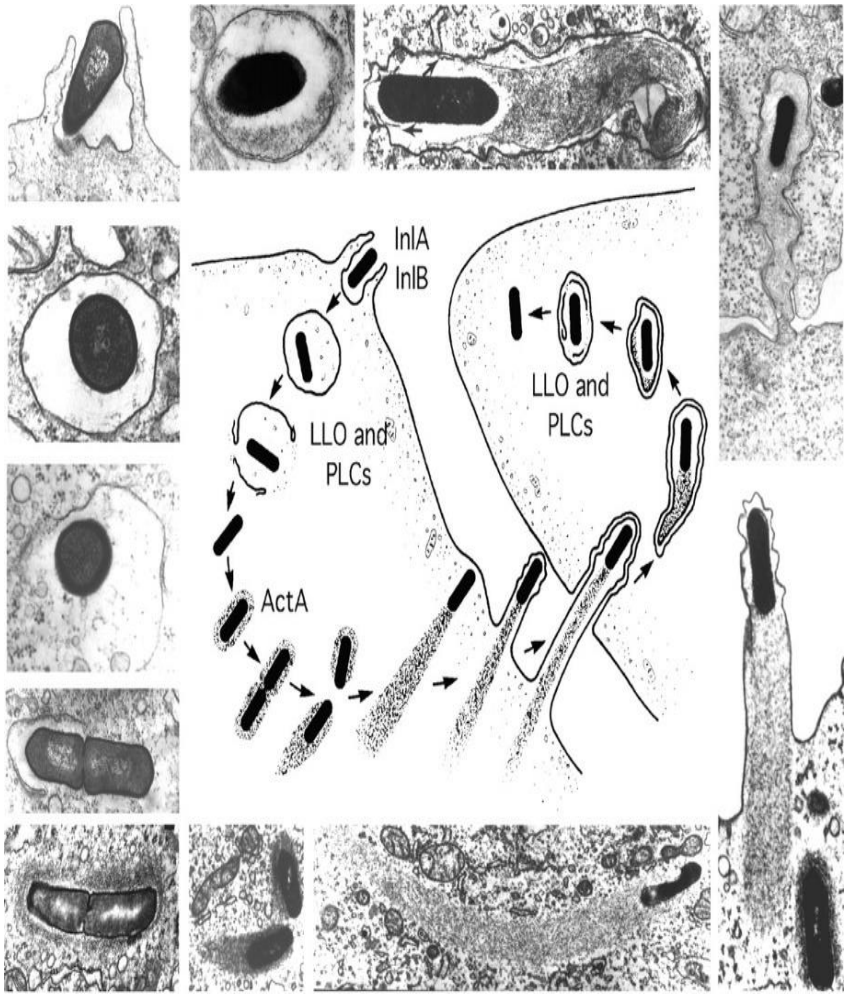

Figure 1. Lifecycle of *Listeria monocytogenes (Tilney & Portnoy, 1989)*

Genetics of *Listeria monocytogenes*:

When *L. monocytogenes* adapts from a free-living saprophyte to an intracellular human pathogen, it must adapt to multiple environmental changes. Several of these changes are thought to upregulate virulence factors. The first is the rise in temperature within a host body, which causes the hair-pin loop to denature, exposing the

thermoswitch aptamer. The activation of the thermoswitch then upregulates the *prfA* transcription activator. This activator has been identified as controlling the regulation of almost all the virulence genes required by *L. monocytogenes* to replicate and spread within the host (Goldfine & Shen, 2007).

Riboswitches are regulatory messenger RNA molecules that contain two domains that are vital to the bacterium's ability to adapt to a changing environment. The first domain is the aptamer domain, and the second is the expression platform domain. The riboswitch can bind to small molecules at the aptamer domain in what is called the ligand binding pocket. This binding can regulate the production of mRNA for gene translation. When an aptamer is bound, the expression platform domain changes conformation and the riboswitch can interact with the transcriptional or translational process and regulate gene activation (Mandal & Breaker, 2004).

The thermoswitch in the UTR of the *prfA* gene is a small hairpin loop. This hairpin loop changes conformation when the environmental temperature is greater than 30^{o}C, and then breaks the nucleic acid hydrogen bonds to reveal the Shine Delgarno (SD) region. This conformation change is what allows the ribosome to bind to the SD region. The *S*-adenosyl methionine **(**SAM**)** molecule then binds and activates the *prfA* gene. This trans-acting riboswitch also influences other genes downstream of the pathogenicity island (Liu, 2008; Xayarath & Freitag, 2009).

L. monocytogenes can gain entry into cells through phagocytosis by macrophages or induce internalization via the internalin virulence factors InlA and InlB.

The internalins belong to a family of leucine rich repeat (LLR) proteins. This family of proteins can be further divided into three classes. The first class, in which inlA belongs, are proteins that tend to express an LPXTG motif (Leu-Pro-any-Thr-Gly) on their C-terminus. The C-terminus is also what anchors InlA to the bacterial cell wall. The second class includes InlB; this molecule is an 80 amino-acid repeat that forms a weak association with the cell wall (Goldfine & Shen, 2007; Liu, 2008). The third class is comprised of internalins that do not seem to present any surface domains and are therefore secreted only in the extracellular space (Luo, Rauch, K. Marr, Müller-Altrock, & Goebel, 2004). The internalins *InlA* and *InlB* are the major effectors that allow the bacterium to enter nonphagocytic cells. (Liu, 2008).

One bacterial mechanism of lateral gene transfer that can occur in *L. monocytogenes* is pathogenicity island spreading (PAI). *L. monocytogenes* has a pathogenicity island known as *Listeria* pathogenicity island 1 (*LIPI-1)*. This gene cluster is thought to be an ancient PAI that has lost the ability to transfer this pathogenic island to other strains of *Listeria*. This pathogenicity island is comprised of a gene cluster of 6 genes including *prfA, plcA, hly, mpl, actA,* and *plcB* (Schmidt & Hensel, 2004). These genes are upregulated by the PrfA protein and are crucial for the intracellular infection and spread of *L. monocytogenes*. *LIPI-1* is only present in *L. ivanovii* and *L. monocytogenes* (Liu, 2008).

Once the bacterium has entered the host cell, it must escape the vacuole (see Figure 1). The *plcA*, *plcB* and *hly* genes facilitate this escape. The *hly* gene transcribes the

virulence factor listeriolysin O (LLO). LLO is a 60 kDa, cholesterol dependent, pore-forming hemolysin toxin. To date, LLO is the most virulent factor in *L. monocytogenes* (Goldfine & Shen, 2007). LLO is essential for escaping both the single- and double-membrane vacuoles. LLO inserts into the cholesterol containing membrane of the vacuole and forms 20 -30nm diameter pores. Bielecki et al. (1990) showed that *L. monocytogenes* with the LLO deletions were unable to escape from the vacuole. LLO is more active at a pH level of 5.5, the same acidity level as a cytosolic vacuole. In contrast, LLO is relatively weak in the neutral pH of the cytosol. This reduced cytotoxicity can explain why it can escape the vacuole but does not cause lesions to the host cell itself (Liu, 2008).

The *prfA* gene is essential for the virulence of *L. monocytogenes* in the host cell. PrfA is a regulatory protein that upregulates more than 18 other genes with at least 63 additional genes being influenced by the Prfa protein. Without the PrfA protein, *hly* is downregulated and LLO is not produced; therefore, escape from the vacuole is unlikely (de las Heras, Cain, Bielecka, & Vázquez-Boland, 2011). The transcription of PlcA forms the previously discussed bicistronic segment of the PlcA and PrfA mRNA, which also includes the hairpin loop riboswitch. The riboswitch is then denatured at temperatures greater than 30^{o}C to activate and upregulate the transcription of monocistronic *PrfA* mRNA segments. This upregulation greatly increases the translation of the PrfA regulatory protein (Böckmann, Dickneite, Goebel, & Bohne, 2000).

The PrfA protein downregulates the flagellin gene *flaA*, causing the loss of the flagella at host temperatures. With the loss of the flagella, all intracellular movement is accomplished through actin filament polymerization, controlled by the *actA* gene. When *L. monocytogenes* loses the *actA* gene, such as in an induced mutant *ΔactA,* the bacterium fails to assemble actin filaments and is therefore not motile in the cell (Goldfine & Shen, 2007; Liu, 2008). The *actA* gene is more active in a host cell than in lab cultures, suggesting other factors may play a role in *actA* upregulation (Bamburg, 2011).

Once the bacterium has formed a double vacuole and successfully infected a nearby cell, it must then escape from a double walled vacuole. Upon initial infection, LLO, PlcA, and PlcB proteins create lesions for the bacterium to escape the single vacuole (Freitag et al., 2009). The *plcB* gene is positively upregulated by the prfA protein, enhancing the bacterium's ability to escape the double vacuole. In murine models with a mutant *ΔplcB*, the bacterium is less likely to escape the double vacuole (Rudnicka et al., 1997). The PlcB protein has the ability to hydrolyze an array of phospholipids to facilitate escape. Because PlcB protein activity is zinc dependent, it relies on the scavenging of zinc by the protease metallopeptidase enzyme coded for by the *mpl* gene. Once the bacterium is free of the double vacuole it is able to replicate, and again recruit actin filaments to begin the cycle over (Goldfine & Shen, 2007; Liu, 2008).

Project Introduction

My attempt to knockout the thermoswitch in the untranslated region of *prfA* consisted of assembling a plasmid vector that would disrupt the 5' UTR of the prfA gene. The plasmid pIMC was acquired from Dr. Sarah D'Orazio at the University of Kentucky. After construction of the pIMC vector, it was electroporated into electrocompetent *L. monocytogenes* cells. Isolates that grew on selective media after electroporation were tested to determine success of the electroporation.

The goal of this research was to examine disruption of downstream genes by knocking out the riboswitches in the 5' UTR of the *prfA* gene. The hypothesis was that by disrupting this region, downstream genes reliant on the riboswitches would in turn be downregulated. This would be detected by quantitative polymerase chain reaction (qPCR). This downregulation would cause a decrease in genes outside the *prfA* regulation box. We could then attempt to identify novel downregulated genes that may assist in the development of safer immunotherapies.

Chapter 3

Materials and Methods

Acquisition of Strain 19111

A Kwikstik™ with the *L. monocytogenes* bacterial strain 19111 ATCC (American Type Culture Collection (ATCC) 10801 University Boulevard Manassas, VA 20110 USA) was aseptically inoculated onto 10 blood agar plates (BAP) that contained 5% sheep's blood. The plates were then incubated at 37°C for 24 hours. A colony from the sample was then Gram stained for tentative confirmation. The isolates were then aseptically transferred to sterile 1.5ml Eppendorf tubes with 500µl of 10% sorbitol and 1% fetal bovine serum (FBS) solution and stored at -80°C overnight. The samples were inoculated on BAP and incubated at 37°C for 24 hours. A single colony was selected for inoculation into brain heart infusion (BHI) broth and incubated for 24 hours at 37°C.

DNA extraction

The ChargeSwitch® gDNA Mini Bacteria Kit (ThermoFisher Scientific, Waltham, MA) was used to collect the genomic DNA via the ChargeSwitch® gDNA Mini Bacteria Kit protocol. 1ml of cultured broth containing *L. monocytogenes* was centrifuged for 10 minutes at 11K RCF, to form a pellet. The supernatant was then pipetted off and discarded. 5µl of the lysozyme solution(50mg/ml) was added to 100µl of the resuspension buffer provided in the kit. This mixture was then added to the bacterial pellet and gently pipetted up and down to form a homogenous mixture of cells in solution, and this was incubated in 37°C water bath for 10 minutes. A solution of 500µl

of lysis buffer and 10µl of proteinase K (one solution per bacterial sample) were added together in a clean sterile 1.5ml Eppendorf tube and mixed together by inverting 6 times. After ten minutes in the 37^{o}C water bath, the 500µl lysis solution was added to each tube and the tubes returned to a water bath for incubation at 55^{o}C for 10 minutes. Following incubation, 40µl of ChargeSwitch Magnetic Beads in solution were added. This was then mixed by gently pipetting up and down before 300µl of binding buffer was added and the mixture incubated at room temperature for one minute. After incubation, the tubes were placed on a magnetic rack and allowed to rest until a pellet formed on the side of the tube. The remaining supernatant was gently removed from the tube. The tubes were removed from the magnetic rack and washed with 1ml of wash buffer by gently pipetting up and down three times. The tube was then placed back onto the magnetic rack and left to rest until a tight pellet had formed. To remove the protein and other cellular material, the supernatant was washed leaving only the DNA. The DNA was collected by adding 100µl of elution buffer to the tube. The tube was placed on the magnetic rack one final time and the elution buffer containing the genomic DNA was collected in a clean sterile 1.5ml Eppendorf tube and placed in the -20^{o}C freezer for storage.

PCR

DNA concentration was determined by Nanodrop (ThermoFisher Scientific, Waltham, MA); it was also used to determine both purity (260nm/280nm) and quality of the sample. The absorbance of the solution at 260nm compared to the absorbance at

280nm is used on the nanodrop to measure purity. After determining the DNA concentration of each sample, 200ng/µl of genomic DNA was chosen as the starting concentration. 21µl of nuclease free sterile water was added to a 0.2ml tube with a puReTaq ready-to-go PCR bead (GE Healthcare and Life Sciences, Pittsburgh, PA). The tube was maintained in a cold rack to prevent premature reactions from occurring. 2µl of DNA (100ng/µl) was added to the tube, after which 10ng/µl forward and reverse primers were added. PCR was set up using a Bio Rad T100 thermocycler (BioRad, Hercules, California) with the initial denature temperature of 90^{o}C for two minutes, followed by 30 cycles of denaturation at 90^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, and finally, elongation at 72^{o}C for 45 seconds. This was followed by a final two minutes of elongation at 72^{o}C. A 2% agarose gel with a 100bp molecular weight ladder was used to identify bands in the 350bp range.

Plasmid Extraction

E. coli with the plasmid pKEN mut2 were obtained via Addgene from the laboratory of Stanley Falkow & Raphael Valdivia, while pIMC was acquired from Dr. Sarah D'Orazio's laboratory at the University of Kentucky. The *E. coli* with the pIMC plasmid was grown on Terrific Broth (TB) agar with chloramphenicol (125µg/ml) overnight. A single colony was transferred to TB and again allowed to grow overnight at 37°C.

A Qiagen plasmid midi prep kit (Qiagen, Hilden, Germany) was used to extract the plasmid. 50ml of cells were collected by centrifugation at 12.5K RCF for 20 minutes

at 4°C. The pellet was then resuspended using a resuspension buffer. The solution was vortexed until the mixture became homogenous. Lysis buffer was then added and then mixed by inverting the tube several times. Ice-cold neutralization buffer was then added and mixed. The tube was then rested on ice for five minutes. The mixture was then centrifuged at 12.5k RCF for 10 minutes and the supernatant was decanted.

A Qiagen tip20 was equilibrated by adding 10ml of equilibration buffer and allowed to empty by gravity flow. The supernatant was transferred to the tip20 and allowed to empty by gravity flow. The tip20 was washed twice with wash buffer. This plasmid was then eluted with elution buffer. Isopropanol was then added to the elution, mixed, and centrifuged at 12.5k RCF for 30 minutes. The supernatant was discarded, and the pellet washed with 70% ethanol before centrifuging for 30 minutes at 12.5k RCF. The supernatant was discarded, and the pellet allowed to dry. 1X Tris EDTA (TE) buffer was added to resuspend the pellet. The mixture was then checked using the Nanodrop for DNA concentration.

Preparing the Vector

To prepare the vector, the pKEN plasmid was cut using the restriction enzymes XbaI and PstI. These two restriction enzymes cut out the GFP cassette of the pKEN plasmid (appendix 5, Fig. 12). The linear plasmid was then amplified by PCR to have one sticky end with PstI and the other with EcoRI. To circularize the plasmid, the DNA insert was then added to a linearized pIMC plasmid along with the T4 DNA ligase. The vector was incubated at 22°C for five minutes.

To insert the knockout into the pIMC plasmid, the plasmid was first cut using the sticky end restriction enzymes PstI and EcoRI. The linear plasmid was then circularized with the knockout sequences using the T4 DNA ligase. The vector was incubated at 22°C for five minutes.

To purify the plasmid, the plasmid was electroporated into *E. coli* and incubated overnight on selective media. The *E. coli* was then transferred to Terrific Broth chloramphenicol (125µl/ml) and allowed to grow overnight. Plasmid extraction was carried out with the Qiagen plasmid mini prep kit (Qiagen, Hilden, Germany) and checked via Nanodrop (ThermoFisher Scientific, Waltham, MA).

Electroporation

To make the *L. monocytogenes* cells electrocompetent, they were first grown on BHI (Brain Heart Infusion) agar overnight at 37°C. A colony was selected and transferred to BHI broth before incubating overnight at 37°C. An aliquot was transferred to BHIS (Brain Heart Infusion Sucrose) broth and grown at 37°C on a shaker on low speed for 2.5 hours. Penicillin (10ng/ml) was then added to the BHIS and returned to the shaker for two hours. The cells were centrifuged at 12.5K RCF and the supernatant removed, after which the cells were promptly washed with a sucrose/glycerol solution before being allowed to rest. The cells were then centrifuged at 12.5K RCF and the supernatant was removed. 30ml of fresh sucrose/glycerol solution was added with lysozyme (10ng/ml) and allowed to activate. The final 30ml of sucrose/glycerol solution was added and the

cells allowed to rest. The cells were centrifuged at 12.5k RCF and a 1ml aliquot of sucrose/glycerol solution was added.

500µl of prepared electrocompetent cells were mixed with 1µl of purified plasmid and then added to a 1cm electroporation cuvette. The GenePulser (BioRad, Hercules, California) was set to 3000V, 400Ω, and 25µf and used to electroporate the cells. The pulse lasted for 4-8mS. After electroporation, the cells were rested in BHIS + IPTG (isopropyl β-D-1-thiogalactopyranoside) (50µg/ml) for an hour at 37°C. The cells were then plated onto BHIS + IPTG + selective media and grown at 37°C for 48-72 hours.

PCR Colony Prep

A single colony was selected using an autoclaved pipet tip. The tip was rubbed onto the bottom of a PCR tube and then into a tube containing BHI. Elution buffer was then added to the PCR tube and placed in boiling water for two minutes. The PCR tubes were then centrifuged and an aliquot added to a new PCR tube. PCR was then performed on the aliquot and analyzed by gel electrophoresis using a 2% agarose gel.

RNA Extraction cDNA Amplification

To begin the extraction, isolates were first grown on BHI agar overnight at 37°C. A colony was selected and transferred to BHI broth and grown at 37°C overnight. The broth was then centrifuged at 12.5K RCF for 10 minutes and the supernatant was discarded**.** The lysis binding solution (50µg/ml) was added to centrifuged cells. A 64% ethanol solution was then added before mixing gently. This mixture was pipetted into a

spin column and centrifuged. The flow through liquid was discarded, and a wash solution was then added to the spin column before repeating centrifugation at 12.5k RCF. Wash solution was added to the tube and centrifuged leaving a pellet. The supernatant was discarded, and the wash step repeated. The spin column was then placed in a clean collection tube and a preheated elution buffer (TE) was added. The elution solution was centrifuged at 12.5k RCF and 100µl of elution buffer was added before repeating centrifugation at 12.5k RCF to maximize RNA precipitation.

The DNase treatment was then applied to the RNA elution. 10x DNase buffer was added to the elution and then incubated at 37°C for 30 minutes. DNase inactivation reagent was then added to the RNA solution. The elution was gently mixed and incubated for two minutes. The mixture was then centrifuged and the supernatant removed. The pellet was then resuspended and transferred to a new tube.

qPCR

The bacterial isolates had been electroporated and grown onto the appropriate selective media containing the antibiotics. RNA was extracted from the isolates and cDNA was produced. The cDNA was then quantified and standardized to 200 ng/µl. The standardized cDNA was added to Promega GoTaq qPCR master mix (Promega corporation 2800 Woods Hollow Road Madison, WI 53711 USA) along with the forward and reverse primers for each virulence gene on a 96-well qPCR plate. The plate was then placed in the ABI 7300 (Applied Biosystems, 850 Lincoln Centre Drive Foster City, CA

94404 USA) at 95°C for 10 minutes, followed by forty cycles at 95°C (15 seconds), 60°C (30 seconds) and 72°C (30 seconds), and finally 10 minutes at 72°C.

Sanger Sequencing

After the DNA was extracted from the electroporated cells using the Charge Switch method, the 5' UTR region of each isolate was amplified using the primer set UTR3_F and UTR_R. The PCR cycle used for the amplification of DNA for Sanger sequencing was 95°C for 5 minutes, followed by thirty cycles at 95°C for 15 seconds, annealing at 54°C for 30 seconds, and elongating at 72°C for 30 seconds, with a final elongation at 72°C for 5 minutes on the Bio Rad T100 thermocycler (BioRad, Hercules, California). This amplicon was electrophoresed on a 2% agarose gel and the bands cut out and extracted using the Qiagen QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). This purified amplicon was then sent to the University of Kentucky's Advanced Genetic Technologies Center for Sanger sequencing. Once the samples were processed and the data retrieved, Bio Edit (Tom Hall, Ibis Biosciences, 2251 Faraday Avenue Carlsbad, CA 92008) was used to create a contig file from the forward and reverse Sanger sequence runs[1]. The contig file was cleaned up using DNA Dynamo (BLUE TRACTOR SOFTWARE, North Wales, UK.).

Chapter 4

Results

Electroporation Results:

The pIMC plasmid with the GFP insert grew as ten colonies on antibiotic selective media. The first five samples that had the insert were grown using kanamycin selective media. The second five samples that had the insert was grown using tetracycline selective media. A total of ten plates of *L. monocytogenes* with the GFP insert produced fluorescence under UV light. No further testing was conducted on these plates.

After proof of concept was achieved, 80 (500µl) aliquots of the bacterial strain 19111 ATCC *L. monocytogenes* were then electroporated over the course of 4 attempts. Each attempt consisted of 5 single insertions and 15 double insertions. Of these 80 aliquots, a total of 62 grew on the antibiotic-containing selective media. There was an average of 105 colonies on each plate (Appendix 2, Table 1-4)[1]. Fourteen attempts at electroporation resulted in an arc fault (a voltage jump between the anode and cathode instead of through the cuvette) that resulted in no colonies being produced since electroporation did not occur. In the first attempt several isolates produced no colonies even though an arc fault had not occurred. In the second attempt, several isolates produced colony growth. In the third and fourth attempts, multiple isolates produced

[1] All tables and figures are presented in the appendices following the thesis.

colonies and were tested via colony PCR method for the correct insert. In the fourth attempt, a single isolate showed the proper bands when colony PCR was performed.

Sanger Sequencing:

The designed PCR products were 425bp, which included base pairs in the plcA and prfA genes to ensure the entire UTR was captured. In the first attempt, colonies grown on antibiotic-containing selective media were sent for Sanger sequencing. The results from this Sanger sequence (Appendix 3, fig. 2 - 10) had multiple errors, large gaps and sequences from the control. No viable data was collected.

The second attempt at Sanger sequencing produced multiple errors and the resultant sequences ranged from 130 to 360bp. Several chromatographs showed poor results with less than the target 350bp. Once the contig files had been compiled and truncated, less than 180bp were viable on most of the sequences.

qPCR Results:

Unfortunately, the qPRC failed. The first qPCR produced no amplification. In the second attempt at qPCR, the machine failed and we were forced to abort this attempt. In the final attempt, data was acquired, but produced results that were not linear, and no relative quantification was seen. This data is not included, due to being performed at the University of Kentucky by a fellow graduate student.

Chapter 5

Discussion

The goal of this project was to downregulate the riboswitch in the 5' UTR region of the *prfA* gene by disrupting the hairpin loop thermoswitch. By downregulating the *prfA* gene, we could then look at virulence factors to confirm downregulation and look for previously unknown genes that would be also be downregulated. While it is known that downregulation can affect virulence factors, this study wanted to examine growth factors also, but we were unable to make it to that point.

L. monocytogenes with the GFP inserts were shown to successfully fluoresce under UV light. This was considered to be an indication of insertion into the genome. However, no further testing was conducted. It was assumed that the knockout of the untranslated region would be successfully transformed. This was a mistake for two reasons. First, the plasmid may have been inserted by the GFP cassette, but not taken up by the genome. The second, even if the GFP cassette had inserted into the genome, it may not have been a stable construct.

Several knockout vectors were then designed that should affect the riboswitch in different ways. The first was to disrupt the riboswitch causing it simply to form incorrectly; the second was an attempt to increase the G and C content to prevent the hairpin loop from opening, and the third was an attempt to disrupt the promoter prior to the riboswitch.

Each knockout vector was electroporated into the electrocompetent *L. monocytogenes* and the bacteria allowed to grow for 72 hours. Because the isolates grew successfully on antibiotic selective media, it is believed the plasmid inserted into the *L. monocytogenes* chromosome, but not at the correct location. Wild type isolates grew successfully after electroporation on LB media within 24 hours, but there was not any growth of wild type on selective media within the 72-hour time frame.

The pIMC plasmid is an excellent choice for insertion of genes into the genomic DNA of *Listeria* because of the phage integrase sequence. The pIMC vector also has three variants each contain a different selectable marker. These selectable marker antibiotics are kanamycin, erythromycin, and tetracycline. This makes the pIMC plasmid versatile in which a double insert can be placed into a single bacterial cell. While pIMC would have been a good choice for the insertion of a gene, it was not appropriate to manipulate the UTR of the prfA gene in the *L. monocytogenes*. pIMC has a default location in which it inserts the gene of interest; this is why insertion of the GFP cassette worked, but the riboswitch knockouts were unsuccessfully transformed in the correct location. A better choice would have been to create an insert for a plasmid such as pKSV7, which does not have the specific location for insertion that pIMC has.

Four attempts were made to insert the pIMC vector containing one of the five inserts into the electrocompetent *L. monocytogenes*. Each attempt resulted in colony growth on the selective media. It was determined that the pIMC vector was inserting at

the wrong location in the genome, and therefore could not be used to disrupt the riboswitch.

On the fourth run, a single isolate produced two positive results when colony PCR was performed. This colony PCR was used to amplify the mutated region of the 5' UTR to inside the prfA gene. It was thought that only a mutant would present with bands. Since it is highly unlikely any mutations occurred within the 5' UTR region, these bands would need to be sequenced to determine why these two colonies produced bands.

Sanger sequencing was performed after the first and third electroporation. Neither sequencing produced usable sequences, as they resulted in sequences that had many gaps and possible erroneous nucleotide insertions and deletion.

qPCR was attempted multiple times, but none were successful. In the first attempt the machine failed and had multiple errors. In the second attempt the reagents may have been out of date, resulting in no data. The final attempt failed because the qPCR machine would not link to the computer and thus had to be aborted.

Our hypothesis was that manipulating the 5' UTR region of the prfA gene could cause further downregulation of genes, leading to potential immunotherapy and cancer vaccination. Further research could include redesigning the correct insert and using a different plasmid, or by using these *prfA* mutants to get a base line of qPCR data for further research. Finally, using illumina miseq to collect, analyze and compare gene data of wildtype *prfA*, mutant *prfa,* and 5' UTR mutant *L. monocytogenes*.

Procedures that should have been performed

One of the first things that should have been performed was to grow the GFP insert on LB media to determine the transformation efficiency. It would have been simple to determine how many colonies on the plate were fluorescent compared to those that were not. This alone would have allowed me to quantify the efficiency of the electroporation. Had I combined this with heat shock I could have determined the efficiency of electroporation versus heat shock.

The plasmid pIMC was not the correct choice for this project. While it is designed to insert a gene into *L. monocytogenes,* it was not designed for the type of knockout I had planned. I feel that the reason the GFP insert was successful and the 5' UTR knockout was due to the location where the insert was placed in the genome. Had I used a vector such as pVS7 with a ~1000bp before and after, I believe that it would have indeed inserted in the correct location.

More data should have been collected on the knockout colonies themselves. The growth process took an extra 24 hours for some isolates. However, I did not account for that in my data collection. I instead just waited 72 hours to collect positive results. I feel that if I had thought that the insertion was at the wrong location and that the insertion could still be affecting the growth of the *L. monocytogenes,* I would have viewed this as more relevant.

Additionally, I would have performed multiple qPCR runs on many of the growth genes to see if any downregulation had occurred after the insert. Had any of the qPCR data been salvageable, it may have shown down regulation of the virulence genes even though the insert was not in the desired location. Unfortunately, the qPCR data was not able to be interpreted.

Finally, Sanger sequencing of the pIMC location could have confirmed that the insert was transformed into the electroporated *L. monocytogenes*. This could have confirmed that the isolates were successfully transformed. While this was not the ultimate goal of this project, conformation of the insert would have been very desirable. This would have provided evidence that the insert was in the *L. monocytogenes*.

Future direction

There are two very appropriate ways to approach future endeavors of this project. The first is of course to use the correct plasmid. This could easily be done by simply using the inserts previously designed in this project and continuing where I left off. I would lay the project out a little different. Knowing what I have learned I feel that inserting the GFP with the new plasmid may not be successful unless the entire cassette is inserted, not just the GFP gene. Since the new plasmid will be designed to insert into *L. monocytogenes,* it would just be a matter of designing the insert to include 1000bp prior to and after the 5' UTR of *prfA*. The regions of *prfA* and *pclA* would be highly conserved and primers quickly designed from the NCBI database. By cloning the 1000bp, designing the new knockout region, and then using Gibson assembly to join, all the

pieces would come together in the insert. Once this new insert was ligated in the pVS7 plasmid, it could then be transformed into the electrocompetent *L. monocytogenes*.

The second future direction of this project is to try to determine if the inserts have indeed affected the growth of the *L. monocytogenes*. Several strains of *L. monocytogenes* contain CRISPR. While the insert was not designed to be a CRISPR guide RNA, it is possible some aspect of CRISPR caused the slowing of growth. This would be of interest because *Listeria* phages could be designed to carry a sequence into a *L. monocytogenes* bacterium. While this project was not a success in that I knocked out the riboswitch, it is worth noting the extended growth time may indicate another method to downregulate the virulence factors of *L. monocytogenes*.

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Appendices

Appendix A:

Primers

Table 1. List of primers used in the experiments.

Name	
prfA_1R	AGCAATGGGAACTCCTGGT
prfA_1F	TGCTGCCGTAAGTGGGAAA
prfA_2R	AAATTCGCCAACATTCCCGC
prfA_2F	ATGTTTGTGCTTGCGCCATT
UTR_1F	Failed
UTR_1R	Failed
UTR_2F	Failed
UTR_2R	Failed
UTR_3	ACGCGTCGACCCCATATATCTAACCCTATTTCAATTTAACATCCTA
UTR_3	CGGGATCCCCGTCTGCTTGAGCGGTTCATGTCTCAT
hly_F 1	ACCTATCCAGGTGCTCCTCGT
hly_R 1	ATCTTTTGCGGAACCTCCGT
hly_F 2	AATGTGCCGCCAAGAAAAGG
hly_R 2	CGTAAGTCTCCGAGGTTGCC
plcA_F	TGGATGTCCGCCTACCTGA
plcA_R	GCAGCATACTGACGAGGTGT
actA_F1	TCATCCGGGAAACAGCATCC
actA_F1	GTTCTGCTAGTTTGGTGCC
actA_F2	CACCGCCTCCAACAGAAGAT
actA_R2	CTTAACGAAGGAACCGGGCT
plcB_F	CTTACAAACACCCGCAGCTC
plcB_R	GCTTTCGCCCTTTTCGCATT
mpl _F1	ATCAAGGTCCACGTCACACC
mpl_R1	AATGAGCATCCACTGCGGAA
mpl_F2	GAACGGGCTGATACCCACAA
mpl_R2	TCCAATCCCGCCGAATACTG
fla_F	ATGACTCAAGCGCAAGAACG
fla_R	AAGGCGACTCATACCCAGCAC
inlA_F	TGCGGTTAAACCTGCTAGGG
inlA_R	TTACAACGCTTCAGGCGGAT
inlB_F	AGACTATCACCGTGCCAACG
inlB_R	GTCGGAGGTTTAGGTGGCAGT
Kn1	GAATTCTCGCCCTTTTTTGGGGCGCCCTAAAAACATTAGGCCCCGGG
Kn2	GAATTCGATATACTAACCCTTTTTGGGGCGCCCTAAAAACATCCCGGG
Kn3	GAATTCTGATGTTTTTTACATATAGGAGGGGCGTATACAAATTCCCGGG
Kn4	GAATTCGGTTCCGAGGTTGCTCGGAGGCCGCCTAACCCTTTTCCCGGG
Kn5	GAATTCAATGCATACATATTTAAAAACGGATGGCGGTAGATGTTAAAATTGAAATAGAGTTCCCGGG
Kn6	CCCGGGTCGCCCTTTTTTGGGGCGCCCTAAAAACATTAGGCGAATTC
Kn7	CCCGGGGATATACTAACCCTTTTTGGGGCGCCCTAAAAACATGAATTC
Kn8	CCCGGGTGATGTTTTTTACATATAGGAGGGGCGTATACAAATTGAATTC
Kn9	CCCGGGGGTTCCGAGGTTGCTCGGAGGCGCGCCTAACCCTTTTGAATTC
Kn10	CCCGGGAATGCATACATATTTAAAAACGGATGGCGGTAGATGTTAAAATTGAAATAGAGTTGAATTC

Appendix B:

Electroporation Tables

Table 2 Electroporation Experiment 1. Colony distribution after each attempt at 3000v

	Preset Voltage	Resistance	Current	Actual Voltage	Time (mS)	Cuvette	Colony
KN1	3000V	400Ω	25µf	Arc	0	1mm	0
KN2	3000V	400Ω	25µf	Arc	0	1mm	0
KN3	3000V	400Ω	25µf	1998	6.7	1mm	1
KN4	3000V	400Ω	25µf	1992	5.7	1mm	0
KN5	3000V	400Ω	25µf	1995	6.4	1mm	0
KN6	3000V	400Ω	25µf	Arc	0	1mm	0
KN7	3000V	400Ω	25µf	Arc	0	1mm	0
KN8	3000V	400Ω	25µf	Arc	0	1mm	6
KN9	3000V	400Ω	25µf	1997	502	1mm	0
KN10	3000V	400Ω	25µf	1998	6.5	1mm	0
KN11	3000V	400Ω	25µf	Arc	0	1mm	0
KN12	3000V	400Ω	25µf	Arc	0	1mm	2
KN13	3000V	400Ω	25µf	1995	5.2	1mm	0
KN14	3000V	400Ω	25µf	1995	4.8	1mm	0
KN15	3000V	400Ω	25µf	Arc	0	1mm	0
KN16	3000V	400Ω	25µf	Arc	0	1mm	0
KN17	3000V	400Ω	25µf	Arc	0	1mm	0
KN18	3000V	400Ω	25µf	1998	6.5	1mm	0
KN19	3000V	400Ω	25µf	Arc	0	1mm	0
KN20	3000V	400Ω	25µf	1992	6.8	1mm	0

Table 3 Electroporation Experiment 2. Colony distribution after each attempt at 3000v

	Preset Voltage	Resistance	Current	Actual Voltage	Time (mS)	Cuvette	Colony
KN1	3000V	400Ω	25µf	1998	7.8	1mm	126
KN2	3000V	400Ω	25µf	1998	7.8	1mm	132
KN3	3000V	400Ω	25µf	1997	7.6	1mm	125
KN4	3000V	400Ω	25µf	1992	7.8	1mm	145
KN5	3000V	400Ω	25µf	1998	7.6	1mm	165
KN6	3000V	400Ω	25µf	Arc	0	1mm	0
KN7	3000V	400Ω	25µf	1687	6.7	1mm	90
KN8	3000V	400Ω	25µf	Arc	0	1mm	0
KN9	3000V	400Ω	25µf	Arc	0	1mm	0
KN10	3000V	400Ω	25µf	1968	6.9	1mm	135
KN11	3000V	400Ω	25µf	Arc	0	1mm	0
KN12	3000V	400Ω	25µf	1658	5.4	1mm	127
KN13	3000V	400Ω	25µf	1667	5.9	1mm	0
KN14	3000V	400Ω	25µf	1988	6.7	1mm	72
KN15	3000V	400Ω	25µf	1992	6.7	1mm	6
KN16	3000V	400Ω	25µf	1998	5.9	1mm	56
KN17	3000V	400Ω	25µf	1784	6.9	1mm	0
KN18	3000V	400Ω	25µf	1657	5.4	1mm	0
KN19	3000V	400Ω	25µf	Arc	0	1mm	0
KN20	3000V	400Ω	25µf	1562	5.2	1mm	0

Table 4 Electroporation Experiment 3. Colony distribution after each attempt at 3000v

	Preset Voltage	Resistance	Current	Actual Voltage	Time mS	Cuvette	Colony
KN1	3000V	400Ω	25µf	1999	7.8	1mm	125
KN2	3000V	400Ω	25µf	1998	7.8	1mm	128
KN3	3000V	400Ω	25µf	1998	7.8	1mm	130
KN4	3000V	400Ω	25µf	1997	7.6	1mm	128
KN5	3000V	400Ω	25µf	1998	7.8	1mm	159
KN6	3000V	400Ω	25µf	1998	7.8	1mm	147
KN7	3000V	400Ω	25µf	1998	7.8	1mm	156
KN8	3000V	400Ω	25µf	1954	4.4	1mm	21
KN9	3000V	400Ω	25µf	1944	4.6	1mm	26
KN10	3000V	400Ω	25µf	1965	4.4	1mm	31
KN11	3000V	400Ω	25µf	1975	7.4	1mm	21
KN12	3000V	400Ω	25µf	1966	7.4	1mm	15
KN13	3000V	400Ω	25µf	1998	7.7	1mm	156
KN14	3000V	400Ω	25µf	1998	7.8	1mm	123
KN15	3000V	400Ω	25µf	1997	7.8	1mm	258
KN16	3000V	400Ω	25µf	1996	7.6	1mm	132
KN17	3000V	400Ω	25µf	1996	7.5	1mm	119
KN18	3000V	400Ω	25µf	1998	7.4	1mm	145
KN19	3000V	400Ω	25µf	1985	6.8	1mm	5
KN20	3000V	400Ω	25µf	1988	6.9	1mm	4

Table 5 Electroporation Experiment 4. Colony distribution after each attempt at 3000v

	Preset Voltage	Resistance	Current	Actual Voltage	Time mS	Cuvette	Colony
KN1	3000V	400Ω	25µf	1998	7.8	1mm	123
KN2	3000V	400Ω	25µf	1998	7.7	1mm	125
KN3	3000V	400Ω	25µf	1998	7.8	1mm	158
KN4	3000V	400Ω	25µf	1994	7.8	1mm	218
KN5	3000V	400Ω	25µf	1992	7.6	1mm	131
KN6	3000V	400Ω	25µf	1992	7.2	1mm	119
KN7	3000V	400Ω	25µf	1996	7.4	1mm	126
KN8	3000V	400Ω	25µf	1995	6.8	1mm	98
KN9	3000V	400Ω	25µf	1996	7.2	1mm	152
KN10	3000V	400Ω	25µf	1996	7.4	1mm	189
KN11	3000V	400Ω	25µf	1998	7.2	1mm	139
KN12	3000V	400Ω	25µf	1992	6.8	1mm	17
KN13	3000V	400Ω	25µf	1990	6.5	1mm	26
KN14	3000V	400Ω	25µf	1992	7.8	1mm	159
KN15	3000V	400Ω	25µf	1998	7.8	1mm	125
KN16	3000V	400Ω	25µf	1996	7.6	1mm	119
KN17	3000V	400Ω	25µf	1998	7.4	1mm	91
KN18	3000V	400Ω	25µf	1998	7.2	1mm	152
KN19	3000V	400Ω	25µf	1994	6.3	1mm	133
KN20	3000V	400Ω	25µf	1992	6.4	1mm	121

Appendix C:

Sanger Chromatograms

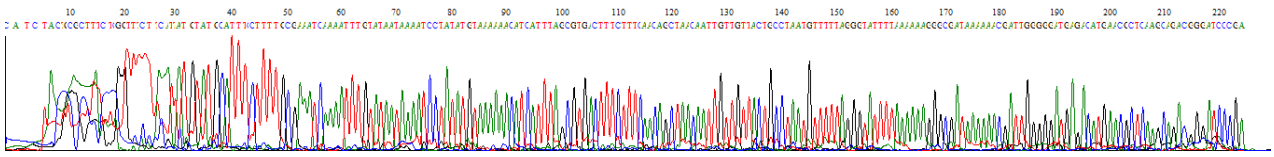

Fig.2 Isolate KN1F Chromatogram using primer UTR3 with expected length of >350bp

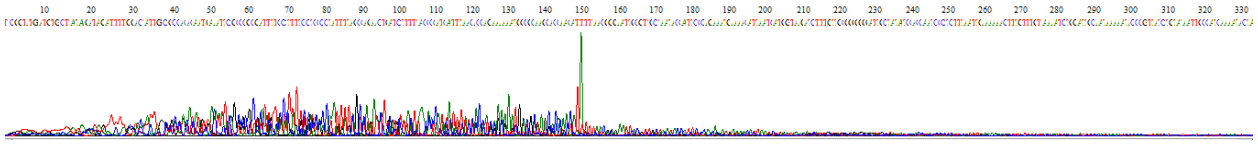

Fig.3 Isolate KN4F Chromatogram using primer UTR3 with expected length of >350bp

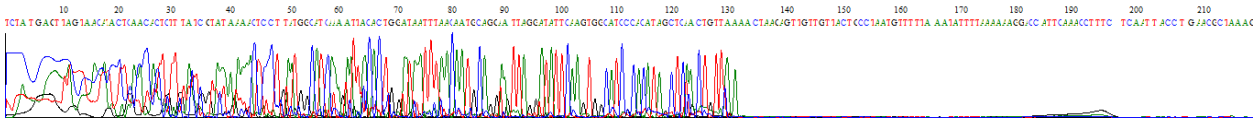

Fig.4 Isolate KN6F Chromatogram using primer UTR3 with expected length of >350bp

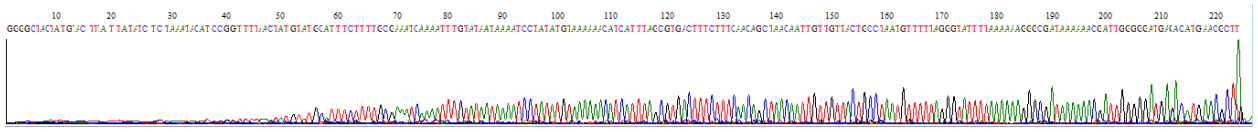

Fig.5 Isolate KN7F Chromatogram using primer UTR3 with expected length of >350bp

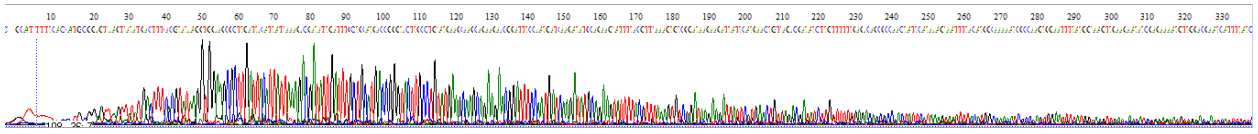

Fig.6 Isolate KN8F Chromatogram using primer UTR3 with expected length of >350bp

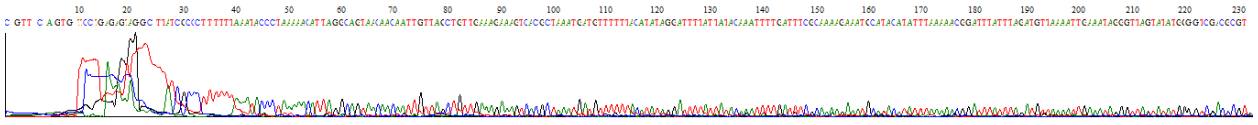

Fig.7 Isolate KN10F Chromatogram using primer UTR3 with expected length of >350bp

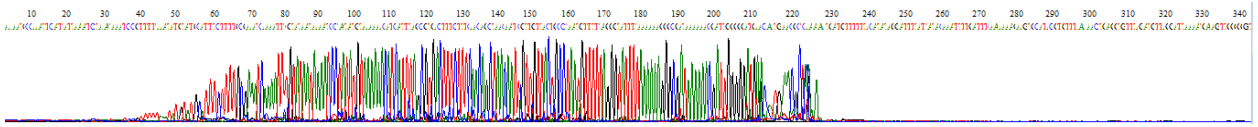

Fig.8 Isolate KN1R Chromatogram using primer UTR3 with expected length of >350bp

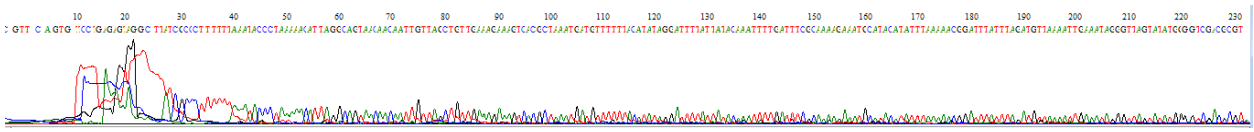

Fig.9 Isolate KN2R Chromatogram using primer UTR3 with expected length of >350bp

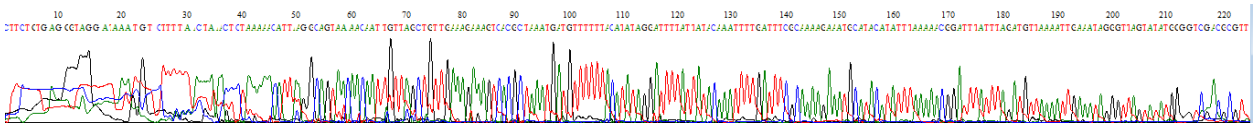

Fig.10 Isolate KN4R chromatogram using primer UTR3 with expected length of >350bp

Appendix D:

Knockout PCR Gel

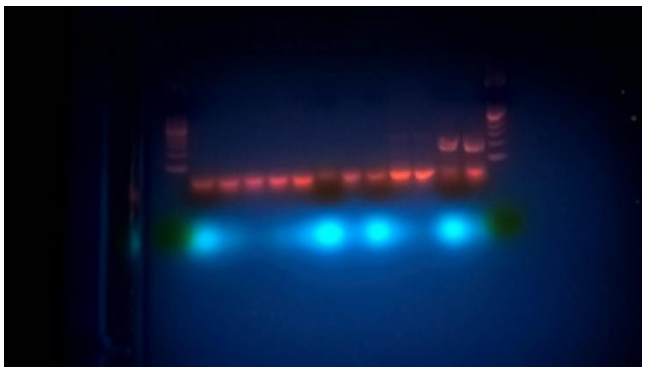

Fig.11 Colony PCR. Isolates were collected and a primer inside the insert and a primer in the pclA gene to look for possible inserts. Wells 12 and 13 show the possibility of an insert. (ladder, KN1_1, KN1_2, KN4_1, KN4_2, KN6_1, KN6_2, KN7_1, KN7_2, KN8_1, KN8_2, KN10_1, KN10_2, and ladder)

Appendix E:

Plasmid Construct

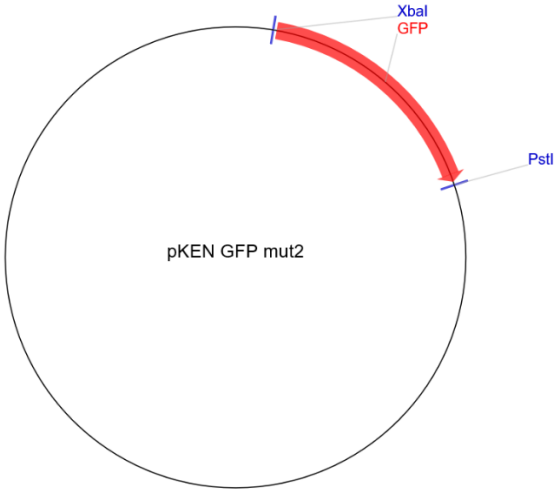

Fig.12 pKEN plasmid construct by Addgene (pKEN GFP mut2 was a gift from Stanley Falkow & Raphael Valdivia (Addgene plasmid # 20409))

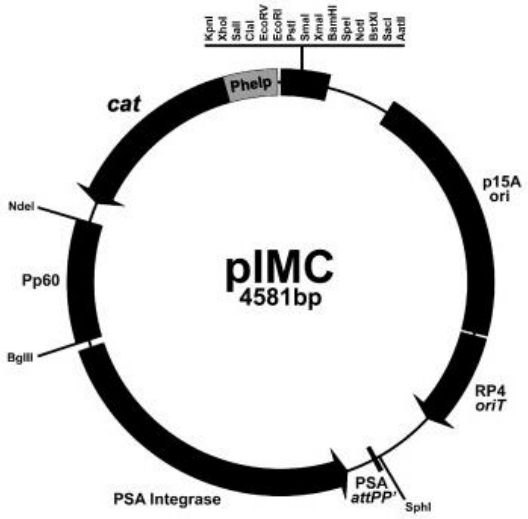

Fig.13 pIMC (Monk, Casey, Cronin, Gahan, & Hill, 2008) given by Dr. D'Orazio lab at the university of Kentucky, Lexington, KY.

Appendix F

5’UTR region of *prfA*

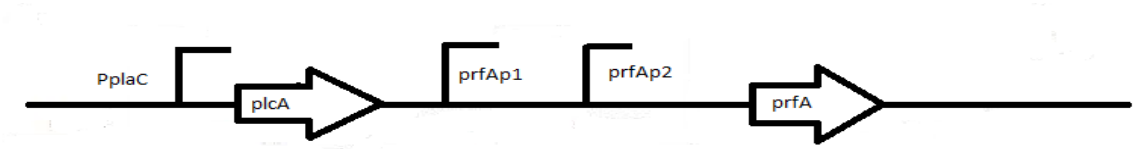

Fig.14 5’ UTR Region of the *prfA* gene with promoters