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Efficiency of the TargeTron Gene Knockout System as a Transformative Protocol for the Mutagenesis of Listeria monocytogenes

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EXPLORATION OF THE TARGETRON GENE KNOCKOUT SYSTEM AS A TRANSFORMATIVE PROTOCOL FOR THE MUTAGENESIS OF *LISTERIA MONOCYTOGENES*

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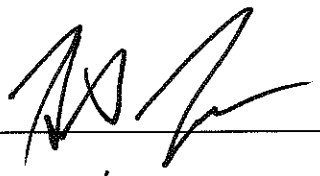
Signature 

Date 4/10/16

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DEDICATION

This thesis is dedicated to my parents,
Ralph and Anita Frazier,
for their unwavering support.

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ABSTRACT

The process of using electroporation to introduce plasmid DNA into host cells is a valuable molecular technique that is increasingly employed in labs worldwide. Electroporators are generally small and relatively inexpensive, making them attractive systems to use for a variety of purposes. Electroporation protocols are numerous in the published literature and encompass all cell types, from prokaryotic bacterial cells to eukaryotic human cells. The TargeTron Gene Knockout System by Sigma-Aldrich is an affordable option for the electroporation of numerous bacterial species. However, its use in Listeria monocytogenes has not been extensively characterized. Here we sought to discuss the effectiveness of the TargeTron Gene Knockout System in transforming Listeria monocytogenes via electroporation along with the challenges this process presents.

We attempted to transfect Listeria monocytogenes with two plasmids constructed through SigmaAldrich as part of the TargeTron Gene Knockout System, pACD4K-C and pNL9164, both of which are designed to induce targeted deletion of genes within the host genome. Electroporation was performed under varying conditions, with voltages ranging from 200 to 1250V. Following shock, cells were grown in blood agar or brain heart infusion media containing kanamycin. Pores were induced in the cell wall prior to electroporation by incubating the bacteria in media containing pencillin, ampicillin, or lysozyme. Results show no colonies on selective media post-electroporation for either plasmid across all conditions.

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CHAPTER 1

Introduction

The process of using electroporation to introduce plasmid DNA into host cells is an increasingly employed method in labs worldwide. Electroporators are generally small and relatively inexpensive, making them attractive systems to use for a variety of purposes. Electroporation protocols are numerous in the published literature and encompass all cell types, from prokaryotic bacterial cells to eukaryotic human cells.

While a significant number of bacterial species have been successfully transformed using this method, the specific protocols for each species vary greatly. In some cases, the bacteria can be difficult to transform. Commercial systems have been developed by manufacturers in order to make the process of transforming bacterial cells more accessible, streamlined, and readily available. The TargeTron Gene Knockout System by Sigma-Aldrich is rapidly becoming a commonly used system for electroporating cells and is an affordable option for smaller, less well-funded laboratories.

In this thesis, I will discuss the methods involved in attempting to transform *Listeria monocytogenes* via electroporation along with the challenges this process presents. I will also assess the effectiveness of the TargeTron Gene Knockout System and whether or not it is a viable option for labs planning on working with this particular species.

CHAPTER 2

Literature Review

Listeria monocytogenes, PrfA, and listeriolysin O

Listeria monocytogenes is an intracellular, food-borne pathogenic bacterium responsible for the disease listeriosis. It is classified as Gram-positive, motile via flagella, and facultatively anaerobic. *L. monocytogenes* is not always pathogenic, but possesses inherent and initially inactive cellular properties, primarily the production of a hemolysin called listeriolysin O, that render it virulent upon activation. Although normally limited to gastroenteritis in healthy individuals, infection with pathogenic *Listeria monocytogenes* can cause up to a 30% fatality rate in certain risk groups, including immunocompromised patients such as those with AIDS, the elderly, or the very young (10). In pregnant women, infection of the fetus is possible, leading to potential abortion or stillbirth (20).

In its extracellular state, *L. monocytogenes* exists as a non-virulent saprophyte and is seen primarily in soil rich with decaying vegetable and plant matter (20). Once ingested by a susceptible host, the bacteria enter epithelial cells of the GI tract. From there, they move to the liver or spleen via macrophages, where they rapidly reproduce within the cytosol of infected cells. If not stopped by the host immune system, they then migrate to the bloodstream causing systemic or central nervous system infections (20). While antibiotics are capable of halting these processes, prolonged use can lead to harmful side effects, such as weight loss and disruption of the normal microbiota of the

host. To combat this, researchers are now investigating the use of bacteriophages as a safer alternative treatment. Promising results have been observed in mice, with no negative effects from the phages being reported (35).

The ability of *L. monocytogenes* to infect and destroy host tissue is mediated by a variety of pathways, genes, and receptors (10). However, a regulatory gene known as *PrfA* has been shown to control most of the virulence factors in pathogenic forms of the bacteria (12). *PrfA* is post-translationally regulated and becomes activated upon arrival in the cell cytosol (55). Synthesis of *PrfA*-regulated proteins has been shown to occur under various types of environmental stress, particularly heat stress, although changes in pH, sugar availability, and carbon metabolism appear to play an important role in activation as well (42). While the environmental conditions required to activate *PrfA* translation are known, the molecular method of activation has not been fully determined. Primary activation appears to occur via the binding of a small cofactor not yet identified, although research suggests that it may be a negatively charged ligand due to the overall positive charge found within the *PrfA* binding pocket (55).

The regulatory and transcriptional pathways leading to *PrfA* translation after initial environmental stress and cofactor binding are also poorly understood. However, recent research indicates metabolic pathways, such as the branch chain amino acid (BCAA) and arginine pathways, may play an important role in the ability of *L. monocytogenes* to replicate within the cell cytosol (31). One study in particular explored

the relationship between the BCAA pathway and the expression of virulence genes, indicating a positive correlation between the two (31).

Of the virulence factors controlled by *PrfA* activation, the production of listeriolysin O (LLO) is arguably the most important. LLO is a cholesterol-dependent, pore-forming toxin and is biochemically similar to other commonly studied bacteria toxins such as streptolysin O and perfringolysin O (61). It is encoded by the *hly* gene locus and is an essential component for virulence. It is only present once the bacteria have successfully entered host cells, although the specifics of this environmentally-driven activation are still unknown. To date, only a theoretical model of the protein's folding exists as no crystal structures have been resolved.

Listeriolysin O is secreted outside of the bacterium as a monomer, although it initially begins as a 529 residue precursor protein before cleavage (61). Upon secretion, LLO migrates to the wall of the phagosome of macrophages where it then blocks the fusion of the phagosome to the lysosome, although the exact mechanism of this action is not currently known (61). This effectively halts destruction of the bacteria allowing it to escape into the cell cytosol. From here, the bacteria is able to replicate and continue proliferation throughout the host (61).

During escape from the phagosome, listeriolysin O works in synergy with lipases such as phospholipase PI-PLC. However, even in the absence of these lipases, the bacteria can still escape from the vacuole using LLO alone (LLO REVIEW). In contrast, the absence of listeriolysin O significantly stunts escape in most cell types. It is for this

reason that I chose to focus on this particular virulence factor and its connection to the translational activator *PrfA* in my study.

Methods of Listeria monocytogenes mutagenesis

Due to lack of thorough information regarding the relationship between metabolism and virulence and its potential to be an effective way of reducing the ability of the bacteria to spread in host cells, further research into this aspect of the bacteria's cellular processes should be conducted. A simple method for exploring this possible connection would be through targeted mutagenesis of the bacterial genome, a process which has been successfully accomplished, but yet still remains somewhat difficult , complex, and many times, costly.

The use of electroporation to transform *Listeria monocytogenes* has been described previously in a multitude of papers (3, 38, 46). However, the specifics of this process vary widely depending on the particular strain, gene locus, and vector being used. Many studies also report varying degrees of effectiveness of the transformation themselves, not only between different techniques, but also between repeated performance of the same protocol and process, even within an individual study. Due to these variations, finding a singular consistently effective protocol is difficult.

Further review of the literature also indicates that the vast majority of successful studies show a strong favoring of the allelic exchange method in lieu of more basic

electroporation procedures. This method also appears to yield a higher number of successful transformants in comparison to other commonly used methods, in particular that of standard electroporation using bare plasmids.

Another popular method for the transformation of *Listeria monocytogenes* is through transduction via the use of listeriophages. This method, as described by Lauer (2002), once again proves to be relatively successful as compared to more standard electroporation procedures. Unfortunately, this method requires more equipment and reagents, making it significantly more expensive and costly.

While allelic exchange and transfection both appear to be viable methods for introducing plasmid constructs into *Listeria monocytogenes*, they are also complex, time consuming, and expensive procedures to perform. Smaller university labs, particularly those with limited research funding or support, often lack the necessary equipment required to implement these methods. The simpler system involving transformations via transposons is a much more realistic venture for a small setup. However, this technique is much more difficult to perform successfully in *Listeria monocytogenes*.

In 2008, Monk *et. al.* conducted a review of the tools available to observe genomic changes in *L. monocytogenes* using the transposon method previously mentioned above. Their results were impressive and a large number of transformants were successfully recovered. However, like most transfection and allelic exchange protocols, the methods used to design the *Listeria* -specific plasmids used in the study were complex, expensive, and difficult to perform. Required reagents included a total of

six different vector constructs, 10 sets of primers, a strain of *E. coli*, and eggs obtained from the frog species *X. laevis* for cytoplasmic culture (35, 38). Unfortunately, this level of vector construction is generally beyond the scope of a smaller lab. Due to these limitations, a simpler and more user-friendly system is desirable.

The TargeTron Gene Knockout System

The TargeTron Gene Knockout System is a simple and streamlined protocol designed by Sigma-Aldrich for the purpose of rapidly and permanently interrupting genes in a variety of prokaryotic organisms (50). The system was primarily designed with *E. coli* and other taxonomically related bacteria in mind; however, the availability of different TargeTron vectors allows the system to be used in a wide variety of bacteria (50). It has been tested in numerous bacterial species, both Gram-positive and Gram-negative, including *S. aureus*, *S. pyogenes*, and *E. coli*.

It is important to note that the TargeTron System protocol is geared toward the design of primers, ligation of plasmids, and the induction of introns present within the available vectors. While it is intended to be used as a way of introducing transposable elements into the genome of cells through the use of electroporation, it does not actually include the specific parameters needed to accomplish this. Instead, the system requires users to find appropriate information using outside resources.

While the efficiency of the TargeTron System has been clearly demonstrated across numerous genera, *Listeria* does not fall under this category. As it stands, only one independent study, (3), has successfully recovered transformants using this system. The study, which was conducted to assess the effect of mutational activation of *PrsA2* on the virulence of the bacteria, used the pNL9164 vector alongside a separate, cadmium-inducible shuttle vector (3). This additional shuttle vector is not an official TargeTron vector and is not commercially available for purchase.

Proposed Project

For my project, I attempted to fill in some of the gaps in knowledge indicated by previous research. As stated earlier, prior studies have suggested that the cellular processes governing metabolism may be connected to those controlling virulence activation (31). Based on this, I designed my project to examine at the effect of interrupting the arginine tRNA ligase gene on production of listeriolysin O. A decision was made to use the TargeTron Gene Knockout System by Sigma-Aldrich to complete this study as our lab was already in possession of the written protocol, all standard reagents required, and an adequate amount of complementary primer purchases.

However, after a considerable amount of time attempting to complete our designed project, it became apparent that the system chosen was not producing results. At this point, my research focus shifted from the effects of transformation to the technical

process of *Listeria monocytogenes* mutagenesis itself. Multiple published studies show that this process is incredibly complex and nuanced with consistent results being somewhat tenuous (38). Only one paper, published by Alonzo in 2009, addresses the TargeTron System's use in this particular species of bacteria. For this reason, I chose to aim this thesis at the overall efficiency of the TargeTron System and its associated vectors and reagents in transforming *Listeria monocytogenes* and whether or not it is a viable option for smaller labs such as ours.

Glossary of Terms

Arginine tRNA ligase - a gene involved in the metabolism and synthesis of arginine

Electrocompetence - a state in which cells are prepared to undergo electroporation; involves specific growth parameters, media, and treatment with antibiotics

Electroporation - the use of an electrical voltage to create small pores in the wall of cells in order to allow the uptake of plasmid DNA

Listeria monocytogenes - a pathogenic bacterial species responsible for listeriosis

Listeriolysin O - a hemolysin used by *L. monocytogenes* primarily to escape host cell vacuoles

PrfA - a gene that controls a significant amount of virulence factors present in *L. monocytogenes*

Saprophyte - an organism that feeds on decaying organic matter

Purpose of Research

For this project, our lab attempted to fill in some of the gaps previous research has indicated. As stated earlier, prior studies have suggested that the cellular processes governing metabolism may be connected to those controlling virulence activation. Based on this, I designed my project to look at the effect of interrupting the arginine tRNA ligase gene on listeriolysin O production. A decision was made to use the TargeTron Gene Knockout System by Sigma-Aldrich to complete this study as our lab was already in possession of the written protocol, all standard reagents required, and an adequate amount of complementary primer purchases.

As transformation of *Listeria* was not possible, we shifted focus onto the viability of the TargeTron Gene Knockout System itself. Thus, this thesis serves as an attempt to document the pitfalls and complications involved with using this system as a mutagenesis protocol for the transformation of *Listeria monocytogenes*.

CHAPTER 3

Methods

Listeria monocytogenes strain

For this project, we chose the *L. monocytogenes* strain EGD-e (Murray, *et. al.*) purchased from ATCC®. Kwik-Stick swabs were kept at 4° C prior to culturing. Bacteria was cultured on blood agar plates (BAP) using isolation streaking and incubated overnight in a 37° C table-top incubator. Individual colonies were subsequently harvested and transferred to sterile Eppendorf tubes containing 1mL of fresh 1% serum sorbitol solution (100 mL distilled water, 1g sorbitol, 1mL bovine solution, mixed and autoclaved. Tubes were stored at -80° C until needed. In total, 15 tubes were prepared and frozen.

Primer design for arginine tRNA-ligase

To locate the insertion site for gene interruption, we used the NCBI GenBank program to choose an appropriate gene locus. The locus containing the arginine tRNA-ligase gene (Accession #YP_007608457) was determined to be the best choice. To design primers for re-targeting of the vector, we used the TargeTron Gene Knockout System automated algorithm. Three sites were generated using this system and ranked according to the estimated percentage of insertion success. The top two matches were chosen for this study. Primers were generated via the TargeTron System.

Testing of DNA extraction protocols for detection of listeriolysin O in samples

Prior to beginning the mutagenesis protocol, we tested the efficiency of the Invitrogen DNA extraction kit in isolating DNA from *Listeria monocytogenes*. Thawed samples were cultured on BAP and incubated at 37° C overnight. Individual colonies were then transferred to tubes containing 9mL of tryptic soy broth (TSB) and again incubated overnight at 37° C. DNA was extracted from the broth cultures using the protocol included with the Invitrogen DNA extraction kit. DNA was extracted via ethanol precipitation. The resuspended DNA pellet was kept in sterile Eppendorf tubes at 4° C overnight or -25° C for long-term storage. Thawed samples of DNA were tested for amount and purity using the NanoDrop™ provided by another lab.

Testing of positive controls for detection of listeriolysin O through PCR

Upon receiving the Invitrogen primers specific to the gene locus *hly* (accession #NC_003210.1), we tested our previously cultured samples for the presence of listeriolysin O in order to confirm our positive controls. Samples of frozen culture were thawed and mixed thoroughly. Each primer was rehydrated with 11.95 µL of RNase free water to make 12 µL of master stock. Rehydrated primers were then further diluted into working stock (10 µl master stock, 90 µl RNase free water) and aliquoted into sterile PCR tubes for storage at 4° C.

Samples from the previous DNA extraction with high yields were chosen for positive controls. A total of 4 tubes were prepared. Upon completion of PCR, samples were removed and analyzed via gel electrophoresis using a 1% agar gel with a run time of 1.5 hours at 80 V. The gel was then observed under UV light for bands at 48 kDa.

Primer-mediated re-targeting of TargeTron System introns via PCR and gel electrophoresis

Two primer sets specific for the arginine tRNA gene locus acquired via the TargeTron System were rehydrated to 100 µM in preparation of PCR (designated 285-285a IBS, EBS1d, EBS2 and 97-97a IBS, EBS1d, and EBS2). A master mix was prepared for each set according to the TageTron Gene Knockout System protocol guidelines for a total of 20 µl (Fig 1). PCR reactions were prepared using GE Healthcare PuReTaq tubes (23 µl RNase free water, 1 µl master mix, 1 µl intron PCR template, and 25 µl JumpStart REDTaq ReadyMix). After PCR cycling was complete, 3 µl of loading dye and 7 µl of water were added to 10 µl of the PCR samples and mixed thoroughly. Samples were then loaded into a 1% agar gel and run using a standard gel electrophoresis protocol. The gels were observed under UV light for the presence of banding at 300 bp.

Purification of PCR products

PCR products were purified with the GenElute PCR Purification kit from Sigma-Aldrich using the protocol provided. A ratio of 50 µl of binding buffer to 10 µl of PCR product was determined to be optimal. Purified products were then tested via the NanoDrop machine as described previously. Gel electrophoresis was performed on purified samples to ensure an increase in purity and adequate yield.

Double digestion of purified PCR products

Samples were digested according to the TargeTron System protocol. 8 µl of purified PCR product, 2 µl of 10x Restriction Enzyme Buffer, 1 µl of the restriction enzyme HindIII, 1 µl of the restriction enzyme BsrGI, and 8 µl of RNase free water were added to an Eppendorf tube and vortexed. Tubes were incubated in a 37° C water bath for 30 minutes, a 60° C water bath for 30 minutes, and an 80° C water bath for 10 minutes. After cooling, samples were then stored at

-20° C overnight.

Preparation of media for electroporation protocol

Brain heart infusion (BHI) broth was prepared using 74 g of powdered BHI per 1 litre distilled water. The mixture was stirred and heated till boiling to ensure complete

homogeneity of solution and subsequently autoclaved for sterilization. A sucrose solution containing 59.9 g of sucrose in 175 mL of distilled water was made for supplementation of BHI broth. The mixture was stirred and heated until all sucrose had dissolved and subsequently filter sterilized into a freshly autoclaved flask using a .45 micron nylon membrane syringe filter. BHI sucrose (BHIS) broth was prepared by mixing BHI broth and sucrose solutions in a 1:1 ratio.

Selective media was prepared by supplementing 500 mL autoclaved BHI agar with 450 µl stock kanamycin solution. Plates were stored in the walk-in freezer at 4° C until needed.

Preparation of electrocompetent cells

Thawed *L.monocytogenes* was cultured on BAP overnight at 37° C. Individual colonies were subcultured in 7.5 mL BHI broth supplemented with 7.5 mL filtered sucrose solution overnight at 37° C. The following day, 5 mL of subculture was transferred into 50 mL of BHIS and incubated for 4 hours at 37° C. At 4 hours, 1 mL of penicillin G stock solution (0.0125 g penicillin sodium salt + 20 mL NaOH) was added to the BHIS broth and incubation continued for a further 2.5 hrs.

The bacterial culture was then transferred to a sterile, disposable centrifuge tube. The solution was centrifuged at 5000 x g, 4° C for 10 minutes. Supernatant was removed and the cells washed with 40 mL of filtered sucrose solution. The tube was again

centrifuged using the same parameters. Supernatant was removed and cells washed with 20 mL of sucrose solution. This was repeated twice. After the final spin, supernatant was removed and the cells were resuspended in 2.5 mL of sucrose solution. 100 µl aliquots were transferred into sterile autoclaved Eppendorf tubes and frozen at -25° C until further use.

Ligation of TargeTron System vector

In accordance with the TargeTron System protocol provided in the kit, the TargeTron vector pACD4K-C was ligated using the QuickLink T4 Ligation Kit (Sigma-Aldrich, cat. #LIG2-1KT). A solution containing 2 µl vector, 6 µl digested primer product as previously described, 10 µl QuickLink Buffer A, and 2 µl QuickLink Buffer B was prepared and mixed in a sterile Eppendorf tube. The mixture was heated to 60° C via water bath for 30 seconds. The tube was then immediately transferred to ice and cooled for 1 minute. Upon removal from ice, 1 µl of QuickLink T4 Ligase was added to the mixture. The tube was left at room temperature for 30 minutes.

Transformation of Listeria monocytogenes

After ligation, 100 µl of thawed electrocompetent cells were transferred into a chilled 1 mm Bio-Rad cuvette. The entirety of the ligation mixture (a total of 20 µl) was added to the cuvette. The cuvette was loaded into the Bio-Rad GenePulser Xcell

electroporator. Cells were pulsed in the electroporator at 1,250 volts (V), 25 mirco farads (μF), and 200 Ohm. This protocol was based off of recommended settings in the user manual for the TargeTron System.

Electroporated samples incubated for 1.5 hrs in a low temperature incubator at 30° C. Upon removal from the incubator, the samples were plated on BAP and BHI+kanamycin media and incubated overnight at 37° C.

Substitution of provided vector with pNL9164

Due to the lack of results, each subsequent transformation attempt was modified in an attempt to confer a higher rate of plasmid uptake and integration. The vector pACD4K-C was replaced with another TargeTron vector (pNL9164, Sigma-Aldrich, Cat. #T6701-2UG), a Gram positive specific vector that had been reported to be successful according to previous research (3).

This substitution required a minor change in the previously reported digestion protocol and is described as follows: 1 μl of the restriction enzyme *Dpn*I was added while the given amount of distilled water was reduced from 8 μl to 7 μl. This resulted in a slightly modified digestion mixture with the total volume remaining 20 μl.

Table 1. Attempted Modifications to the TargeTron System Protocol. (*represents inclusion of lysozyme.)

Growth Media	Antibiotic	Plasmid	Wash Buffer	Cells	Electroporation Parameters	Inc. Time	Plate Media	Colonies
250 mL BHI + sucrose	1 mL penicillin G stock (0.0125 g/20 mL)	pACD4K-C	Sucrose	Thawed	1250 V, 25 µF, 200 Ohms	2 hrs	BAP, LB + kan	None
250 mL BHI + sucrose	2.5 mL penicillin G stock (100 mg/mL)	pACD4K-C	Sucrose	Thawed	1000 V, 25 µF, 200 Ohms	1 hr	BAP, LB + kan	None
100 mL BHI + sucrose	5 mL penicillin G stock (1 mg/mL)	pACD4K-C	Sucrose	Thawed	1000 V, 25 µF, 200 Ohms	1 hr	BAP, LB + kan	None
*500 mL BHIS autoclaved	100 µl ampicillin stock (50 mg/mL)	pACD4K-C	SGWB	Thawed	1000 V, 25 µF, 400 Ohms	1.5 hrs	BAP, BHI + kan	BAP only
*50 mL BHIS autoclaved	10 µl ampicillin stock (50 mg/mL)	pACD4K-C	SGWB	Thawed	1000 V, 25 µF, 400 Ohms	20 min	BAP, BHI + kan	BAP only
*50 mL BHIS autoclaved	10 µl ampicillin stock (50 mg/mL) + 10 µl kanamycin stock	pNL9164	SGWB	Thawed	200 V, 25 µF, 400 Ohms	20 min	BAP, BHI + kan	None
50 mL BHIS autoclaved	10 µl ampicillin stock (50 mg/mL)	pNL9164	SGWB	Thawed	1000 V, 25 µF, 400 Ohms	1.5 hrs	BAP, BHI + kan	BAP and BHI + kan*
50 mL BHIS autoclaved	10 µl ampicillin stock (50 mg/mL)	pACD4K-C	Sucrose	Thawed	500V, 25 µF, 400 Ohms	1.5 hrs	BAP, BHI + kan, LB + kan	BAP only
50 mL BHIS autoclaved	10 µl ampicillin stock (50 mg/mL)	pNL9164	BHI	Fresh	750 V, 25 µF, 400 Ohms	1.5 hrs	BAP, BHI + kan, LB + kan	BAP only

Other changes to standard protocol

In addition to the substitution of pACD4K-C with pNL9164, other modifications were made to each attempted transformation. Due to the high number of attempted transformations, all modifications made are categorized in the table on the following page (Table 1).

CHAPTER 4

Results

Gel electrophoresis of positive controls and re-targeted TargeTron primers

Positive control samples amplified at the *hly* gene locus showed banding at approximately 250 bp (Fig 2). This is consistent with expected results of the electrophoresis, with the amplified portion of the *hly* gene being approximately 200-300 bp in size (29).

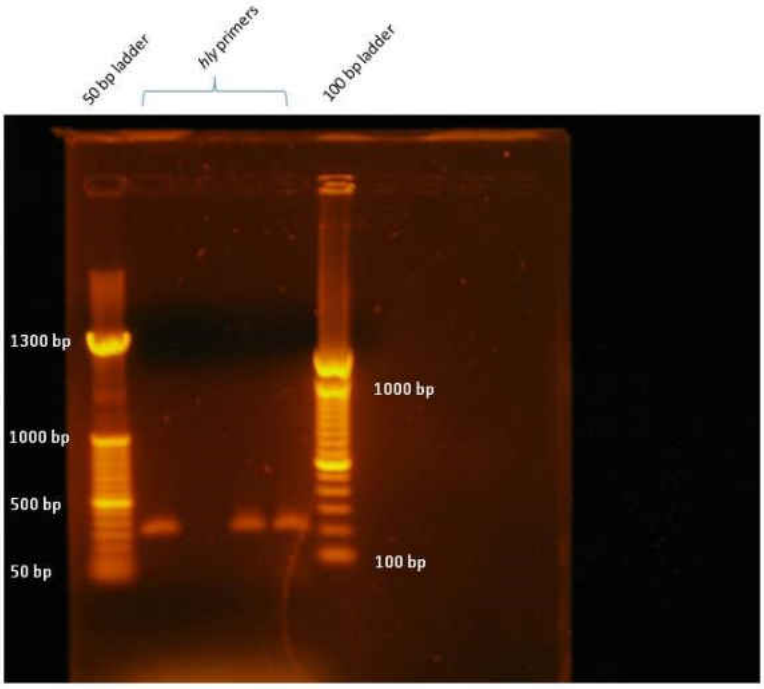

Fig 1. Gel Electrophoresis of Positive Controls

Re-targeted primers showed clear, bright banding at 300 bp (Fig 3). This is consistent with predicted results according to the TargeTron System protocol (50).

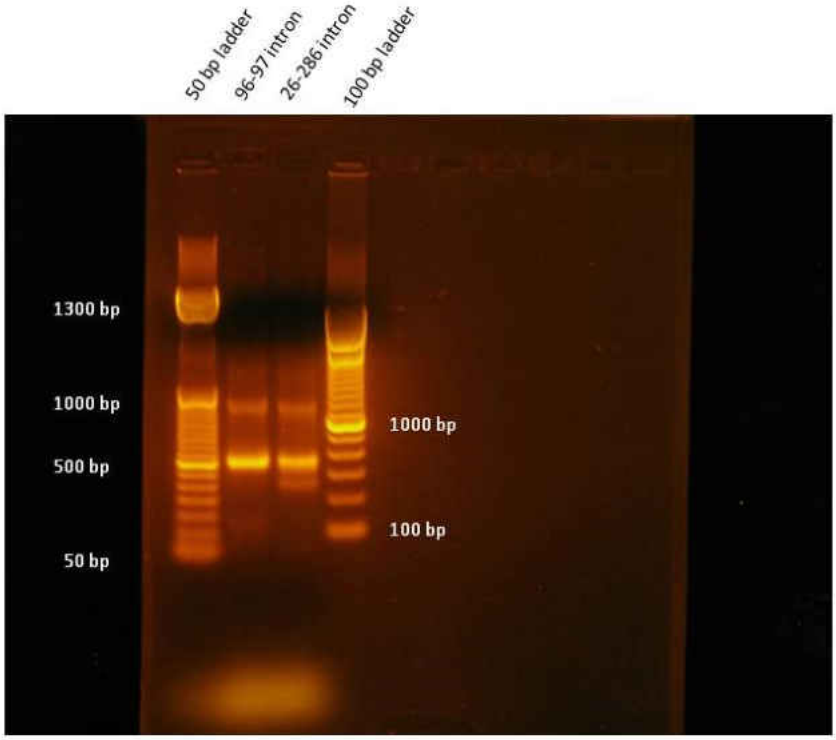

Fig 2. Gel Electrophoresis of Re-Targeted TargeTron Primers

Purified re-targeted primers also showed banding at 300 bp as expected (Fig 4). However, one of our primer sets (95-96, shown in wells 4 and 5 in the figure below) did not stay in their designated wells. Two new samples were mixed with loading dye and re-entered before running the gel.

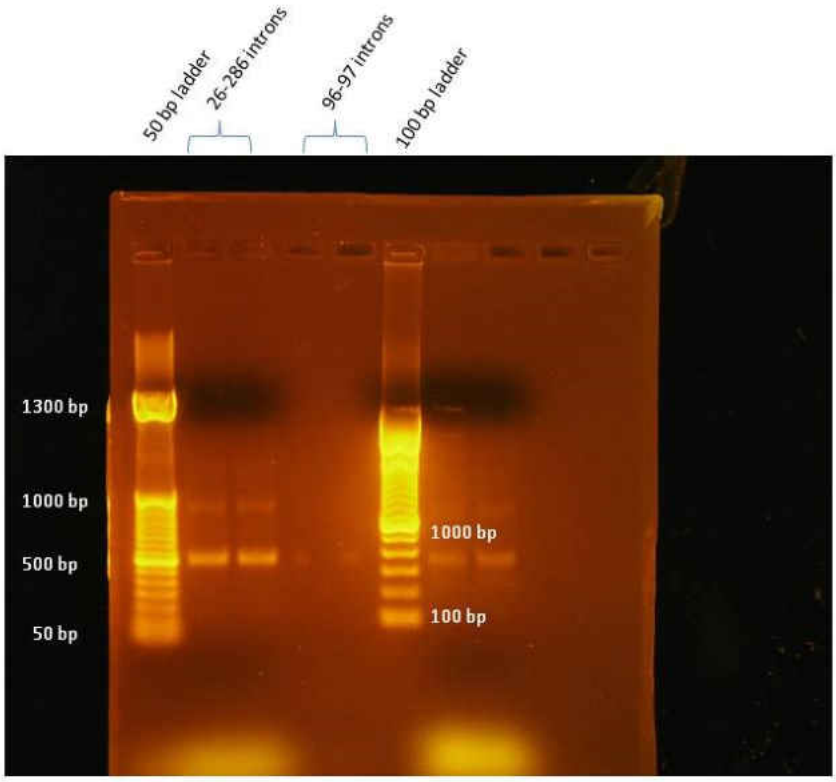

Fig 3. Gel Electrophoresis of Purified Re-Targeted TargeTron Primers

Electrocompetent cell growth and transformations

Multiple attempts to transform *Listeria monocytogenes* proved unsuccessful using the provided reagents, protocols, and vectors that were included with the TargeTron System. Modifications to the protocols compiled from outside sources were implemented with varying results (Table 1). Some changes, in particular the transition from a sucrose wash buffer to SGWB (without the addition of lysozyme), dramatically

increased growth of electroporated cells on BAP. When using only the sucrose solution, few colonies were seen on the plates with most concentrated in the area of the primary streak. Upon switching to SGWB, complete growth comprising all streaked segments of plate were observed. When supplementing the SGWB with lysozyme according to the protocol put forth by Park, growth was significantly hindered, although still more pronounced than that seen using only sucrose.

Growth of transformed bacteria on selective media appeared to be virtually nonexistent. A small amount of growth was observed after run #7 on the selective media BHI+kanamycin (indicated in Table 1 by the asterisk), but upon performing a Gram stain, these colonies were shown to be *Staphylococcus epidermidis*. We concluded that this was due to contamination of the sample tubes prior to electroporation. This resulted in viable *S. epidermis* cells taking up the pNL9164 ligated vector present in the cuvette during electroporation. These results are consistent with the advertised use of the vector, which is described as species-specific for *Staphylococcus* and more generally for all Gram positive species as a whole.

CHAPTER 5

Discussion

First Hypothesis

Upon beginning our originally planned project, our goal had been to introduce a mutation in the arginine-tRNA ligase gene locus of the *L. monocytogenes* genome. This gene was chosen based on its involvement with the bacterial metabolism of this species, specifically in the catabolism of environmental arginine. As previous studies had shown a possible link between active metabolism genes and the synthesis of virulence factors, we believed that a connection could exist between certain metabolic genes and the production of listeriolysin O, a highly important hemolysin required for the pathogenesis of the bacterium (31).

Originally, our proposed outline had included multiple metabolic genes, including pyridoxine kinase and the thiT thiamine transporter gene (45). However, exploring these other genes was contingent upon successful transformation of *L. monocytogenes* through the mutation of arginine-tRNA ligase.

It is important to note here that the gene for arginine-tRNA ligase, which is a critical element of the translation machinery, is vital in regard to protein synthesis and cellular function. In many instances, this gene could very easily be considered to be essential to the cellular function of the bacterium. However, due to the discussion of arginine biosynthesis potentially being involved in activation of *PrfA*-mediated virulence

in the paper by Lobel, *et. al.*, we proceeded with mutagenesis attempts under the assumption that this paricular gene was non-essential in *L. monocytogenes*. This assumption could very well be erroneous and is discussed in the section titled "PROBLEMS, TROUBLESHOOTING, AND IMPROVEMENTS."

Protocol Selection

As stated in the introduction of this thesis, we chose the TargeTron Gene Knockout System as our protocol for a variety of reasons, including the availability of reagents, the accessibility with which the user manual was designed, and primers included with the kit. In addition, on-going experiments conducted by other students in the lab had led to numerous successful transformations and plasmid integrations in multiple different species. This led us to believe that it could potentially be a viable and useful method of introducing mutations in *Listeria monocytogenes* as well.

For the preparation of electrocompetent cells, a simple protocol titled "Transformation of *Listeria monocytogenes*" was obtained via an online database of user-submitted protocols (51). Adjustments to volume were made to accommodate a smaller lab size, although ratios of reagents, antibiotics, and live culture remained the same, as did incubation times and temperatures.

Initial Transformation Attempts

Repeated attempts were made to transform our samples using the protocol cited above (51). It is assumed that these transformations all failed, as no growth was observed on any of the plates. Gel electrophoresis results indicated that the plasmids were being properly retargeted to our gene of interest through PCR (Fig. 3 and 4). However, as these PCR products were never sequenced, it is possible that the bands were indistinguishable from those of non-retargeted plasmid. Arginine-tRNA ligase is known to be a very small gene of only about 300 bp. On a gel with a ladder for constructs upwards of 7,000 bp, this is an incredibly incremental size difference that would lead to very similar bands. If this were the case, it could easily be attributed to simple human error and a lack of appropriate controls, issues which will be discussed further in the section "PROBLEMS, TROUBLESHOOTING, AND IMPROVEMENTS."

In theory, however, an improperly targeted plasmid should not necessarily lead to a lack of growth on media provided it did not disrupt additional genes required for *L. monocytogenes'* survival *in vitro*. In general, un-incorporated, empty vector is relatively harmless to bacteria and is often lost during subsequent replication. If an empty vector containing an antibiotic resistance gene were to remain within a population of bacteria, growth on selective media should be observable. For this reason, I believe that an unsuitable or harmful empty plasmid would not be a viable explanation for the lack of transformants recovered during these initial attempts.

Revision of Protocols

After repeated attempts to transform our samples using the initial protocols we had chosen failed, we began to make modifications to both the TargeTron System as well as our preparation of cells prior to electroporation. We abandoned the online protocol that outlined electrocompetency in lieu of a more thorough and peer-reviewed system by Monk (2008). This new protocol made significant changes to both the antibiotics used and the media used to grow our cultures (39). However, while this change significantly improved the viability of our cells after electroporation and led to increased growth on our non-selective BAP media, it did not contribute to the recovery of transformed cells on our selective media.

After calling Sigma-Aldrich and speaking with a consultant, we were informed of a paper by Alonzo that used the TargeTron Gene Knockout System to transform *Listeria monocytogenes* (3). As of now, this paper appears to be the only published example of this system successfully inducing observable mutations through this system within this particular species of bacteria. As opposed to using the standard pACD4k-C linear vector provided in the base TargeTron System kit, this study used the TargeTron vector pNL9164, which has been optimized for Gram-positive bacteria (3).

A comparison of the two vectors shows significant differences including the type of selective resistance markers, promotors, origin of replications, and size of the construct itself (Fig 5). Any of these changes could potentially modify the behavior of the plasmid

once integrated into the bacterium. For example, one promoter could be more powerful, leading to a higher replication rate.

Using this new vector alongside the modifications in our electrocompetency protocol, we again attempted to transform our samples (*see Problems, Troubleshooting...)*. Additional failed attempts led to more modifications to our previous protocol all to no avail. A final modification was considered involving the use of a different vector entirely unrelated to the TargeTron System. As stated previously in the literature review, there exists a wide variety of options for performing mutagenesis in *L. monocytogenes* using allelic exchange, horizontal gene transfer via conjugation, and transduction through the use of listeriophages (30, 38, 40). However, very few papers focus on transformations using bare plasmid as was being performed in this study. Those that did choose this method used very complex plasmids constructed from the ground up, targeted and designed to integrated only within their specific gene of interest. An example of this type of system can be seen in Lyska (2013) where researchers used a multitude of oligonucleotides and promoters to build a working vector series for their species of interest. This procedure started with a commercial vector which was then modified via PCR and ligation multiple times to reach a viable construct.

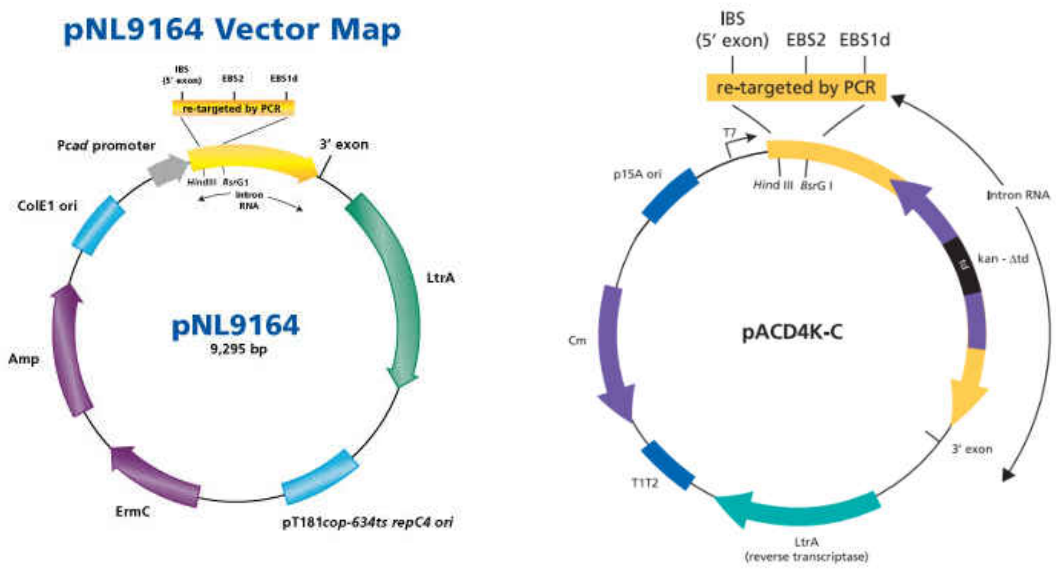

Fig 4. Comparison of pNL9164 and pACD4K-C. Source: "TargeTron Gene Knockout System User Guide." Sigma Aldrich, 2008.

Unfortunately, manufacturing this type of plasmid is incredibly complicated, costly, and time consuming. Additionally, designing the final, usable vector requires a starting base construct with its own set of requirements that are equally as complex (35, 38). For a smaller lab, constructing these plasmids with the available resources is unrealistic, especially in the event of a closing deadline, as was the case in our study. Attempts were made to contact a private company in the event that they could construct the vector for us, but their own reservations about the complexity of the project eliminated the possibility of receiving the vector in a timely manner. Due to increasing time constraints and the inability to find a supplier of our preferred plasmid, a decision

was made to reframe our original hypothesis rather than continuing to make modifications to our transformation protocol.

Final Hypothesis

In light of our unsuccessful attempts at transforming *Listeria monocytogenes*, we decided to alter our focus from the virulence, metabolism, and genetics of the bacterium to the techniques and available systems used to generate mutations in this particular species. As stated earlier, only one paper has been published on the use of the TargeTron Gene Knockout System in *Listeria monocytogenes*. This paper (3) was successful in its attempt to transform the bacteria, but showed a lack of efficiency and a lower yield than that of other studies using different methods. As no other papers attempting to replicate these results were discovered, we have decided to explore the data, modifications, and results collected in this thesis to discover whether or not the TargeTron Gene Knockout System and its available vectors is a viable method for the transforming *Listeria monocytogenes* in a small laboratory setting.

Assessment of protocols, vectors, and the TargeTron Gene Knockout System

In this particular study, we used multiple types of antibiotic treatment to introduce the previously described pores. Specifically, we chose penicillin G and ampicillin. This selection was chosen based on prior research which referenced these

antibiotics on multiple occasions (3, 38). Stock solutions created with these antibiotics were roughly 10 mg/mL in concentration. While it is possible that the amount of antibiotic used during incubation could have negatively affected the survival rate of our samples, repeated growth of the samples on BAP indicate that this is unlikely.

Electroporation parameters were also chosen based on those described in prior research with modifications implemented after multiple failed attempts. Once again, it is unlikely that these changes, specifically the increase in voltage to 1500V or the extended duration of pulses, contributed to the lack of growth on selective media due to the repeated growth on BAP that occurred with most samples that were shocked.

Having eliminated other explanations, we concluded that the lack of successful transformants could only be explained by the inability of our ligated plasmids to enter the cells. There are two points at which this failure could be occurring: entry of the vector into the host cell or integration of the ligated plasmid into the genome of the bacteria.

The inability of the vector to be taken up by the bacteria could be caused by an insufficient breakdown of the cell wall prior to electroporation. When using a standard transformation protocol in which the bacteria is incubated with bare plasmid DNA, it is crucial that the cells be properly prepared beforehand. Broadly, this requires a temporary and repairable disintegration of small areas of cell wall large enough to allow entry of the plasmid yet small enough to not cause cell lysis. This process tends to be more difficult in Gram-positive bacteria due to the thick peptidoglycan layer present.

Many *Listeria* species also possess the ability to produce a capsule which could potentially further complicate the induction of these pores, although these capsules are rarely observed in controlled laboratory environments and may be negligible in their effect.

Provided that the plasmid is able to successfully enter cells, it must then integrate into the host genome. It is at this point that the re-targeting accomplished by the TargeTron Gene Knockout System becomes particularly important. If the intron has been re-targeted appropriately, the plasmid should easily insert into the gene of interest; however, if the plasmid was not properly targeted, it will remain in the cytosol of the cell where it is vulnerable to disintegration or migration out of the cell itself.

It is difficult to confirm at which point in the process our system was experiencing difficulty. The lack of growth on our BHI+kanamycin and BHI+ampicillin plates indicate that there were no viable transformants generated by our electroporation, but it does not indicate whether or not the cells initially received the vector. As the TargeTron System plasmids require successful integration for activation of their resistance genes, potential vectors free-floating in the cells could confer growth on selective media, making it impossible to distinguish between cells with intake plasmids versus those without. However, as plasmids are prone to being lost after multiple cellular divisions and as numerous different electrocompetency protocols were attempted, we have concluded that the most likely explanation is the inability of the generalized TargeTron System plasmids to properly integrate into the host cell genome

as compared to plasmids designed and specifically constructed for *Listeria monocytogenes*.

In conclusion, these consistently negative results suggest that while the TargeTron Gene Knockout System is a simple, user-friendly, and affordable system for many other bacterial species, the limited vectors and lack of sufficient trials in *Listeria* species' make it a poor choice for the transformation of *Listeria monocytogenes*.

Problems, Troubleshooting, and Improvements

The first write-up of the results and discussion sections reported in this thesis were compiled in August, 2014. A detailed review of these initial findings in the following months showed a significant number of issues, mistakes, and unaddressed potential solutions within the methods used to conduct these experiments. This section will attempt to address some of these mistakes as well as provide improvements that could have been implemented.

TargeTron System Plasmid Re-Targeting

As stated earlier in the discussion section, it is possible that the pACD4K-C plasmid I was attempting to retarget for our gene of interest was not correct and was instead empty, non-specific vector. While the bands observed on my electrophoresis

gels appeared to be in the correct spot, this does not guarantee a correct plasmid. Gel electrophoresis is a quick, easy, and useful method for determining the relative size of PCR products but is not an absolute indicator of plasmid size, sequence, or target. As the gene of interest for this study is incredibly small (~300 bp), the difference between a plasmid size of approximately 7,700 bp and 8,000 bp is almost impossible to distinguish using a gel alone. In hindsight, relying on this method for retargeting my plasmid was, frankly, naive and ineffective. However, had additional steps been taken alongside the electrophoresis, the technique might have been much more reliable

The easiest way to improve upon this method for differentiating retargeted plasmid from empty plasmid would be to simply run empty vector alongside ligated vector in the same gel as a control. While the differences between the two would be very small, seeing them both side by side on the same gel could have shown an observable size shift between bands. If this size shift was large enough to be seen by the naked eye, it could indicate that the plasmid had been altered to incorporate the targeted section specific for our gene of interest. This size shift would not have given any additional details such as direction of the targeted section or the plasmid's sequence, but it would be an essential first step in determining if our retargeting protocol was working effectively.

In addition to this simple electrophoresis control, sending off the samples for sequencing would be the best way to ensure that the plasmid was not only re-targeted to our gene of interest, but also oriented properly. Orientation of the variable region of

a plasmid can play an important role in the function of said plasmid. Improperly oriented variable regions can place promoter regions in inappropriate directions, leading to no transcription of the genes under their control. Misplaced promoter regions can also lead to an increase in transcription of potentially dangerous genes or a decrease in genes which are essential, both of which could adversely affect the survival rate of the transformed bacteria.

Media Preparation and Inappropriate Antibiotic Choice

When preparing selective media for recovery of transformed bacteria, use of a proper antibiotic is essential. The two plasmids used in this study, pACD4K-C and pNL9164, have resistance genes for ampicillin and kanamycin, respectively. Unfortunately, the selective media used to plate the bacteria after it had been electroporated with these plasmids did not always contain appropriately matching antibiotics.

During the first half of the study, I attempted to transform *Listeria monocytogenes* with the pACD4K-C. However, instead of using ampicillin in my BHI plates, I used kanamycin. The reason for this is unknown, even after consulting the lab notebook associated with these experiments. In lieu of a specific, documented reason for this choice, it is my assumption that it was a mistake due to either a lack of understanding of the plasmid itself or a mix-up between the two plasmids. Use of an

inappropriate antibiotic would have been crippling to the study, as even properly transformed bacteria that had integrated plasmid present would not have been able to grow on antibiotic plates without the associated resistance gene.

During the second half of the study, I continued to use kanamycin plates while also using the pNL9164 plasmid in my transformations. However, even though this antibiotic and the resistance gene present in the plasmid are complimentary to one another, I still saw no growth on my selective media. Considering that the pNL9164 plasmid was designed specifically for Gram-positive bacteria and that I was seeing substantial improvements in growth on my non-selective media, it seems unlikely that the major issues in this study can all be attributed to using the wrong antibiotic plates while attempting to transform the bacteria with pACD4K-C. There is a small chance that the initial transformation attempts with the pACD4K-C plasmid were actually effective and simply not being recovered due to my mistake, but I feel that this is unlikely simply because proper transformations with pNL9164 should have been more effective than those performed with pACD4K-C due to its specificity for Gram-positive bacteria.

Arginine t-RNA Ligase

The most glaring issue of this thesis is the target gene itself. A survey of the literature lead me to a paper that provided a brief list of metabolic targets whose relationships with the regulatory *PrfA* gene needed further characterization. Mentioned in this list was the gene encoding arginine tRNA ligase. At the time, my knowledge and

understanding of metabolism, genetic replication, and vector-mediated knockouts was minimal. Due to my lack of thorough understanding of arginine tRNA ligase's function, I chose to begin with this particular gene.

However, months later, after beginning my Ph.D. study and taking more in-depth classes on genetics and cellular metabolism, I realized that choosing to knockout a gene that codes for a tRNA ligase was an incredibly poor choice. During DNA replication, the various tRNAs are responsible for locating appropriate amino acids to the replication machinery during translation. tRNA ligase is then facilitates binding of the amino acid to the synthesizing strand of DNA that is being produced during translation.

Arginine, an amino acid that is present in numerous, if not nearly all, proteins is required for proper function of cellular mechanisms. By knocking out the ligase that allows this amino acid to attach to replicating DNA, we would effectively halt translation of potentially essential proteins, or, adversely affect their ability to properly fold due to changing the hydrophobicity or charge present in their domains. In the event that these proteins were not able to be synthesized properly or be folded into their normal confirmation, we could very easily have induced lethal cellular defects or halted growth and replication of the bacteria.

From this line of thought, we can assume that in the event of our plasmid being successfully been retargeted and inserted into the genome of *Listeria monocytogenes*, disruption or knockout of the arginine tRNA ligase could have attributed to the lack of growth seen on both our selective and non-selective media. While we did observe small

amounts of growth on non-selective BAP plates after some electroporation attempts, it is more likely that the colonies found were simply bacteria that did not pick up the plasmid due to the lack of growth on selective media from these same attempts.

However, ideally, when growth is observed on non-selective media, the next step would be to plasmid purify the colony through performing a mini-prep, plasmid extraction, and gel electrophoresis and clean-up. The DNA could then be sent off for sequencing using primers that specific to our insertion site in an attempt to confirm that the plasmid was present. Unfortunately, due to my own inexperience and lack of confidence in these positive results, this step was never taken.

Nevertheless, I still believe that the poor choice of target gene is the most likely explanation for our lack of results as an inappropriately targeted plasmid or one that was unable to cross the cell wall into the cytoplasm of the bacterium would likely not halt all growth on non-selective media. Instead, we would most likely find colonies lacking plasmid entirely or containing empty or inactive vector.

Future research

While these results indicate that the TargeTron System should be avoided in lieu of more complex methods when attempting to induce mutations into this particular species, it does not mean that it could not eventually become an effective system with the proper modifications made. Due to the vast number of mistakes and potentially

incorrect assumptions made during the course of this study, the results presented should not be taken as reliable. In addition, as stated previously in the literature review, Alonzo, *et. al.,* successfully used this system to introduce genetic deletions in *Listeria monocytogenes*, suggesting that with the proper vectors and parameters, the TargeTron protocol could still be a viable method of transformation. If this were the case, it would be especially helpful for smaller labs where access to expensive constructs and equipment is not readily available.

However, in light of this study, it may be more applicable to use alternative methods for silencing or deleting genes of interest that do include electroporation of this bacteria. One such method that could prove useful is siRNAs, also known as silencing RNAs. These small molecules are usually 20-30 bp long and can be targeted to a specific mRNA transcripts. Upon base pair-binding to these transcripts, the complex will be degraded by endogenous cell processes, halting translation of the transcript and thus production of the protein of interest. Previous studies have already used this method to knockdown genes in other bacterial species, such as methicillin-resistant *Staphylococcus aureus*, although its use in *Listeria* appears to be limited to simple screening methods for genes of interest rather than robust studies of the gene knockdown themselves (62). However, the fact that siRNA delivery has been confirmed for *Listeria* is a promising sign that this method could be viable for a project such as this one.

While not as novel, transduction is also another method that could be worth consideration in this particular species of bacteria as it once again does not require pore-formation on the surface of the cell wall. Instead, it uses synthesized viral constructs containing a plasmid to deliver the DNA into the host cell. This could be useful if a marker for selectivity, such as antibiotic resistance, was needed as the original plasmids featured in this study could still be used with the addition of some modifications. Unlike siRNAs, colonies recovered after infection with virus could be screened by simple plating the bacteria on selective media rather than requiring additional techniques such as Western blots or rtPCR to test for mRNA or protein production.

Future research should focus on redoing the experiments presented here with the appropriate controls in place. Other avenues that could be explored include improving upon the existing protocol by using other commercially available vectors, different electroporation parameters, and a variety of electrocompetence methods alongside the general TargeTron Gene Knockout System protocol, as well as trying new techniques for DNA delivery entirely. While the results here are discouraging, it is possible that with the proper changes, a fast, effective, and inexpensive TargeTron protocol could be devised for this particular bacteria. These changes should be explored in order to gain a better understanding of this system and its potential to become a possible transformation system for *Listeria.*

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