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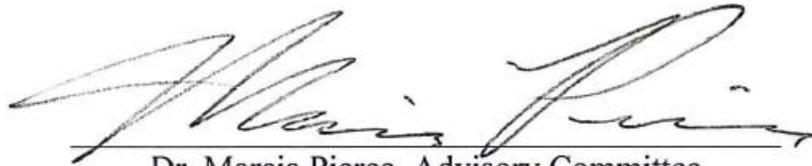
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GENE KNOCKOUT OF *PRFA* AND THE ARGININE DEIMINASE PATHWAY IN
LISTERIA MONOCYTOGENES

BY

KEARSTIN L. EDMONDS

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A handwritten signature in black ink, reading "Hearsting J. Edmonds". The signature is written in a cursive style with a large, stylized initial "H".

Date _____

April 25, 2018

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IN *LISTERIA MONOCYTOGENES*

BY

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Bachelor of Science
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Eastern Kentucky University
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ABSTRACT

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen. This species is a common cause of food-borne illness outbreaks with high fatality rates. *L. monocytogenes* is known to produce a number of virulence factors, including enzymes and toxins, that are involved in the disease process. The arginine deiminase pathway has the ability to regulate acidic environments by converting arginine to ammonia, ornithine, and ATP. With the help of a membrane-bound antiporter, the ornithine is transported out of the cell while the ammonia is converted to ammonium. The ATP by-product can also be used with the F₀F₁-ATPase system to help maintain homeostasis. The *prfA* gene, which codes for the PrfA protein, is considered to be a crucial virulence regulator in *L. monocytogenes*. The PrfA protein has the ability to bind to palindromic promoter boxes within the chromosome in order to recruit necessary components for transcription of ten crucial virulence factors. This study attempted to produce genetic knockouts of the *prfA* gene and ADI pathway. The splicing overlap extension polymerase chain reaction method was used for genetic manipulation in this study. The 6.9 kb shuttle vector pKSV7 was used to introduce the spliced genetic insert into the wild type strain. The pKSV7-AD vector for the Δarc was successfully introduced into *L. monocytogenes*. The integration of the plasmid into the host chromosome was also successful; however the plasmid did not integrate with the gene of interest. The attempted Δarc was unsuccessful due to the inability of the spliced SoeAD-pKSV7 insert to bind to the gene of interest. The pKSV7-AD vector for the attempted $\Delta prfA$ successfully incorporated into the *L. monocytogenes* chromosome. The *prfA* SoeAD-pKSV7 inserted in the correct gene of interest, resulting in a successful mutant strain.

Key words: Listeria monocytogenes; transformation; virulence; prfA; arginine deiminase; SOEing; listeriosis; pKSV7, knock-out

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION.....	1
1.1 <i>Listeria monocytogenes</i> background.....	1
1.2 Morphology and physiology.....	3
1.3 Virulence.....	4
1.4 Arginine deiminase (ADI) pathway molecular background.....	6
1.5 The <i>prfA</i> gene and PrfA regulon.....	9
1.6 The splicing overlap extension PCR technique.....	13
II. PROJECT DESIGN.....	15
2.1 Project aim one.....	15
2.2 Project aim two.....	15
III. METHODS.....	16
3.1 Primer design.....	16
3.2 Stock <i>L. monocytogenes</i>	17
3.3 Vent PCR for SoeAB and SoeCD.....	18
3.4 Vent PCR for SoeAD.....	19
3.5 Incorporating SoeAD into pKSV7.....	19
3.6 Transformation of <i>E. coli</i>	20
3.7 Electrocompetent <i>L. monocytogenes</i>	22
3.8 Transformation of <i>L. monocytogenes</i>	23
3.9 Chromosomal integration.....	23
3.10 Verification of construct integration.....	24
IV. RESULTS.....	25
4.1 Vent PCR product for SoeAB and SoeCD.....	25
4.2 Vent PCR product for SoeAD.....	26
4.3 Ligation and transformation into <i>E. coli</i>	26
4.4 Transformation of <i>L. monocytogenes</i>	27
4.5 Chromosomal integration.....	28

V. DISCUSSION.....	31
REFERENCES.....	35
APPENDICES.....	A1
A. SUPPLEMENTAL TABLES.....	A2

LIST OF TABLES

TABLE	PAGE
1. Foods associated with <i>L. monocytogenes</i> infection.....	3
2. Arginine deiminase genes.....	8
3. SOEing primers for targeting the <i>prfA</i> gene and the ADI gene complex.....	17
4. Thermocycler conditions for vent polymerase chain reactions.....	18
5. Thermocycler conditions for colony polymerase chain reactions.....	22
6. Primer design guidelines.....	A3

LIST OF FIGURES

FIGURE	PAGE
1. <i>L. monocytogenes</i> intracellular infection.....	12
2. Diagram of SOEing primer orientation.....	13
3. Gel electrophoresis: SoeAB and SoeCD.....	25
4. Gel electrophoresis: SoeAD.....	26
5. Gel electrophoresis: pKSV7-AD.....	27
6. Gel electrophoresis: chromosomal integration Δarc	29
7. Gel electrophoresis: chromosomal integration $\Delta prfA$	30

CHAPTER 1

INTRODUCTION

1.1 *Listeria monocytogenes* background

Listeriosis, a condition caused by *L. monocytogenes* infection, has been ranked as the third most fatal food-borne illness in the United States (1). After a series of food-borne illness outbreaks during the 1980s, *L. monocytogenes* became a widely studied pathogenic species (2). The disease has a 20-40% fatality rate in humans, which is higher than most food-borne illnesses (3, 4). As of 2011 in the U.S., *L. monocytogenes* was the third most fatal food-borne illness, with a 19% fatality rate (1). The two most fatal food-borne illnesses recorded were *Salmonella* (28%) and *Toxoplasma gondii* (24%) (1).

Listeriosis can occur in various mammals, affecting the central nervous system, bloodstream, or the uterus during pregnancy. Other species of *Listeria*, *L. inovii* and *L. seeliger*, have been known to cause listeriosis, but this event rarely occurs (2, 5). In most human cases of infection, individuals are immunocompromised or pregnant (2, 6–8). Pregnant women infected with the disease usually exhibit mild flu-like symptoms or are asymptomatic (2). The infected fetus, however, is often lost to miscarriage or born with a neonatal case of listeriosis (2). Patients in the neonatal stage have also been known to contract the bacterium via cross-contamination (9). Pregnant women make up about 17-24% of clinical cases with about 28% of cases resulting in abortion or stillbirth (2). Human immunodeficiency virus (HIV)-infected individuals have a high infection rate with *L. monocytogenes*, due to their immunocompromised state (9). This fact results in many underprivileged countries with high HIV rates also having elevated listeriosis rates (9).

Human cases are primarily contracted via ingestion, although contact with infected animals or humans can sometimes result in infection and disease (2). Clinical presentation of the diseased state varies between 1-90 days post infection, with an average of 30 days (2, 10). Cases of human infection have a peak season in the fall or late summer. However, livestock peak season usually occurs in the spring (2). Infection can

also occur in livestock, including sheep and goats, and most commonly results in encephalitis, miscarriage, or septicemia (2). This can result in significant economic loss due to rapid transmission through livestock. The disease is easily treated with combined antibiotic therapy. The accepted form of antibiotic therapy is combined treatment with ampicillin and gentamicin (2). *Listeria* species show resistance to cephalosporins, aztreonam, piperimic acid, and dalfopristin/quinupristin sulfamethoxazole (2).

Although most individual infections with *L. monocytogenes* are sporadic, there have been concentrated food-borne outbreaks documented around the world (2, 4, 6, 7). Many foods have been associated with listeriosis outbreaks (Table 1). The infectious dose of *L. monocytogenes* is unknown and likely varies among patients (2). The average contamination level of foods ingested by affected patients is 10^2 - 10^6 CFUs/ml/g (2, 11). Some countries accept foods containing below 100 CFUs/g (2). The United States, however, has a no-tolerance policy for the presence of *L. monocytogenes* in any food (2, 11). Commonly contaminated foods can be seen in Table 1. *L. monocytogenes* is ubiquitous in the environment, and contamination can occur in foods grown in soil (7). *Listeria* survive as saprophytes in soil, vegetation, and water, and upon ingestion can move to a replicating phase (2).

Table 1. Foods associated with *L. monocytogenes* infections

Dairy	Meats	Fish	Vegetables
Soft Cheese	Cooked Chicken	Shellfish	Coleslaw
Milk	Turkey Frankfurters	Shrimp	Vegetable rennet
Ice Cream	Sausages	Smoked Fish	Salted Mushroom
Butter	Pate and Rillettes	Cod Roe	Alfalfa

Foods that have been correlated with *L. monocytogenes* contamination and food-borne illness (2, 4, 6, 12). *L.monocytogenes* is ubiquitous in the environment; however most mass outbreaks occur due to contaminated foods. *Source(s)*: Liu D. 2008. The Handbook of *Listeria Monocytogenes*. Taylor Fr. Gr. 6.

1.2 Morphology and physiology

All members of the genus *Listeria* are Gram-positive rods, with a cell size of about 0.4-0.5 µm wide by 1-2 µm long (2, 13–15). The cell wall is comprised of a peptidoglycan multilayer with mesodiaminopimelic acid (meso-DAP), which is typical of Gram-positive bacteria (8, 16). The ribitol, lipotechoic acids, and flagella antigens determine the various serotypes of *L. monocytogenes* (2). The bacteria are motile via 5-6 peritrichous flagella that are uniformly distributed around the cell (2, 7, 16). Motility is most active between 20-25°C, and lost at 37°C (2). *L. monocytogenes* is a facultative anaerobe and a neutrophile (2, 7, 16). *Listeria* are generally unchallenging to grow as they only require biotin, thiamine, riboflavin, thioctic acid, and some amino acids (2). Therefore the species grows well on non-selective media (2). However, it has been shown that brain-heart infusion broth provides the best nutrients for growth (8, 12, 13, 17, 18).

Blood agar plates are the best solid selective medium for the species and help identify *Listeria* due to hemolytic virulence factors (2). The production of virulence factor phospholipase-C creates blue-turquoise colonies on media containing L- α - phosphatidylinositol, which is another identifying characteristic of the bacterium (14, 18). *L. monocytogenes* has the ability to survive extreme conditions including high pH, extreme temperatures, and high salt concentrations (4, 6, 8, 12, 18). The bacterium has efficient stress response mechanisms that allow regulation and survival in extreme situations (6, 12).

L. monocytogenes is a widely studied bacterial species because of its virulence capabilities, but not all serotypes express the same virulence (18). Only three serotypes are associated with human clinical cases: 1/2a, 1/2b, and 4b (19). The French *Centre National de Référence des Listeria* explored the infection levels of *L. monocytogenes* serotypes between 2001-2003 in human clinical cases by characterizing the serotypes from multiple sources, and they found that serotype 4b caused 49% of human clinical cases (2). The 1/2a serotype was the second highest cause of clinical cases at 27% (2). The *Agence Française de Sécurité Sanitaire* in Maisons-Alfort, France combined voluntary submissions of strains isolated from foods to determine percentages of serotypes. They discovered that the 1/2a strain was the most frequent contaminating serotype found in foods (64%), and was significantly higher than the second highest (1/2c) at 12% (2). The 1/2a strain is usually responsible for human clinical cases related to food-borne illness (19).

1.3 Virulence

Virulence factors are crucial for the ability of *L. monocytogenes* to proliferate in a mammalian host (4, 8, 12, 14, 20). When the bacteria are ingested they enter host immune cells, multiply, and spread to neighboring cells (7, 12, 14). *L. monocytogenes* has several virulence factors that allow it to survive environments other species cannot (7, 12). When the host ingests *L. monocytogenes*, the bacterium moves to hostile pH levels in

the stomach, and then to the intestinal lumen (7, 8, 21). Here the bacterium invades intestinal mucosa by route of M cells within Peyer's patches and intestinal endothelial cells (2). The bacteria can multiply there before entering the circulatory system. In the blood and lymph systems, the bacterium comes into contact with macrophages of the liver, where most invading cells are destroyed (2, 18). However, those that survive move to phagocytic cells of the spleen, where they are internalized (2, 21).

Phagocytic cells take in foreign particles and place them in an intracellular vacuole that will degrade them (2, 12, 14). *L. monocytogenes* escapes this vacuole and multiplies in the cytoplasm (2, 6, 7). The bacterial cell induces polymerization of actin, causing structures known as actin comet tails to form (18). These comet tails are used to propel the bacterium from the cytoplasm to the cell membrane, where it is propelled out of the phagocytic cell, while acquiring a portion of the host membrane before entering the next phagocytic cell (2, 18). As it enters the second phagocytic cell, it is engulfed by an internal vacuole, resulting in a double membrane surrounding the bacterial cell (6). When the outer layer of the double membrane is lysed, a new infection cycle will occur. The ability of *L. monocytogenes* to evade the immune system results in a disease state in the host.

There are three important stress factors that *L. monocytogenes* can withstand: heat, acid, and osmotic stress (4–6). The ability to withstand these obstacles allows the bacteria to survive many common food processing measures, which leads to food-borne outbreaks (2). The virulence factors are particularly important in surviving the host infection cycle. Heat stress occurs when *L. monocytogenes* moves from an external environment into the host body, and again at numerous stages in its infectious cycle (5). This stress can be regulated by a series of heat-shock proteins (HSPs) that work to re-fold proteins during heat degradation (8, 14, 21). Unique to *L. monocytogenes* is its ability to regulate highly acidic conditions.

The bacterium faces acidic stress due to low pH and weak organic acids in its surroundings (6, 7). *L. monocytogenes*, like many other bacteria, is a neutrophile with an optimum pH of 6 or 7 (2). The species has evolved the ability to regulate pH to maintain

stability using a variety of factors (6). *L. monocytogenes* primarily uses glutamate decarboxylase, F₀F₁-ATPase system, and the arginine deiminase pathway (6, 20). The glutamate decarboxylase (GAD) system works by irreversibly converting a molecule of external glutamate to γ -aminobutyrate (GABA) (6, 20). This removes some intracellular protons, helping to maintain pH homeostasis. The F₀F₁-ATPase system in *L. monocytogenes* actively moves protons out of the cell to regulate pH (4–7). The final acid stress mechanism is the regulation of the arginine deiminase (ADI) system (6). High levels of arginine are secreted by the host cell into the phagosome where the pathogen is sequestered (6). This pathway allows the conversion of arginine to ornithine, carbon dioxide, and ammonia (6, 12, 16, 20, 21). This prevents death of the bacteria in hostile pH levels because it produces a less acidic environment.

1.4 Arginine deiminase (ADI) pathway molecular background

Three primary molecules are used in the ADI pathway regulation: arginine deiminase, catabolic ornithine carbamoyltransferase (cOTCase), and carbamate kinase (CK) (6, 20). For every mole of arginine catabolized by cOTCase, two moles of ammonia and one mole of ATP are produced (6, 20). These products can combine with intracellular protons to create ammonium ions, increasing the pH inside the cell (6, 20). The ornithine that is produced in the pathway is moved out of the cell in an energy-independent mechanism via a membrane-bound antiporter (6, 20). The ATP produced in the pathway can be fed into the F₀F₁-ATPase system, furthering the intracellular pH balance (6, 20).

The genes that control the arginine deiminase pathway are referred to as *arc* genes. These genes have a unique arrangement as follows: *arcB*, *arcD*, *arcC*, and *arcA* (20). The *arcA* gene is physically separate from the other *arc* genes by three uncategorized genes (20). There are also accessory genes on both sides of the gene cluster. The precise function of the accessory genes is not known, but research suggests they aid in pathway regulation (2). A fifth gene, *argR*, has been associated with regulation of the pathway by coding for a dual regulator molecule, argR (2). Research

also suggests another physically separate gene, *argG*, plays a role in arginine production and is up-regulated to increase production under stress response (2).

Each of the genes in the *arc* cluster code for a protein needed in the ADI pathway; a summary can be seen in Table 2. The arginine deiminase enzyme is encoded by the *arcA* gene, catabolic ornithine carbamoyltransferase (cOTCase) by the *arcB* gene, and carbamate kinase (CK) by the *arcC* gene (20). These three enzymes are the main components of the arginine deiminase pathway (2, 6, 20, 22). The primary function of this pathway is to generate resistance to acidic environments. This is achieved, in part, by converting arginine to ornithine. There are two sources of arginine; the first is naturally formed within the cell from glutamate, and the second is the arginine:ornithine antiporter molecule, encoded by the *arcD* gene (20). This antiporter moves molecules of arginine into the cell to be catabolized. The arginine deiminase enzyme will catabolize arginine to citrulline, after which cOTCase will further break down the molecule to produce ornithine (2, 6, 23). As a byproduct of this process, 2 moles of ammonia are produced along with carbamoyl-P, which is dephosphorylated by CK to yield one mole of ATP (20, 23). Carbon dioxide is also a byproduct of the catabolic reaction. The ammonia combines with intracellular hydrogen ions to produce ammonium ions that will increase the pH in order to maintain homeostasis (2). The ornithine is moved out of the cell using the antiporter molecule. The ATP can be fed into the F₀F₁-ATPase system to extrude protons from the cell in order to regulate homeostasis, or to aid with general microbial growth (2, 20). The dual regulator molecule, *argR*, aids survival by simultaneously up-regulating expression of the ADI pathway and down-regulating arginine biosynthesis (20). As previously mentioned, *argG*, which aids in arginine synthesis, is up-regulated under stress response (6). This seems counterproductive; however, research by Ryan et al. (2009) shows that an *argG* mutant is actually more sensitive to acid stress (6). This suggests that both intracellular arginine produced by *L. monocytogenes* and extracellular arginine imported to the cell are crucial to acid stress regulation (6).

Table 2. Arginine deiminase genes

	Abbreviation	Encoding Gene
Arginine deiminase	ADI	<i>arcA</i>
Catabolic Ornithine carbamoyltransferase	cOTCase	<i>arcB</i>
Carbamate kinase	CK	<i>arcC</i>
Arginine:Ornithine Antiporter	-	<i>arcD</i>

The arginine pathway encodes for three enzymes and an antiporter protein. The enzymes work to break down arginine molecules into ornithine, NH₃, ATP, and CO₂. The antiporter is an intramembrane molecule that imports arginine and exports ornithine.

In a study by Ryan et al. (2009), the arginine deiminase pathway (ADI) was disrupted at different loci for virulence factors. Genes were characterized by inducing mutant strains that had deficient forms of *argD*, *argG*, *argA*, and *argR*. The *ArgD* and *argG* mutant cells were starved of arginine, then briefly re-exposed to arginine medium, before being introduced into epithelial cells (7). The *argD* mutant had increased acid sensitivity, but the *argG* mutant did not show significant change (7). The mutant strains were then introduced into mice, where it was found that the *argA* and *argR* mutants had 10x less survival than the parent strain (7). These results indicate disruption of the overall virulence of *L. monocytogenes*. However, when the mutants were introduced *in vivo* to epithelial cells, no change in pathogen survival from parent strains was observed (6). This indicates some other virulence factor must be involved *in vivo*.

1.5 The *prfA* gene and PrfA regulon

Virulence factors are critical to the ability of *L. monocytogenes* to carry out the host infection cycle. One particularly important component of this species is the *prfA* gene, which codes for the PrfA protein. PrfA is a central virulence regulator used during every phase of the bacterial life cycle (2, 6, 7, 13, 19). The *prfA* regulon can act as an activator or repressor of its own gene cluster as well as controlling transcription of several other genes (2, 19, 24). The ten core genes subject to PrfA regulation include: *hly*, *mpl*, *actA*, *plcB*, *plcA*, *prfA*, *inlA*, *inlB*, *inlC*, and *hpt* (2, 4, 14, 19, 25). When *L. monocytogenes* is exposed to high stress environments such as high acidity, host proteolytic enzymes, bile salts, and non-specific inflammatory attacks, the stress response genes are turned on by sigma factor beta (σ^B) (2). This factor is an accessory molecule to the PrfA protein and is encoded by the *sigB* gene (2).

The *hly* gene codes for the hemolysin protein that is the determinant of listeriolysin O (LLO) (4, 7, 24). LLO is a hemolytic protein that is a part of the cholesterol dependent pore-forming toxin (CDTX) family (2, 19). Cholesterol is the receptor for LLO and is required for pore creation. When LLO binds to erythrocytes, toxin monomers oligomerize in the target cell membrane to form pores (2). Pores are also formed for escape from the host cell vacuole via lysis during the infection cycle (2, 19). When cholesterol levels are low, the ability of the protein to function is limited (2, 19). Studies have shown that mutants lacking the *hly* gene are entirely avirulent in mouse models (2).

There are two phospholipases and a metalloprotease (Mpl) that work with LLO to disrupt phagosome membranes (2, 7, 12). Phosphatidylinositol-phospholipase C (PI-PLC) is an enzyme that hydrolyzes phosphatidylinositol (2, 7, 12). This enzyme is encoded by the *plcA* gene, which can be found upstream of the *hly* gene (2). The enzymatic activity of this protein aids LLO in the escape from the host vacuole. This molecule is characterized by a barrel domain with an active site on its c-terminal area (2, 7, 12). The second phospholipase hydrolyzes phosphatidylcholine and is known as phosphatidylcholine-phospholipase C (PC-PLC) or lecithinase (2, 7, 12). This enzyme is

not as substrate-specific as PI-PLC. The gene *plcB* that codes for this enzyme is downstream from the *hly* gene (2). Mpl helps aid in cell-to-cell spread by activating PC-PLC with proteolytic activity (2). The PC-PLC relies on this activation by metalloprotease Mpl, coded by the gene *mpl*, to function.

A cell-surface protein, actin-polymerization protein (ActA), aids in the intracellular proliferation of *L. monocytogenes* and is encoded by the gene *actA* (7, 12, 14). The primary function of ActA is to induce an actin-based motive force through internalin-dependent uptake of *L. monocytogenes* (2, 26). After escape from the phagosome, a network of actin filaments and actin-binding proteins surrounds *L. monocytogenes* (2, 26–28). The protein is distributed evenly across the cell and begins to concentrate on one pole while more filaments are consistently growing toward the surface (2). This creates a motive force that pushes *L. monocytogenes* through the host cytoplasm at speeds of up to 1.5 $\mu\text{m/s}$ (2). Polymerized actin structures are used to propel the bacterium from the cytoplasm to the cell membrane, where it is jettisoned out of the host cell while acquiring a portion of the host cell membrane before entering the next host cell (2, 7, 12).

There are three components to ActA: the N-terminal, a central proline-rich repeat region, and the c-terminal (2). When *L. monocytogenes* enters the host, the ActA protein aids in its cellular uptake (2). The ActA N-terminal consists of positively charged particles which, upon host cell entry, bind to the negatively charged heparin-sulfate (HS) moiety (2). The HS moiety is part of HS proteoglycans (HSPG) found on most mammalian cells (2). This binding process encourages uptake by host cells, either by chemical signal or sufficient presentation of bacterial factors triggering phagocytosis (2). Mutants lacking the N-terminal of the ActA protein could not polymerize actin, showing that the component was necessary (29). The second component, the central proline-rich repeat region, is a heavily proline-saturated area. When a mutant of this portion of the protein was produced, the actin filaments were shorter in length and showed slower movement (29). The third portion of ActA is the C-terminal, which includes a membrane

anchor but is not involved in actin polymerization (2, 28). When mutants lacking the C-terminal were created there was no significant impact on actin activity (29).

Another group of proteins called internalins aid in the entry of *Listeria* into the host cell as well as in its infectious life cycle. *L. monocytogenes* can enter the host cell both passively through phagocytosis and actively through the aid of ActA and internalins (2, 4). InlA, encoded by the gene *inlA*, is a covalently bound surface protein with a C-terminal LPXTG motif, which reacts to adhesion E-cadherin proteins to allow cell entry into epithelial cells via local cytoskeletal rearrangements (2, 4, 26). The *inlB* gene codes for the InlB protein, which is non-covalently bound to the cell surface and helps facilitate entry into host hepatocytes, fibroblasts, and epithelioid cells (2, 26). The final internalin that is regulated by PrfA is InlC, which is coded by *inlC* and aids in the post-intestinal stage of the *L. monocytogenes* life cycle (2).

The final virulence factor is hexose phosphate, which is a transporter molecule (30). This protein is encoded by the *hpt* gene and is required for rapid bacterial growth in the host cytosol (2, 30). The protein transports phosphorylated hexose sugars to aid in the replication process. Mutants have shown a strong decrease in virulence capability (30). The gene is completely controlled by the PrfA protein (30).

Upon host cell entry, PrfA acts as a transcriptional activator (1, 3, 5, 12). The core ten genes are down-regulated during extracellular growth and are usually weakly active until host infection (2). The PrfA dimer activates the virulence genes using the palindromic promoter box, known as the “PrfA box” (7, 12, 25). The binding process of PrfA to the PrfA box recruits required proteins for transcription. A co-factor binds to the N-terminal beta-roll domain causing a conformational change that is allosterically transmitted to the C-terminal helix-turn-helix motif (2). This conformational change triggers the transcription of virulence genes (2). Usually at 30°C there is a 5' UTR (untranslated region) that blocks the ribosome binding site (2). At 37°C the secondary structure melts to allow for PrfA translation (2).

There are multiple PrfA boxes that are used to regulate non-virulence associated homeostasis functions from about 145 genes (2). The virulence genes associated with the *prfA* regulon can be found in relation to the life cycle in Figure 1. The majority of the core ten virulence factors can be found close to one another on the chromosomal *Listeria* pathogenicity island 1 (LIPI-1). The island is made up of *inlA*, *inlB*, *inlC*, *hpt*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*. Although *mpl*, *actA*, and *plcB* are part of the LIPI-1, they also make up the lecithinase operon (2).

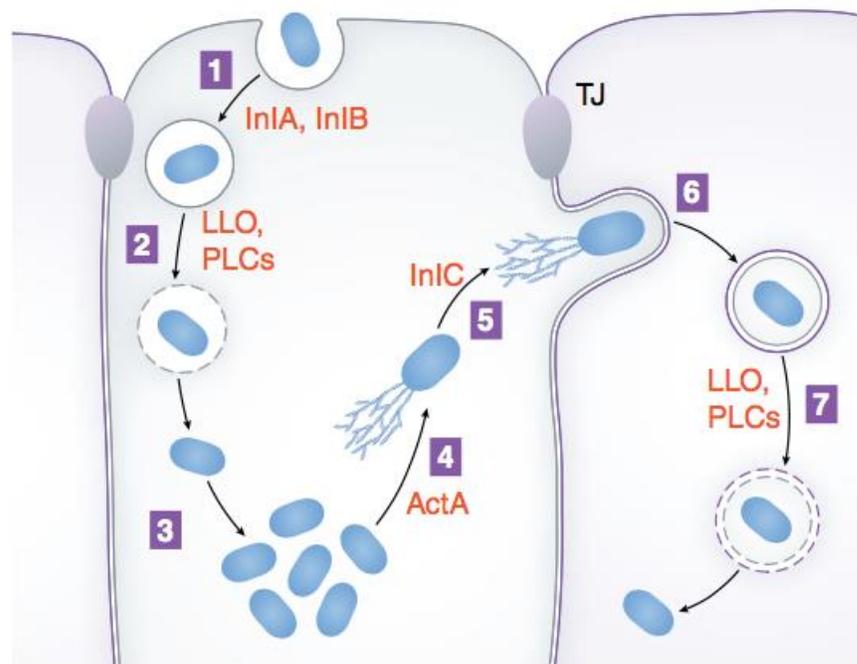


Figure 1. *L. monocytogenes* intracellular infection. Upon host cell entry [1] the bacterium is taken into a phagosome, which it then escapes [2]. The bacteria begin to replicate [3] followed by polymerization of actin comet tails [4]. The bacteria begin to take a portion of the host membrane while entering another host cell [5], resulting in the double vacuole [7]. *Source(s):* Ireton K, Rigano LA, Dowd GC. 2014 Role of host GTPases in infection by *Listeria monocytogenes*. *Cell Microbiol.* 16:1311-1320.

1.6 The splicing overlap extension PCR technique

There are many methods available for the manipulation of the genomic makeup of bacterial species. One of the most common forms of DNA alterations is the polymerase chain reaction (PCR). Splicing by overlap extension PCR is a method carried out by a series of sequential PCRs designed to excise a gene of interest. Two sections of DNA that flank the gene of interest are chosen for amplification (31, 32). The primers for those two sequences are designed with overlapping extensions that facilitate subsequent splicing (31, 32). Those two amplified products are then mixed together in a PCR that creates the final spliced product (31, 32). That product can then be incorporated into a vector, such as a plasmid, for transformation and gene knockout. Visual representation of this process can be seen in Figure 2.

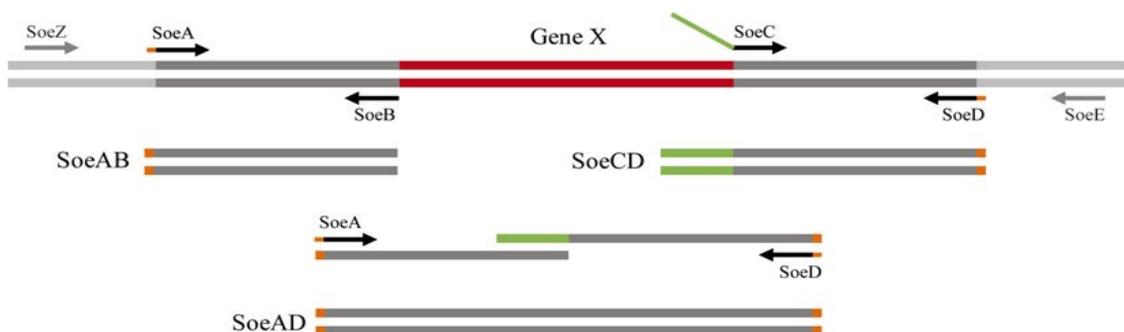


Figure 2. Diagram of SOEing primer orientation This diagram is a representation of the orientation of primers used in the SOEing procedure. The SoeA, SoeB, SoeC, and SoeD primers bind to regions flanking the gene of interest. The final product is a spliced amplicon of the two flanking regions. *Source(s)*: Kathrin Rychli , Caitriona M. Guinane , Karen Daly , Colin Hill and PDC. 2014. Generation of Nonpolar Deletion Mutants in *Listeria monocytogenes* Using the “SOEing” Method. *Methods Mol. Biol.* 1157:85–93.

The vector used in this study, pKSV7, was originally created for use in *Bacillus subtilis* by Smith and Youngman (33). This vector is a hybrid between pUC18-*ble* and

pBD95ts. It is a 6.9 kb shuttle vector that confers both ampicillin and chloramphenicol resistance (31, 33). The pKSV7 plasmid was used to create a specific protocol of the SOEing method for use in *L. monocytogenes* by Rychli *et al.* (31). After the sequential PCR amplifications, the spliced product is integrated with the pKSV7 shuttle vector. The plasmid is forced back into its supercoiled conformation and ligations are confirmed with a transient transformation into *E. coli* DH5 α cells (31). Electrocompetent *L. monocytogenes* are made using the penicillin treatment method, which degrades the Gram-positive cell wall to increase transformation efficiency (leaky-cells) (34). Cells prepared with this method result in transformation efficiencies up to $4 \times 10^6/\mu\text{g}$ of DNA (34). The confirmed ligation plasmids are then introduced into *L. monocytogenes* to generate the desired mutant strains (31).

CHAPTER 2

PROJECT DESIGN

2.1 Project aim one

The arginine deiminase pathway is a source of virulence capability for *L. monocytogenes*, as previously mentioned. Due to evidence that the ADI pathway controls a large portion of the *L. monocytogenes* acid survival mechanisms, this study attempted to create a viable arginine deiminase pathway knockout. This knockout could also be used in further studies exploring virulence capabilities of the species. This study attempted to generate a Δarc strain using the splicing by overlap extension PCR procedure to manipulate the *L. monocytogenes* genome.

2.2 Project aim two

As previously mentioned, *L. monocytogenes* is an infectious species due to abundant virulence factors (4, 8, 12). The gene regulator PrfA controls ten crucial virulence factors associated with *L. monocytogenes* (2, 7, 12, 25). A knockout mutant lacking the virulence regulator PrfA could be useful for further studies. This study attempted to generate a $\Delta prfA$ strain using the splicing by overlap extension PCR procedure to manipulate the *L. monocytogenes* genome.

CHAPTER 3

METHODS

3.1 Primer design

Primers were designed using the *L. monocytogenes* genome accession AJ002742. The SoeA primer is a forward primer that binds upstream of the SoeB primer (31, 32). SoeA also contains a 5' enzyme restriction site that correlates with a pKSV7 restriction site (31, 32). SoeB is a reverse primer that binds to a region flanking the gene targeted for deletion (31, 32). SoeC is a forward primer that binds to a region flanking the gene targeted for deletion, and has a 5' region of ~20 base pairs (bp) that is the reverse complement of SoeB (31, 32). Lastly, SoeD is a reverse primer that binds downstream of SoeC, and contains a 5' enzyme restriction site that correlates with a pKSV7 restriction site (31, 32). A version of SoeC was ordered without any of the alterations made and is referred to as SoeC2. SoZ is a forward primer that binds upstream of SoeA and SoeE is a reverse primer downstream of SoeD. When primers required a restriction site addition, a leader sequence was added to ensure proper incorporation. All primers were edited to optimal conditions by assessing GC concentration and ensuring GC clamps were present when possible. A summary of guidelines for creating primers can be found in Table A.1 and specific primer design can be seen in Figure 2.

Primers for the *prfA* gene were designed using the *L. monocytogenes* genome accession AJ002742. The SoeA primer includes a restriction enzyme site for BamHI, and SoeD includes a restriction enzyme site for HindIII. Primers designed for the ADI gene complex (*arc*) were designed using the PubMed accession AM412558. For this gene SoeA had a restriction enzyme site for EcoRI and SoeD had one correlating to KpnI. The final sequence for primers can be seen in Table 3.

Table 3. SOEing primers for targeting the *prfA* Gene and the ADI gene complex

Target	Primer	Sequence 5'-3'	Length
<i>prfA</i>	SoeA	AGAGATGGATCCGAGCAACCATCGGAACCAT	31
<i>prfA</i>	SoeB	CGTTCATGTCTCATCCCCCAATC	23
<i>prfA</i>	SoeC	TGGGGGATGAGACATGAACGATTAGCGAACAGGCTACCGC	40
<i>prfA</i>	SoeD	GTATCCAAGCTTAAATAGAGCCGAGCTTCCCG	32
<i>prfA</i>	SoeZ	TATTCCTACAAAAAAGGGT	20
<i>prfA</i>	SoeE	TGCTTCTGTAAGTTCTTTAT	20
<i>arc</i>	SoeA	GATGCGAATTCTGTGCTTGCGCCATTTGTTT	31
<i>arc</i>	SoeB	TGTCTGCCGAGCGAATAAAG	21
<i>arc</i>	SoeC	TTATTCGCTCCGGCAGACATTCGGAAGTTTGATCCGCGTG	41
<i>arc</i>	SoeD	ATGTAGGTACCTACGACCTGTGGCACCGATA	31
<i>arc</i>	SoeZ	CCAAGTGTGAACATTCTTTT	20
<i>arc</i>	SoeE	CAGCTCCCCATTTTTTTTACA	20

Original primers were designed using the NCBI Primer-Blast program (35). Restriction enzyme binding sites for BamHI were incorporated into *prfA* SoeA. Restriction sites for HindIII were added to *prfA* SoeD. Restriction enzyme binding sites for EcoRI were incorporated into *arc* SoeA. Restriction sites for KpnI were added to *arc* SoeD. Primers were altered to create optimal PCR conditions.

3.2 Stock *L. monocytogenes*

A KWIK-STIK™ (ATCC ® 19111™) of *L. monocytogenes* was plated on blood agar plates (BAP) and incubated overnight at 37°C. After the incubation period, isolated colonies were chosen from the BAP plate, and grown in overnight cultures of BHI broth. 500 µl of overnight culture was moved to microfuge tubes with 500 µl of 50% glycerol.

All tubes were then stored at -80°C for stock. *L. monocytogenes* DNA was also extracted from overnight cultures using the ChargeSwitch® DNA extraction kit.

3.3 Vent PCR for SoeAB and SoeCD

A 50 µL vent polymerase chain reaction was made with the following reagents (31): 5 µL of 10X Thermopol reaction buffer (1x), 1 µL of 10mM dntp solution mix, 1.5 µL of each primer (20 µM), 0.5 µL of vent DNA polymerase (1 unit), 2 µL of 100mM MgSo4 (6mM), 5 µL DNA template (determined by concentration), and PCR-grade H2O up to 50 µL. Cycling conditions for the reaction can be seen in Table 4.

Table 4. Thermocycler conditions for vent polymerase chain reactions

	Temperature (°C)	Time
Initial denaturation	95	5 minutes
	95	30 seconds
Annealing (30 cycles)	55	30 seconds
	72	1 minute
Final extension	72	5 minutes

Vent polymerase chain reaction thermocycler conditions for SOEing reactions. *Source(s)*: Kathrin Rychli, Caitriona M. Guinane, Karen Daly, and Colin Hill. 2014. Generation of Nonpolar Deletion Mutants in *Listeria monocytogenes* Using the “SOEing” Method. *Methods Mol. Biol.* 1157:85–93.

Due to the complexity of the SoeC primer an extra vent PCR step was used. A version of the SoeC primer (SoeC2) without the additional features was placed in the vent PCR sample with the SoeD primer. That product was used as a DNA template, with the SoeC and SoeD primers, to create the desired product containing restriction sites. Products were verified with a 1.5% agarose gel (750 mg agarose and 50 mL of 1X TAE buffer) and purified using ChargeSwitch® PCR purification kit.

3.4 Vent PCR for SoeAD

A 50µL vent polymerase chain reaction was made with the following reagents (31): 5 µL of 10X Thermopol reaction buffer (1x), 1 µL of 10mM dntp solution mix, 1.5 µL of each primer (20 µM), 0.5 µL of vent DNA polymerase (1 unit), 2 µL of 100mM MgSo4 (6mM), 5 µL SoeCD, 3 µL SoeAB, and PCR-grade H2O up to 50 µL. Cycling conditions for the reaction can be seen in Table 4. Products were verified with a 1.5% gel (750 mg agarose and 50 mL of 1X TAE buffer) and the sample was purified with ChargeSwitch® PCR Purification kit.

3.5 Incorporating SoeAD into pKSV7

E. coli DH5α containing pKSV7 was grown in LB-amp⁵⁰ overnight at 37°C with consistent shaking and the plasmid was extracted using QIAprep spin miniprep kit. All DNA concentration measurements were performed using the NanoDrop 2000 per manufacturer protocol (36). Restriction digests were set up for the PrfA SoeAD product and pKSV7 using HindIII and BamHI FastDigest enzymes from ThermoFisher. The reaction for vector digestion included 1 µg of DNA, 2 µL of FastDigest buffer, 1 µL of each enzyme, and H₂O (reaction total of 30 µL). The reaction proceeded at 37°C for 20 minutes per manufacturer protocol. Heat inactivation was carried out at 80°C for 10 minutes. The PCR product digestion reagents were 0.2 µg of DNA, 2 µL of FastDigest buffer, 1 µL of HindIII, 1 µL of BamHI, and H₂O (reaction total of 30 µL). The reaction

proceeded at 37°C for 25 minutes per manufacturer protocol. Heat inactivation was carried out at 80°C for 10 minutes.

The *arc* SoeAD and pKSV7 were digested using EcoRI and KpnI FastDigest enzymes. The pKSV7 digestion called for 1µg of DNA, 2 µL of FastDigest buffer, 1 µL of each enzyme, and H₂O (reaction total of 30 µL). The reaction was incubated for 20 minutes at 37°C and heat inactivated for 10 minutes at 80°C. The PCR product digestion reagents were 0.2 µg of DNA, 2 µL of FastDigest buffer, 1 µL of EcoRI, 1 µL of KpnI, and H₂O (reaction total of 30 µL). The reaction proceeded at 37°C for 25 minutes per manufacturer protocol. Heat inactivation was carried out at 80°C for 5 minutes. All DNA measurements were done using the NanoDrop system. The samples were then purified using the ChargeSwitch® PCR Purification kit.

For ligation the digested SoeAD and pKSV7 were mixed at three different molar ratios over vector: 5:1, 3:1, and 1:1 (31). Each ligation included 2 µL of 10X ligation buffer and 1 µL (5 Weiss U) of T4 DNA ligase. Each reaction incubated for one hour at 22°C. The ligation reactions were purified using the microdialysis technique where a MF-Millipore™ membrane filter was placed in a sterile petri dish containing 20 mL of sterile H₂O (31). The reaction was incubated at room temperature for 20 minutes, purifying the sample via osmosis. DNA purity and concentrations were measured on the NanoDrop.

3.6 Transformation of *E.coli*

Electrocompetent DH5α *E.coli* (NEB #C2989K) were thawed on ice for 10 minutes. Cuvettes (1mm) were placed on ice while cells were prepared. 1 µL of the ligated pKSV7 SoeAD product (10 pg/µL) was added to 25 µL of cells. The mixture was carefully transferred to a chilled cuvette. The sides of the cuvette were wiped down with a Kimwipe and the cuvette was tapped on the countertop twice before electroporating using the BioRad electroporator set at 1.7 kV, 200 Ω, and 25 µF. 975 µL of pre-warmed (37°C) SOC, a specialized recovery broth for *E. coli*, was immediately added to the mixture and pipetted twice. The contents were then transferred to a 15 mL tube and

rotated at 250 rpm for one hour. Unaltered pKSV7 was used as a control in conjunction with every transformation. From all cultures 200 μL was plated on LB-amp⁵⁰ overnight at 37°C.

Integration of SoeAD into the pKSV7 backbone was verified by two methods: plasmid extraction followed by digestion or colony PCR. For the former method plasmids were extracted from single colonies using the QIA spin mini-prep kit. The *arc* samples were digested with EcoRI and KpnI. The *prfA* samples were digested with BamHI and HindIII. The reaction for vector digestion included 1 μg of DNA, 2 μL of FastDigest buffer, 1 μL of each enzyme appropriate to gene, and H₂O (reaction total of 30 μL). The reaction proceeded at 37°C for 20 minutes per manufacturer protocol and verified with a 1.5% gel. For colony PCR, single colonies were placed in 10 μL of 10% IGEPAL-G630 and heated at 95°C for 10 minutes. That reaction was then used as the DNA template in the following PCR: 5 μL of 10X Thermopol reaction buffer (1x), 1 μL of 10mM dntp solution mix, 1.5 μL of each primer (20 μM), 0.5 μL of vent DNA polymerase (1 unit), 2 μL of 100mM MgSo₄ (6mM), 5 μL of DNA template, and PCR-grade H₂O up to 50 μL . Thermocycler settings for this reaction can be seen in Table 5. Products were verified on a 1.5% gel and positive plasmid extracts were also frozen (-80°C) as stock for later use.

Table 5. Thermocycler conditions for colony polymerase chain reactions

	Temperature (°C)	Time
Initial denaturation	94	3 minutes
	94	30 seconds
Annealing (30 cycles)	50	30 seconds
	72	1.5 minute
Hold	10	∞

Colony polymerase chain reaction thermocycler conditions. *Source(s)*: Kathrin Rychli , Caitriona M. Guinane , Karen Daly , Colin Hill and PDC. 2014. Generation of Nonpolar Deletion Mutants in *Listeria monocytogenes* Using the “SOEing” Method. *Methods Mol. Biol.* 1157:85–93.

3.7 Electrocompetent *L. monocytogenes*

L. monocytogenes was grown in 5 mL of BHI overnight at 37°C with shaking. Two mL of the overnight culture was added to 100 mL BHI and 0.5 M sucrose in a 500 mL flask (31, 34). The culture was grown at 300 rpm for 5-6 hours at 37°C until an OD₆₀₀ of 0.2 was reached (31, 34). Optical density measurements (OD⁶⁰⁰) were taken on the NanoDrop 2000 using the cell culture software, per manufacturer protocol (36). 100 µL of 10mg/mL penicillin stock was added and the culture was incubated for 2 hours under the same conditions (31, 34). Cells were centrifuged for 10 minutes at 8,700 x g and the supernatant was removed (31, 34). The pellet was resuspended in 90 mL of 1mM HEPES and 0.5 M sucrose (31, 34). Cells were centrifuged for 10 minutes at 8,700 x g and the supernatant was removed (31, 34). The pellet was resuspended in 45 mL of 1mM HEPES and 0.5 M sucrose (31, 34). Cells were centrifuged for 10 minutes at 8,700 x g

and the supernatant was removed. 200 μ L of 1mM HEPES and 0.5 M sucrose was added to resuspend the pellet. Cells were frozen at -80°C . When transformed with puc19 (10 $\text{pg}/\mu\text{L}$) prepared electrocompetent *L. monocytogenes* reached transformation levels of 7×10^5 / μg of DNA.

3.8 Transformation of *L. monocytogenes*

For the electroporation reaction 4 μL of the plasmid was added to 50 μL of electrocompetent cells. The cells were electroporated in a pre-chilled cuvette (2mm) at the following parameters: 2 kV, 400 Ω , and 25 μF (31). 1mL of BHI-0.5M sucrose was immediately added to the cells. The contents were then transferred to a 15 mL tube and incubated at 30°C . A control of unaltered pKSV7 was used as a control in conjunction with every transformation. The cells were plated on BHI-amp⁵⁰ for 48 hours at 30°C .

3.9 Chromosomal integration

Three colonies displaying antibiotic resistance were selected from the transformation plates. The colonies were inoculated in 10 ml BHI-cm¹⁰ broth and then plated on BHI-cm¹⁰. Those cultures were incubated for 24 hours at 42°C . One mL of the broth was moved to a fresh 9 mL of BHI-cm¹⁰, and again plated on BHI-cm¹⁰. The cultures were incubated for 24 hours at 42°C . This process was repeated 3 more times. Liquid cultures from the 5th passage were plated on BHI plates and incubated at 30°C for 24 hours. Three to five colonies were screened for chromosomal integration using colony PCR with combinations of SoeZ, SoeE, SoeA, and SoeD. The colonies were also inoculated into 10 mL of BHI and incubated at 30°C for 24 hours.

If no mutants were detected during colony PCR the passaging process continued as follows: 1 mL of the overnight culture was moved to a fresh 9 mL of BHI and incubated at 30°C for 24 hours in parallel with BHI plates. This process was repeated until a mutant could be detected. Colony patching was repeated every 5th passage until

chromosomal integration was detected. Single colonies were chosen and plated onto a BHI plate and a BHI-cm¹⁰ plate, using a template. Colonies that did not show resistance to chloramphenicol were extracted, amplified by colony PCR, and analyzed on a 1.5% gel for mutant verification. The $\Delta prfA$ and Δarc colonies were frozen at -80°C in 50% glycerol once integration was achieved.

3.10 Verification of construct integration

Colony PCR was used to confirm chromosomal integration of the plasmid into the targeted gene of interest. Single colonies from chromosomal integration passages were isolated for examination. Single colonies were placed in 10 μ L of 10% IGEPAL-G630, heated at 95°C for 10 minutes. That reaction was then used as the DNA template in the following PCR: 5 μ L of 10X Thermopol reaction buffer (1x), 1 μ L of 10mM dntp solution mix, 1.5 μ L of each primer (20 μ M), 0.5 μ L of vent DNA polymerase (1 unit), 2 μ L of 100mM MgSo4 (6mM), 5 μ L of DNA template, and PCR-grade H2O up to 50 μ L. Thermocycler settings for this reaction can be seen in Table 5. Combinations of primers SoeZ, SoeE, SoeA, and SoeD, which flank the gene of interest, were used for verification (Table 3). Products were verified on a 1.5% gel and positive samples were also frozen (-80°C) as stock for later use.

CHAPTER 4

RESULTS

4.1 Vent PCR product for SoeAB and SoeCD

The purified vent PCR products for both genes of interest were verified successfully by electrophoresis on a 1.5% agarose gel. The amplicon for the *prfA* gene SoeAB product was ~256 bp and the SoeCD product was ~201 bp. For the *arc* gene the SoeAB product was ~119 bp and the SoeCD was measured at ~417 bp. In order to get SoeCD for the *arc* gene a Vent PCR amplification using a SoeC primer without restriction sites had to be used (Primer C2). This product is referred to as SoeC2D. The SoeC2D product showed an amplicon at ~384 bp and was purified then used as the DNA template with primers SoeC and SoeD to create SoeCD. All gel readings were appropriate to the amplification regions and were repeated with multiple trials to verify results. The gel electrophoresis photos can be seen in Figure 3.

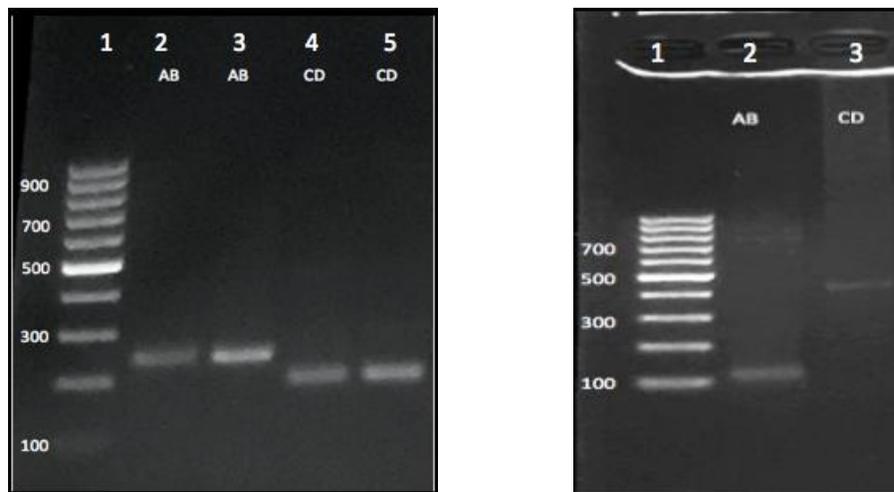


Figure 3. Gel electrophoresis: SoeAB and SoeCD. The first well on each gel is a 100 bp ladder. The gel on the left shows vent PCR results for the *prfA* gene. Wells 2 and 3 show *prfA* SoeAB (~256 bp) and wells 4 and 5 SoeCD (~201 bp). The gel image on the right shows results for the *arc* gene vent PCR results. Well 2 shows *arc* SoeAB (~119 bp) and well 3 shows SoeCD (~417 bp).

4.2 Vent PCR product for SoeAD

The purified vent PCR products for both genes of interest were verified successfully with electrophoresis on a 1.5% agarose gel. The SoeAD product for the *prfA* gene had an amplicon at ~447 bp, which was correct based on gene mapping. The SoeAD product for the *arc* gene showed the expected amplicon at ~537 bp. The gel electrophoresis photos can be seen in Figure 4.

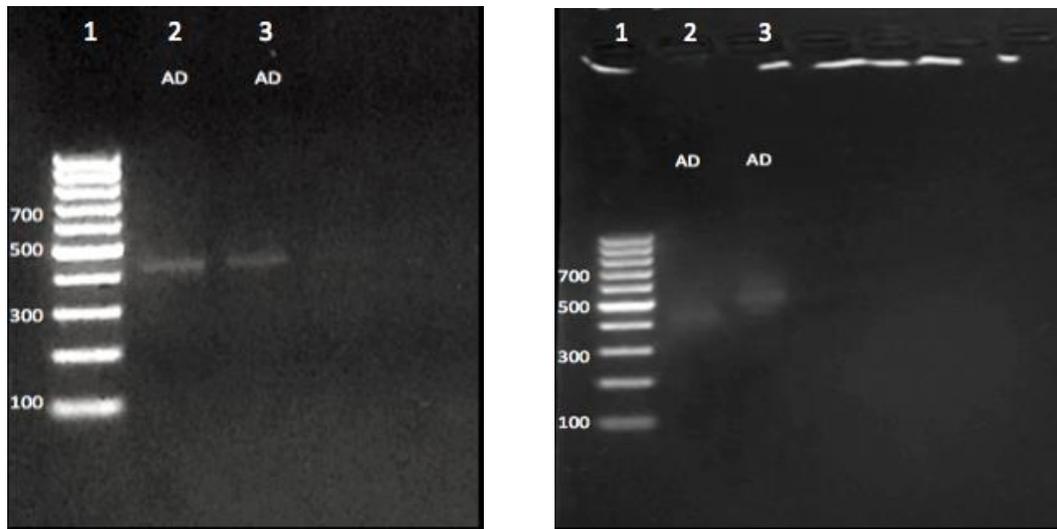


Figure 4. Gel electrophoresis: SoeAD. The first well on both pictured gels is a 100 bp ladder. The gel on the left shows vent PCR results for the *prfA* gene. Wells 2 and 3 show the SoeAD product at ~447 bp. The gel image on the right shows results for both the *prfA* gene and the ADI pathway. Well 2 shows SoeAD for *prfA* and well 3 shows SoeAD for the ADI gene at ~537 bp.

4.3 Ligation and transformation into *E. coli*

The ligation product (pKSV7-AD) was electroporated into *E. coli* and grew on overnight plates of LB-amp⁵⁰. The ability to grow on this media shows successful uptake of the pKSV7 plasmid containing ampicillin resistance. Colonies from the plates were

selected for plasmid extraction using the QIAprep spin miniprep kit followed by amplification of the SoeAD gene via PCR. The gel for *prfA* pKSV7-AD showed an amplicon of ~440 bp released from the plasmid. The gel for the *arc* pKSV7-AD sample showed an amplicon at ~520 bp. These results confirmed that the ligation product was correctly assembled. The gel electrophoresis photos can be seen in Figure 5.

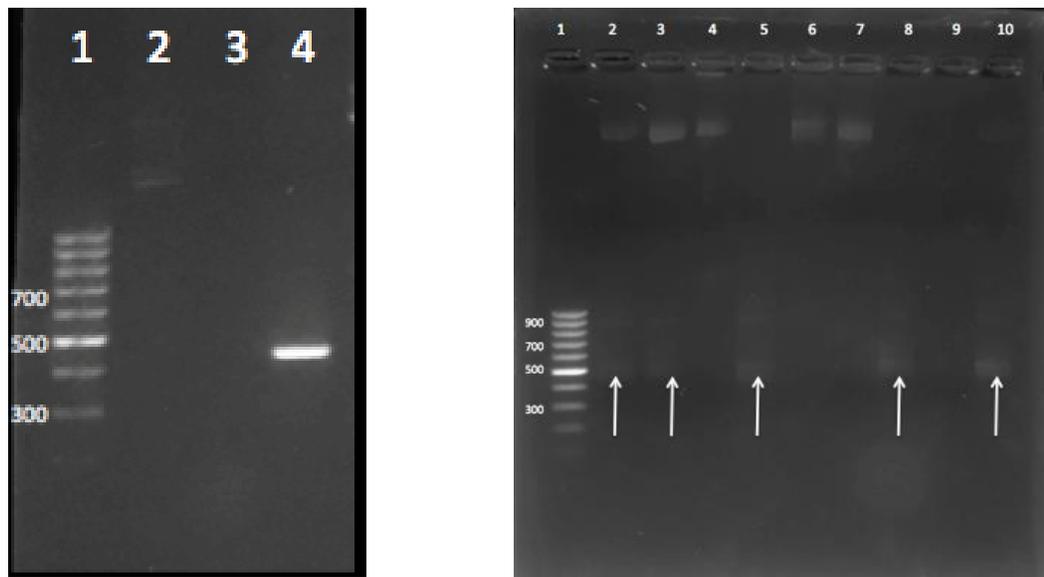


Figure 5. Gel electrophoresis: pKSV7-AD. The first well on each gel is a 100 bp ladder. The gel on the left shows the positive *prfA* SoeAD insert at ~440 bp in well 4. This sample is derived from miniprep pKSV7 from transformed *E. coli* and amplified with PCR using SoeA and SoeD primers. The gel image on the right shows colony PCR samples from pKSV7-AD ADI. The samples that show an amplicon at ~520 bp are considered positive. The positive samples were wells 2,3,5,8, and 10. The other wells were negative for AD inserts. Dim bands at the top, which are not addressed, are non-specific genomic DNA binding common in PCR.

4.4 Transformation of *L. monocytogenes*

The pKSV7-SoeAD plasmid was electroporated into electrocompetent *L. monocytogenes* by electroporation and the samples grew on overnight BHI-cm¹⁰ plates at 30°C. This display of chloramphenicol resistance showed the successful uptake of the plasmid. Three colonies displaying resistance were moved to 42°C on BHI-cm¹⁰ plates.

4.5 Chromosomal integration

Cultures of Δarc transformants grew in the presence of chloramphenicol at 42°C. The pKSV7 plasmid, which confers chloramphenicol resistance, cannot remain extrachromosomal at this temperature. The fact that cells survived on chloramphenicol plates, at this temperature, signifies the plasmid was incorporated into the chromosomal DNA. To see if the plasmid was incorporated into the targeted *arc* gene of the chromosome, colony PCR was performed. The Δarc DNA was extracted from colonies that were isolated from the 5th cell passage. The colony PCR samples were designed using primers that flank the *arc* gene of interest. Wild type colony PCR samples were run in parallel, as a negative control. The deletion target for the *arc* gene was ~1901 bp. Colony PCR was performed on Δarc DNA samples with the primer pair SoeZ and SoeD, as well as the primer pair SoeE and SoeA. All primers are shown in Table 3 and experimental design methods are shown in Figure 2. The use of both sets of primers allows examination of either side of the *arc* gene to check for pKSV7 integration.

The amplicon of a wild type colony, or an Δarc I colony with no chromosomal integration, amplified with SoeZ and SoeD would be ~2925 bp. The Δarc colony DNA amplified with SoeZ and SoeD showed an amplicon at ~2925 bp. The amplicon of a wild type colony, or an Δarc colony with no chromosomal integration, amplified with SoeE and SoeA would be about 2667 bp long. The Δarc colony DNA amplified with SoeE and SoeA showed an amplicon at ~2667 bp. The resulting colony PCR samples were consistent with the expected amplicon size of the *arc* gene with no pKSV7 integration. This suggests that the construct did not integrate into the correct location on the chromosome. The pKSV7 plasmid is untranslatable at high temperatures, such as 42°C. The fact that the Δarc transformation cells were able to translate antibiotic resistance at 42°C indicates pKSV7 had incorporated into the *L. monocytogenes* chromosome. The results of the Δarc colony PCR samples and the wild type negative controls can be seen in the gel image found in Figure 6.

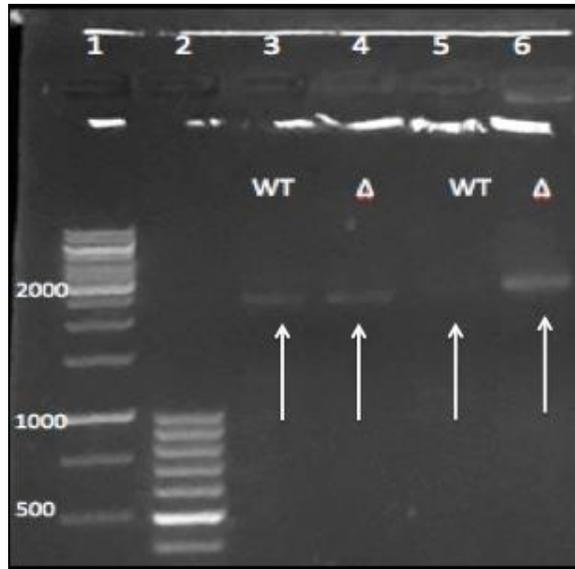


Figure 6. Gel electrophoresis: chromosomal integration Δarc . Wells 1 and 2 contain a 1 kb and 100 bp ladder, respectively. Well 3 shows the wild type colony DNA was amplified with the SoeZ and SoeD primer set at ~2925 bp, which is correct. Well 4 contains Δarc colony DNA amplified with the same primers at ~2925 bp. No change between the wild type and Δarc sample was observed. Well 5 shows wild type DNA was amplified with SoeA and SoeE at ~2667 bp, which is the expected band size. Well 6 shows Δarc colony DNA amplified with the same primers at ~2667 bp. No change between the wild type and Δarc sample was observed.

Cultures of $\Delta prfA$ transformants grew in the presence of chloramphenicol at 42°C. As previously mentioned, pKSV7 chloramphenicol resistance is untranslatable at 42°C as an extrachromosomal unit. The fact that the cells survived on chloramphenicol plates, at this temperature, signifies the plasmid was incorporated into the chromosomal DNA. In order to analyze the samples for chromosomal integration of pKSV7, a colony PCR was designed. The deletion target for the *prfA* gene was ~255 bp long. The $\Delta prfA$ DNA for colony PCR was isolated from colonies during the 5th passage and wild type colony DNA was used as a negative control. DNA samples were amplified with SoeZ and SoeD primers, which flank the *prfA* deletion target. Primers are shown in Table 3 and experimental design is shown in Figure 2. The expected amplicon for a wild type sample, or a $\Delta prfA$ sample with no chromosomal integration, would be ~735 bp in length. Seven samples of $\Delta prfA$ colony PCR showed a amplicon at ~480 bp, which is a decrease of

~255 bp in comparison to the wild type strain. One $\Delta prfA$ colony PCR sample showed an amplicon identical to the wild type strain, signifying a non-mutant sample. These results can be seen in Figure 7. The knockout of the 255 bp region in the *prfA* gene was successful and $\Delta prfA$ colonies were frozen down as stock.

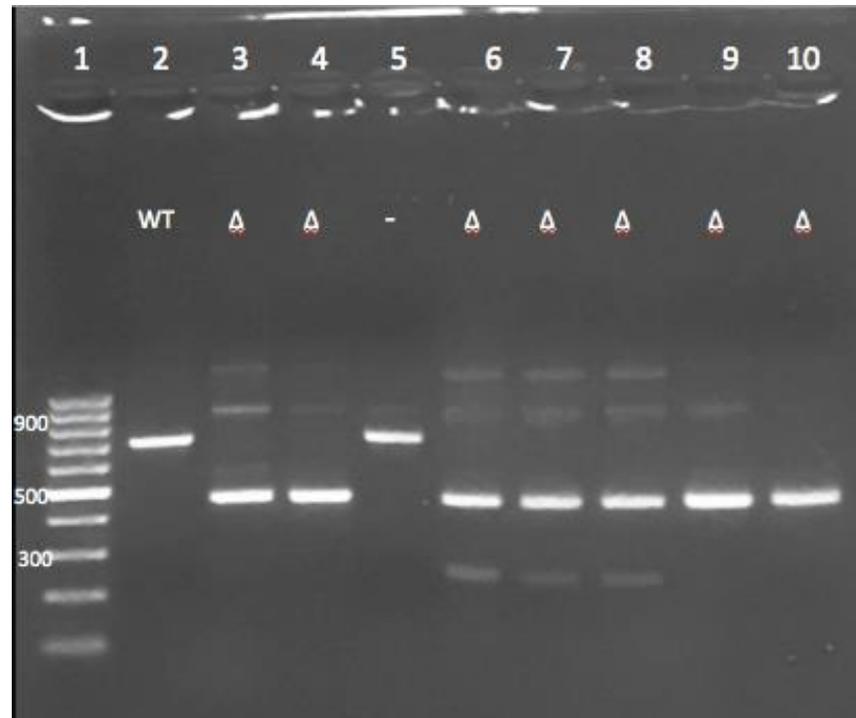


Figure 7. Gel electrophoresis: chromosomal integration $\Delta prfA$. Well 1 contains a 100 bp ladder. Well 2 contains a wild type colony PCR sample amplified with the SoeZ and SoeD primer set. The wild type sample matches the expected amplicon of ~735 bp. Wells 3 through 10 were $\Delta prfA$ samples amplified with the SoeZ and SoeD primer set showing an amplicon at ~480 bp. All colonies were positive for mutant strain except for well 5, which matched the wild type. Dim bands in the background, which are not addressed, are non-specific genomic DNA binding common in colony PCR.

CHAPTER 5

DISCUSSION

L. monocytogenes is a virulent species due to many factors produced during the host infection cycle (7, 16, 19). *Listeria* has the ability to survive and propagate efficiently within a host (19). Many virulence factors are responsible for survival during the host infection cycle. The arginine deiminase pathway helps regulate acidic environments to which *L. monocytogenes* is exposed (6, 20). This pathway has the ability to convert arginine to ammonia, ornithine, and ATP (6, 20). With the help of a membrane-bound antiporter, the ornithine is transported out of the cell while the ammonia is converted to ammonium, both of which help balance intracellular pH (6, 20). ATP by-products can also be used with the F₀F₁-ATPase system to help maintain homeostasis (6, 20).

The *prfA* gene, which codes for the PrfA protein, is considered to be a crucial virulence regulator in *L. monocytogenes* (6, 16, 37). The *prfA* regulon is both an activator and repressor of its own gene cluster (2, 19, 24). The PrfA protein has the ability to bind to palindromic promoter boxes within the chromosome in order to recruit necessary components for transcription (2, 19, 24). There are a total of 145 genes in the *Listeria* chromosome regulated by PrfA; however, there are only ten associated with virulence capabilities (2). The ten core genes subject to PrfA regulation are *hly*, *mpl*, *actA*, *plcB*, *plcA*, *prfA*, *inlA*, *inlB*, *inlC*, and *hpt* (2, 4, 14, 19, 25). The PrfA protein is responsible for regulating virulence expression at every stage of the *L. monocytogenes* infectious life cycle.

L. monocytogenes is highly immunogenic and has the potential to be used in vaccine production (2). Several studies have attempted to develop a killed vaccine with *L. monocytogenes* (2). Killed vaccines are bacterial cells that have been inactivated by chemical or physical reactions. This process has been known to destroy some bacterial antigens but many species treated with this method still result in immunity (2). Some diseases that have effective killed type vaccines include typhoid fever, cholera, plague,

lyme disease, and pertussis (2). However, studies using *L. monocytogenes* for production of a killed vaccine have been well documented and unsuccessful (2). There was minimal resistance to listeriosis in mouse models infected with high doses of heat killed *L. monocytogenes* (2).

Live attenuated vaccines have a better chance of eliciting immune resistance to *Listeria* infection. Attenuated *Listeria* cells have less virulence factors for infection while still maintaining the ability to replicate in the host. This confers immunity to listeriosis at relatively low doses. *Listeria* infects antigen-presenting cells, monocytes, macrophages, and dendritic cells (2). *Listeria* bacterial antigens are presented via the MHC class I pathway to cytotoxic T- lymphocytes (CD8⁺) (2). Antigens are also presented via the MHC class II pathway to T-lymphocyte helper 1 cells (CD4⁺) (2). The ability of *L. monocytogenes* to interact with both MHC classes increases the efficiency of the live vaccine.

Another potential use for attenuated *Listeria* could be a *Listeria*-based cancer vaccine. Anti-cancer vaccines use the host body to fight off disease. Transforming attenuated *L. monocytogenes* with cancer antigen genes can induce targeted self-reactive T-cells (2). Attenuated *Listeria*, containing cancer antigen genes, are taken up and degraded by phagocytic cells (13). The antigens would be presented on phagocytic cell surfaces via the Major Histocompatibility Complexes I and II (2). Cytotoxic T-lymphocytes and T-lymphocyte helper 1 cells would react with the cancer antigens and retarget the host immune system components. CD8⁺ and CD4⁺ T-cells have both been found to have highly increased levels upon interaction with *L. monocytogenes* (2). This fact makes the species a good candidate for immunotherapy for cancer patients. This method is a relatively new approach to cancer and has the potential to be a much less debilitating form of treatment for patients.

This study aimed to create a spliced *arc* gene (ADI pathway) mutant using the splicing by overlap extension PCR procedure. The production of SoeAB and SoeCD products for the *arc* gene were successful with amplicons at ~119 bp and ~417 bp, respectively. Those products were successfully spliced to create the *arc* SoeAD PCR

product with an amplicon at ~537 bp. The *arc* SoeAD insert was incorporated into pKSV7 via digestion and ligation. The *arc* SoeAD insert was effectively verified after transformation into *E. coli*. The *arc* pKSV7-AD plasmid was successfully introduced to the *L. monocytogenes* strain via transformation. The pKSV7, which confers chloramphenicol resistance, cannot be maintained as an extrachromosomal unit at 42°C. The fact that the cells grew in the presence of chloramphenicol at this temperature signifies that pKSV7 was incorporated into the chromosome.

The colony PCR results, however, showed no change compared to the wild type strain. The wild type DNA amplified with primers SoeZ and SoeD are expected to produce an amplicon at ~2925 bp. If pKSV7 were incorporated into the chromosomal DNA sample, at the gene of interest, and the Δarc sample would be expected to yield an amplicon at ~9825 bp. If pKSV7 had left the chromosome with the *arc* deletion target, a mutant would yield an amplicon at ~1024 bp. The wild type DNA amplified with the SoeA and SoeE primers are expected to produce an amplicon at ~2667 bp. The pKSV7 integrated with the targeted *arc* gene would yield an amplicon of ~9567 bp while a successful mutant would produce an amplicon at ~766 bp. The Δarc colony PCR amplification was identical to the negative control wild type amplification. This data signifies the plasmid successfully incorporated into the chromosome; however, it was not incorporated into the *arc* gene deletion target.

This resulted in an unsuccessful attempted Δarc mutant. The failure of the *arc* pKSV7-AD to incorporate into the *arc* gene deletion target is likely due to an inefficient SoeAD product. Small mutations in the SoeAD insert sequence could have been introduced during the sequential PCR amplifications. This would cause correct amplicon bands on gel verification but would not be specific for incorporation into the gene of interest. The project data can still be helpful to further studies by provide an example of potential errors in the procedure, which could be used to make the protocol more efficient.

The second goal of this study was to create a spliced *prfA* gene mutant using the splicing by overlap extension PCR procedure. The vent PCR amplification of SoeAB and

SoeCD produced a correct amplicon at ~256 bp and ~201 bp, respectively. Those products were successfully spliced to create the *prfA* SoeAD PCR product with an amplicon at ~447 bp. The *prfA* AD product was incorporated into the pKSV7 backbone via digestion and ligation. The *prfA* pKSV7-AD vector was verified to be correct after transformation into *E. coli*. The *prfA* pKSV7-AD vector was successfully introduced to *L. monocytogenes* and transformants grew in the presence of chloramphenicol at 42°C. The fact that the *L. monocytogenes* samples were able to translate chloramphenicol resistance at this temperature signified that the *prfA* pKSV7-AD was incorporated into the chromosome.

Colony PCR using primers designed to flank the *prfA* gene deletion target was used to verify chromosomal integration. The *L. monocytogenes* wild type DNA amplified with primers SoeZ and SoeD would produce an amplicon at ~ 735 bp. If *prfA* pKSV7-AD were still present in the chromosome, at the gene of interest, the $\Delta prfA$ sample would produce an amplicon at ~7635 bp. If *prfA* pKSV7-AD had left the chromosome, with the gene of interest, a mutant would yield an amplicon at ~480 bp. The colony PCR $\Delta prfA$ reactions showed a decrease of ~255 bp in comparison to the wild type strain at ~ 735 bp. The colony PCR of $\Delta prfA$ samples corresponded directly with expected results for a mutant strain containing a spliced *prfA* gene. These results signify the plasmid left the host chromosome with the gene of interest. The $\Delta prfA$ strain was isolated and will be useful in further studies on this pathogen.

These further studies could to examine the change in virulence capability of the wild type strain in comparison with successful mutant strains. This property could be assessed using tissue culture assays that monitor infection and survival rates. This comparative analysis would reveal how the loss of virulence factors affects the ability of *L. monocytogenes* to propagate *in vitro*. Due to thermo-activated virulence up-regulation, a study could also be designed to specifically investigate the differences in mutant strains *in vitro* versus *in vivo* in murine models, as compared to wild type *L. monocytogenes*. Comparing fatality and infection rates post-infection of each group could reveal effects of mutations on virulence.

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APPENDICES

APPENDIX A: SUPPLEMENTAL TABLES

Primer Design Guidelines

Primer	Location	Requirements
SoeA	Forward primer Binds upstream of SoeB	Contains a 5' enzyme restriction site
SoeB	Reverse primer Binds to a region flanking the gene targeted for deletion	N/A
SoeC	Forward primer Binds to a region flanking the gene targeted for deletion	Contains a 5' region of ~20 bp that is the reverse complement of SoeB
SoeD	Reverse primer Downstream of SoeC	Contains a 5' enzyme restriction site
SoeZ	Forward primer Binds upstream of SoeA	N/A
SoeE	Reverse primer Downstream of SoeD	N/A

Table A.1. Primers used in the SOEing method, use a method of overlap extension to incorporate the gene of interest into the vector of choice for gene knockout. *Source(s)*: Kathrin Rychli , Caitriona M. Guinane , Karen Daly , Colin Hill and PDC. 2014. Generation of Nonpolar Deletion Mutants in *Listeria monocytogenes* Using the “SOEing” Method. *Methods Mol. Biol.* 1157:85–93.