ABSTRACT

PRICE, ROBERT, E. Characterization of Selected Transposon-mediated Mutants of *Listeria monocytogenes* Regarding Survival and Growth on Cantaloupe. (Under the direction of Sophia Kathariou)

In 2011, *Listeria monocytogenes* was implicated in a major multistate outbreak of listeriosis in the United States involving whole cantaloupe. To date, the genetic features of *L. monocytogenes* that allow adherence and growth on produce remain largely uncharacterized. In this thesis, we characterized two non-hemolytic mutants of one of the outbreak strains, 2011L-2858 (serotype 1/2b) for attachment, growth and survival on the surface of cantaloupe rind. These two mutants harbored single *mariner*-based transposon insertion in *hly*, encoding the hemolysin Listeriolysin O and prfA, encoding a positive regulator for *hly* and numerous other virulence genes, as well as two motility impaired mutants; one harboring an insertion in the DEAD-box RNA helicase gene (*lmo0866* homolog), while the other, harbored an insertion in a gene from a flagellum biosynthesis and chemotaxis gene cluster. When grown independently, none of the mutant strains displayed any significant impact on the adherence, growth or survival of the bacteria on cantaloupe rind at either 25 or 37°C. Over a 72-h period, the DEAD-box RNA helicase mutant displayed a competitive disadvantage relative to the parental strain when grown on the cantaloupe in a mixed culture. This competitive disadvantage was not seen in the three other mutant strains. When the helicase mutant was genetically complemented, the competitive disadvantage was not observed, verifying that the inactivation of DEAD-box RNA helicase gene was responsible for the results. The findings suggest that two key virulence determinants of *L. monocytogenes* as well as the chemotaxis gene cluster responsible for flagellum biosynthesis are not critical for the ability of this pathogen to survive and grow on cantaloupe while the DEAD-box RNA helicase gene is potentially critical for the survival and competitive fitness of *L. monocytogenes* on the rind of cantaloupes.

Characterization of Selected Transposon-mediated Mutants of *Listeria monocytogenes* Regarding Survival and Growth on Cantaloupe

By

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DEDICATION

I would like to dedicate this thesis to my parents, Derek and Debbie Price, and my siblings Anthony, David and Jennifer. Without their unconditional love and support none of this would have been possible. They have always pushed me to do my best at everything I do in life and that has been my motivation thus far. Everything I have accomplished in life is because of them.

BIOGRAPHY

Robert Price was born in Monterey, California. He attended Southern Guilford High School in Greensboro, North Carolina before completing his undergraduate studies in Food Science with a minor in Nutrition at NC State University in 2013. During his undergraduate career, he joined the USDA-ARS Food Science Research Unit lab under the supervision of Dr. Ilenys Perez-Diaz. He joined the lab of Dr. Sophia Kathariou in 2013 to receive a Masters in Food Science in 2016. During his graduate career, he joined the USDA-ARS Food Science Research Unit as a full time Biological Science Research Technician in 2015.

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1.1 *Listeria monocytogenes* and Listeriosis

1.1.1 History of *L. monocytogenes*

Listeria monocytogenes is a food-borne pathogenic bacterium first identified as the causative agent of monocytosis in laboratory rodents in 1926 in Cambridge, United Kingdom by Murray, Webb and Swann (Liu, 2008). It was originally named *Bacterium monocytogenes* because of its monocytosis properties found in infected laboratory rabbits and guinea pigs and later renamed *Listerella hepatolytica* in 1927 (Farber and Peterkin, 1991, Hof, 2003). It was finally given its present name by Pirie in 1940 (Farber and Peterkin, 1991). The first confirmed isolates of *L. monocytogenes* from humans came from the blood cultures of infected individuals that were collected by Nyfeldt (Liu, 2008, Farber and Peterkin, 1991). Compared to other human pathogens recognized to have plagued humans for centuries, the discovery of *Listeria monocytogenes* is relatively recent.

1.1.2 Characterization of *L. monocytogenes*

Listeria monocytogenes is a Gram-positive, facultative anaerobic rod-shaped bacterium which is ubiquitous in soil and other environments. *L. monocytogenes* can grow between 0°C and 50°C making it problematic for the food production industry, in particular ready-to-eat foods that are refrigerated after processing (Farber and Peterkin, 1991**).** The cells can appear as single rods, short chains or in small clusters of multiple rods. It is catalase positive and oxidase negative and possesses peritrichous flagella with a distinct tumbling motility (Farber and Peterkin, 1991). *L. monocytogenes* motility is primarily expressed following growth up to 28-30℃ but is significantly reduced at 37°C (Farber and Peterkin,

1991**)**. The minimum pH allowing growth of *L. monocytogenes* ranges from 5.0 to 5.7 at 4°C and from 4.3 to 5.2 at 30°C (Farber and Peterkin, 1991).

L. monocytogenes has three lifestyles, the first being an intracellular pathogen that utilizes actin-based motility for cytoplasmic movement and cell-to-cell spread. The second is an extracellular free-living, motile bacterium found in the environment and the third a member of a multicellular microbial ecosystem within biofilms (Lemon et al., 2007). *L. monocytogenes* is unique because it can spread directly from cell to cell in the host instead of having to move through the narrow spaces between cells, tissues or parts of an organ (FDA, 2012). *L. monocytogenes* can reproduce upon entry into the host's monocytes, macrophages, or polymorphonuclear leukocytes (FDA, 2012).

In regards to infection, there are two types of infections by *L. monocytogenes*; invasive and noninvasive. *L. monocytogenes* is commonly referred to as an invasive food-borne pathogen that affects individuals most susceptible to infection (elderly and immunocompromised individuals, infants, and pregnant women) and targets the internal organs beyond the gastrointestinal tract in its host potentially leading to life-threatening symptoms. Noninvasive *Listeria* infections, can involve previously healthy individuals; they are less severe and usually involve diarrhea and fever without the bacteria spreading past the gastrointestinal tract (CDC, 2015).

The onset of gastroenteritis caused by *L. monocytogenes* has a relatively short incubation period, ranging from a few hours to 2 to 3 d for the noninvasive form and 3 d to 3 months for the invasive form (FDA, 2012)

The minimum infectious dose of *L. monocytogenes* cells for illness in either healthy or susceptible individuals is not known at this time. It is expected that the number of cells required to cause illness will vary depending upon a variety of factors at the time of ingestion. The two most important factors are the bacterial strain differences and the host susceptibility (Farber and Peterkin, 1991). One explanation for the inability to determine the infectious dose of *L. monocytogenes* is that most people carry T cells with reactivity to *Listeria* spp., most likely a result of subclinical exposure to *Listeria* spp. or other Gram-positive bacteria that share antigens with *Listeria* spp. (Farber and Peterkin, 1991).

1.2. Serotypes and Epidemic Clones of *L. monocytogenes*

1.2.1 Serotypes

Serotyping is a subtyping method that is widely used for *L. monocytogenes*. Within *L. monocytogenes,* two major phylogenetic lineages have been identified using multiple molecular subtyping techniques, with a third less common division being identified for two less common serotypes (Rasmussen et al., 1994, Borucki and Call, 2003). The serotypes that belong to linage I include 1/2b, 3b, most of the serotype 4b, 4d and 4e, and lineage II consists of 1/2a, 1/2c, 3a, and 3c (Brosch et al., 1994, Aarts et al., 1999, Borucki and Call, 2003). The third lineage (III) consists of less common serotype 4a and 4c and certain serotypes 4b strains (Rasmussen et al., 1994). Strains are serotyped based on variation in the somatic and flagellar antigens (Seeliger and Hohne, 1979; Borucki and Call, 2003). Of the 13 serotypes that have been identified, only three (1/2a, 1/2b, and 4b) attribute to most outbreak cases of listeriosis (Kathariou, 2002, Borucki and Call, 2003).

1.2.2 Epidemic Clones of *L. monocytogenes*

While the incidence of most listeriosis outbreaks is sporadic and uncommon, the most publicized events are outbreaks associated with food. Implicated foods in those outbreaks have included dairy products, RTE meats, fish and seafood products and produce (Kathariou, 2002). Strains of *L. monocytogenes* that have been repeatedly involved in outbreaks are referred to as epidemic clones (ECs). ECs are defined as genetically related isolates derived from a common ancestor and involved in different temporally and geographically unrelated outbreaks (Lomonaco et al., 2012, Lomonaco et al., 2013). The use of multivirulence locus sequence typing (MVLST) revealed 5 ECs of *L. monocytogenes*, ECI-V (Lomonaco et al., 2012, Lomonaco et al., 2013). The EC designations have now been replaced by standardized clonal complex (CC) terminology based on multiple locus sequence typing (Cantinelli et al., 2013).

1.2.3 Epidemic Clone I (CC1)

Many of the 4b serotype strains associated with epidemics appears to be genetically distinct from other strains of the 4b serotype (Kathariou, 2002). Several of the strains shared a unique restriction fragment length polymorphism in a genomic region necessary for low-temperature growth of *L. monocytogenes* (Zheng and Kathariou, 1995, Kathariou, 2002). It has also been suggested that ECI strains methylate cytosine at GATC sites in their DNA, which makes the DNA resistant to digestion by the restriction enzyme Sau3AI (Zheng and Kathariou, 1997). It appears that ECI are a cosmopolitan clonal group composed of serotype 4b strains involved in several major outbreaks including coleslaw, soft cheese, and pork (Kathariou, 2002). ECIa was identified as another serotype 4b cluster, implicated in a pate

outbreak in the United Kingdom, 1988, a vegetable outbreak in Boston, MA, 1983, and a milk outbreak in Boston, MA, 1983 (Chen et al., 2007).

1.2.4 Epidemic Clone II (CC6)

Epidemic clone II (ECII) was first observed in a U.S. multistate outbreak associated with contaminated hot dogs in 1998-1999 and a 2002 outbreak associated with turkey deli meat (Kathariou, 2002, Chen et al., 2005, Chen et al., 2007). Isolates had unique ribotype and PFGE patterns that had rarely been identified before (Kathariou, 2002, Kim and Kathariou, 2009). Genomic sequencing of one of the ECII isolates (*L. monocytogenes* H7858) from the 1998-1999 hot dog outbreak showed the presence of a plasmid which harbored genes that mediated resistance of *L. monocytogenes* to the heavy metal cadmium and resistance to the quaternary ammonium disinfectant benzalkonium chloride (Elhanafi and Kathariou, 2007, Kim and Kathariou, 2009, Elhanafi et al., 2010).

1.2.5 Epidemic Clone III

Epidemic clone III (ECIII) differ from ECI, ECIa, and ECII isolates in that they are serotype 1/2a isolates associated with a single hot dog-associated case in 1988 and a turkey deli meat outbreak in 2000 (Kathariou, 2002, Chen et al., 2007). The products associated with both of those events were from the same food processing facility and it was suggested that the outbreak strain had persisted within the plant for at least 12 years without detectable genotypic changes (Kathariou, 2002).

1.2.6 Novel Epidemic Clones

It has been suggested that there may be two novel ECs of *L. monocytogenes* (ECVI and ECVII) (Lomonaco et al., 2013). ECVI isolates were associated with outbreaks involving

imitation crab meat s in Canada in 1996, chicken plants in the U.S. in and cantaloupe in the U.S. in 2011(Lomonaco et al., 2013). They were all serotype 1/2b and shared a common MVLST profile. ECVII isolates were associated with outbreaks involving whipping cream in Canada in 2000, U.S. chicken plants in 2002 and 2006, and U.S. cantaloupe in 2011 (Lomonaco et al., 2013). They were all serotype 1/2a and shared a common MVLST profile. It has been suggested that ECVI and ECVII might have co-colonized harboring sites within the cantaloupe processing facility involved in the 2011 listeriosis outbreak, resulting in multiple strains associated with that outbreak (Lomonaco et al., 2013).

1.3 Foodborne Outbreaks of *L. monocytogenes*

1.3.1 Early Foodborne Outbreaks of *L. monocytogenes*

The majority of cases of human listeriosis appear to be sporadic with the sources and route of transmission usually unknown. What is known is that the majority of human listeriosis cases involve individuals with an underlying medical condition and compromised immune systems. The first major foodborne outbreak of listeriosis that was linked to a common food source, coleslaw, occurred in the Maritime Provinces of Canada between March and September of 1981 (Schlech et al., 1983, Farber and Peterkin, 1991). The outbreak involved 41 total cases including 34 perinatal and 7 adults. Of the 34 perinatal cases, nine resulted in stillbirths, 23 of the live births resulted in ill infants with a 27 % mortality rate and only two live births resulted in healthy infants (Farber and Peterkin, 1991). A survey collected through 1996-1998 by the CDC showed that listeriosis was responsible for approximately 2,500 illnesses and 500 deaths in the U.S annually (FDA, 2012). This

number decreased by 36 percent by 2008. In 2014, it was estimated that approximately 1600 illnesses and 260 deaths in the U.S. were related to listeriosis annually (CDC, 2011, Scallan et al., 2011).

One of the earliest US outbreaks took place in 1985 in Los Angeles, California and involved contaminated Mexican-style soft cheese. The outbreak strain was serotype 4b. Of the 142 human listeriosis cases reported, 93 involved pregnant women or their offspring with 48 cases resulted in deaths. It was suggested that the cheese was contaminated by unpasteurized milk (Linnan et al., 1988, FDA, 2012).

In 1988 and 2000, listeriosis cases associated with hot dogs and turkey deli meats, respectively, involved a serotype 1/2a strain. These events were unusual because the contaminated food was processed by the same processing plant (Kathariou, 2002, Olsen et al., 2005, FDA, 2012).

In 1998-1999, a large multistate outbreak of listeriosis was linked to contaminated hot dogs. This outbreak resulted in at least 50 cases in 11 states and caused six deaths and two pregnant women to have spontaneous abortions. It was determined that the isolates of *L. monocytogenes* from the outbreak were serotype 4b (FDA, 2012).

In 2002, a multistate outbreak occurred in the 8 Northeastern U.S. states resulting in 46 cases, including 7 deaths and 3 stillbirths. The food associated with the outbreak was turkey deli meat with the outbreak serotype being 4b. It was suggested that the processing plant was the source of the outbreak because two of the isolates from the environmental samples has identical PFGE patterns as the outbreak strain (CDC, 1999, Mead et al., 2006, FDA, 2012). It was determined in 2006 that the strains associated with the 1998-1999

contaminated hotdog outbreaks were genetically similar to the strains in the 2002 outbreak. This suggests that the two outbreaks involved strains from the same epidemic clonal group, Epidemic Clone II (ECII) (Kathariou et al., 2006).

1.3.2 Listeriosis Outbreaks Associated with Produce

L. monocytogenes has been associated with numerous foodborne outbreaks, most of which involved cheese and dairy products and meat products. Fresh produce was considered a less common source of listeriosis. One listeriosis outbreak involved the consumption of raw celery, tomatoes, and lettuces by 23 patients from eight Boston hospitals in 1979 (Ho et al., 1986, Beuchat, 1996). However, no *L. monocytogenes* isolates were obtained from vegetables at the time of the outbreak. Another outbreak was documented in 1981 in the Maritime Provinces of Prince Edwards Island, Nova Scotia and New Brunswick, Canada (Schlech et al., 1983). It was revealed that the infected persons had consumed contaminated coleslaw and isolates from coleslaw were positive for *L. monocytogenes*. Serotype 4b isolates from both the coleslaw and from patients had the same genotypes. Two unopened packages of the coleslaw also had serotype 4b *L. monocytogenes* (Beuchat, 1996, Harris et al., 2003).

The Maritime outbreak caused researchers to re-evaluate the significance and prevalence of *L. monocytogenes* on produce. One study determined that out of the 60 pre-packed RTE salads sampled in the United Kingdom, 4 were positive for *L. monocytogenes* (Beuchat, 1996). Other studies determined that 11 of 25 samples of fresh cut vegetables in the Netherlands and 7 of 66 samples of salad vegetables and prepared salads in Northern Ireland were contaminated with *L. monocytogenes* (Beuchat, 1996). A high percentage of

bean sprouts, sliced cucumbers, and leafy vegetables in Malaysia were positive for *L. monocytogenes* (Beuchat, 1996).

1.3.3 Listeriosis Outbreak Associated with Cantaloupe

In 2011, a major multistate outbreak was determined to be associated with cantaloupe produced by a single Colorado farm (McCollum et al., 2013). Even though there have been studies previously done on the growth and survival of cantaloupe of *L. monocytogenes* on the surface of fresh fruit, including cantaloupe (Ukuku et al, 2004), this outbreak of listeriosis was the first to be documented with cantaloupe as the implicated food. Even more significant was the fact that this was the largest U.S. listeriosis outbreak to date (McCollum et al., 2013). There were 147 cases, resulting in 33 deaths and 1 miscarriage (McCollum et al., 2013). It must also be mentioned that while the majority of listeriosis outbreaks have been associated with serotype 4b, this particular outbreak was associated with the less common 1/2a and 1/2b serotypes (McCollum et al., 2013). Of the 28 foodborne outbreaks reported to the CDC associated with cantaloupe in the past, eleven were cause by *Salmonella*, seven by norovirus, one by *Campylobacter jejuni* and one by *Escherichia coli* O157:H7 (McCollum et al., 2013). Of the samples collected from the patient's homes, several contained one or more of the *L. monocytogenes* outbreak-related strains. It was determined that the introduction of a used water wash system with brush and felt rollers for cleaning the cantaloupes was one of the causes for the contamination. It was suggested that the used equipment was contaminated with *L. monocytogenes* before it was bought by the company. Of the 39 environmental swabs collected from food-contact or adjacent surfaces of the implicated processing facilities, 12

swabs yielding *L. monocytogenes* isolates with similar PFGE patterns as three of the outbreak-related subtypes were identified (McCollum et al., 2013).

1.4 *L. monocytogenes* Genetic Characterization

1.4.1 Hemolysin

L. monocytogenes has been linked to serious illness in immunocompromised individuals and pregnant women (Farber and Speirs, 1987, Portnoy, 1988)**.** One of the key determinants of *L. monocytogenes* virulence is the hemolysin, Listeriolysin O (LLO) (Kathariou et al., 1987, Portnoy, 1988). LLO is a member of the family of sulfhydryl-activated pore-forming cytolysins (Hamon, 2012, Portnoy, 1988). LLO was determined to be essential for virulence by the isolation of non-hemolytic transposon mutants which were non-virulent in comparison to hemolytic strains (Gaillard et al., 1986, Kathariou et al., 1987, Kuhn et al., 1988, Portnoy, 1988). LLO was determined to be a virulence factor that is required for bacterial escape from the primary vacuole or the secondary vacuole formed by bacterial spreading to neighboring cells (Hamon, 2012). LLO-negative mutants used in previous studies have usually been found in host vacuoles and have been unable to grow intracellularly (Portnoy, 1988). The association between LLO and virulence in *L. monocytogenes* was further confirmed when the cloned LLO gene on a plasmid was inserted into a mutated strain which resulted in a restoration of virulence in the mutated strain (Portnoy, 1988). LLO differs from other sulfhydryl-activated pore-forming cytolysins in that its activity is regulated by pH with an optimal pH of 5.7 (Glomski et al., 2002, Hamon et al., 2012). LLO is transcriptionally regulated within the pathogenicity gene cluster, LIPI-1 by the transcriptional activator, PrfA at 37°C (Johansson et al., 2002).

Aside from its primary role during bacterial internalization, new roles for LLO have been suggested in recent years. One of said roles is the control of autophagy upon vacuolar escape. Autophagy is an intracellular catabolic process in which cellular or foreign materials are targeted for degradation through fusion with lysosomes (Hamon et al., 2012). In *Listeria*, mechanisms have evolved to avoid autophagy via expression of surface proteins (Hamon et al., 2012). It has also been suggested that autophagy induction is LLO-dependent and that the autophagy machinery recognizes LLO-damaged phagosomal membranes (Hamon et al., 2012).

Another recently determined role of LLO is the ability to suppress reactive oxygen species (ROS) produced by the NOX2 NADPH oxidase produced in response to *L. monocytogenes* infection in infected macrophages (Hamon et al., 2012, Lam et al., 2010). It has been suggested that the combination of LLO and phospholipase C (PLC) allows *L. monocytogenes* to escape the phagosome and replicate within the host cytosol (Lam et al., 2010). The exact mechanism by which this occurs is unclear, but it has been hypothesized by Hamon et al. that LLO inhibits localization of the oxidase to phagosomes without globally disrupting ROS production.

1.4.2 PrfA

L. monocytogenes has the ability to change from an extracellular bacterium propelled by flagella to an intracellular pathogenic bacterium that utilizes actin-based motility for cytoplasmic movement (Lemon et al., 2010). In *L. monocytogenes*, the virulence transcription factor PrfA regulates the switch between extracellular and intracellular lifestyles (Lemon et al., 2010**,** Bruno and Freitag, 2010**).** Transcriptional control of *prfA* is the first

mechanism used by *L. monocytogenes* to regulate the expression of virulence genes (Gray et al, 2006). Three promoter regions contribute to the regulation of *prfA* expression (Gray et al, 2006). Two of these, P*prfA*$_{P1}$ and P*prfA*$_{P2}$, are located upstream of the coding sequence of *prfA* and direct the production of single *prfA* transcripts (Gray et al, 2006). The other promoter (P*plcA*) directs a single *plcA* transcript as well as a bicistronic transcript encoding both *plcA* and *prfA* (Gray et al, 2006). The transcript of *prfA* directed by P*prfA*$_{P1}$ contains a thermosensitive structure that inhibits translation of PrfA at temperatures lower than 30°C, while at higher temperatures (37°C and above), the structure melts and allows translation to occur (Gray et al, 2006). P*prfA*$_{P2}$, which also directs single protein transcripts, contains a putative PrfA binding box, which provides an autoregulatory loop (Gray et al, 2006**;** Freitag, 2009).

1.4.3 *L. monocytogenes* Motility

L. monocytogenes has four to six peritrichous flagella per cell, each consisting of thousands of flagellin monomers modified by β-O-linked glycosylation (Schirm et al., 2004, Lemon et al., 2007). At 37°C, *L. monocytogenes* flagellar motility genes are transcriptionally repressed *in vitro* (Peel et al., 1987, Grundling et al., 2004, Way et al., 2004, O'Neil and Marquis, 2006). There was however, variation in down-regulation from strain to strain. The temperature-dependent regulation of motility and chemotaxis genes is partially attributed to the regulator of motility gene expression, MogR; deletion of this gene, resulted in virulence attenuation of the mutant in mice infected intravenously, while nonmotile mutants were not attenuated (Grundling et al., 2004, Way et al., 2004, Shen and Higgins, 2006). This means that at the internal temperature of mammalian host, 37°C, most *L. monocytogenes* strains do

not produce flagella and are non-motile. However, at 30°C and below, *mogR* is inhibited by the antirepressor, *gmaR*, which allows the flagellar genes to be transcribed resulting in *L. monocytogenes* motility at those temperatures (Peel et al., 1987, Shen et al., 2006, Lemon et al., 2007).

1.4.4 DEAD-box RNA Helicase Genes

DEAD-box RNA helicases are highly conserved with 12 characteristic sequence motifs present in all eukaryotic cells and many bacteria (Markkula et al., 2012, Vakulskas et al., 2014). DEAD-box proteins get their name from the shared amino acids, Aspartic acid (D)-Glutamic acid (E)-Alanine (A)-Aspartic acid (D), abbreviated DEAD for the single letter code of each amino acid. These enzymes unwind secondary RNA structures and are believed to be important during the growth of *L. monocytogenes* at low temperatures (Markkula et al., 2012 a, b, Netterling et al., 2012). In the *L. monocytogenes* EGD-e genome, there are four predicted DEAD-box RNA helicase genes (*lmo0866*, *lmo1246*, *lmo1450*, *lmo1722*) (Glaser et al., 2001, Markkula et al., 2012 a, b, Netterling et al., 2012). All four of the DEAD-box protein-encoding genes have a role in cold-temperature growth and deletion of three of the DEAD-box RNA helicase genes resulted in impaired growth at temperatures below 10°C (Azizoglu et al., 2010, Markkula et al., 2012 a,b, Netterling et al., 2012). The minimum growth temperatures were higher for three of the mutants (Δlmo0866, Δlmo1450, Δlmo1722) compared to the wild-type strain; in addition, motility of the DEAD-box RNA helicase mutants was impaired, indicating that those genes play a significant role both in cold tolerance and in motility of *L. monocytogenes* (Azizoglu et al., 2010, Markkula et al.,2012 a, b).

1.4.5 Biofilm Formation

Biofilm formation of *L. monocytogenes* has been a major concern for the food industry for years (Chae and Schraft, 2000). *L. monocytogenes* can easily contaminate food during processing because of its ability to adhere and persist on food contact surfaces (Farber and Peterkin, 1991, Chae and Schraft, 2000). It is believed that the primary reservoirs of *L. monocytogenes* in processing plants are the floors, drains, walls and air ventilation systems (Chae and Schraft, 2000). Biofilms are matrix-enclosed bacterial populations that are adherent to each other and/ that attach and proliferate on solid surfaces (Costerton et al., 1995, Chae and Schraft, 2000). It is now clear that biofilms constitute a distinct physiological state for bacteria that is profoundly different from the planktonic growth phase (Costerton et al., 1995). During adhesion, bacterial cells alter their phenotypes in response to the proximity of a surface (Costerton et al., 1995). During the initial stages of biofilm formation, immobile bacteria find themselves in a stable environment with cells of the same species and with those of differing species, as single-species and mixed-species microcolonies are formed (Costerton et al., 1995). This results in different biofilm bacteria responding to their specific microenvironmental conditions with different growth patterns, leading to a structurally complex, mature biofilm (Costerton et al., 1995). The ability of certain strains of *L. monocytogenes* strain to produce high levels of extracellular carbohydrates may contribute to biofilm formation (Chae et al., 2005). *L. monocytogenes* strains vary in their ability to produce biofilm after various incubation times (Chae and Schraft, 2001, Harvey et al., 2006).

1.4.6 Antimicrobial Resistance

Previous studies have shown that *L. monocytogenes* are naturally susceptible to penicillin, aminoglycosides, trimethoprim, tetracycline, macrolides, and vancomycin (Troxler et al., 2000, Swaminathan and Gerner-Smidt, 2007). There is reduced susceptibility or resistance to sulfamethoxazole, cephalosporin, and some quinolones (Troxler et al., 2000, Swaminathan and Gerner-Smidt, 2007). One major concern regarding *L. monocytogenes* is the potential for antibiotic resistance gene transfer from other *Listeria* species. It is recognized that the non-pathogenic *L. innocua* is a potential reservoir of antibiotic resistance (AB^R) for *L. monocytogenes* (Bertrand et al., 2005). The first documented AB^R in *L. monocytogenes* was reported in 1990 (Poyart-Salmeron et al., 1990). In that study, resistance genes to chloramphenicol, erythromycin, streptomycin, and tetracycline were transferred to other *L. monocytogenes* cells, enterococci-streptococci, and to *Staphylococcus aureus* (Poyart-Salmeron et al., 1990). It was suggested that the emergence of multiple-antibiotic resistance in *L. monocytogenes* is likely due to the transfer of replicons from enterococci-streptococci since the resistance genes that were transferred were closely related to plasmid-borne determinants that were common in enterococci-streptococci (Poyart-Salmeron et al., 1990). In a study by Bertsch et al. (2014), all the novel resistance elements examined were transferable from a *L. innocua* donor to *L. monocytogenes*. One of the antibiotics that were used in the study that was on the borderline for resistance was ampicillin, which is used to treat human diseases, suggesting the need to monitor the changes in antibiotic susceptibility of *L. monocytogenes* as often as possible (Bertsch et al., 2014). Resistance was not noticed for two other first-choice antibiotics, amoxicillin and gentamicin (Bertsch et al., 2014).

1.5 Factors that Influence Growth on Cantaloupe

1.5.1 Influence of Growth Temperature on Attachment of *L. monocytogenes*

The influence of strain and growth temperature on attachment of *Listeria* spp. on intact and cut cabbage was reported in 2006 (Ells et al., 2006). Twenty-four strains of *Listeria* were examined for their ability to attach and colonize to cabbage tissue. While all strains exhibited the ability to adhere and colonize both cut and uncut surfaces, some strains exhibited higher levels of attachment (Ells et al., 2006). There was no association between attachment levels and the species or serotypes of the strains suggesting that this attribution is individual and dependent on individual strains. There was a significant difference in the strength of attachment of cells during the first 4 h of exposure at differing temperatures (10, 22 and 37°C) (Ells et al., 2006). No differences were seen after 24 h at any temperature and at least 80 % of the attached cells remained on the cut cabbage after successive washes, regardless of temperature (Ells et al., 2006).

1.5.2 Behavior of *L. monocytogenes* on Cantaloupe Surfaces

The attachment of *Listeria* spp. to cantaloupe surfaces has been previously documented (Ukuku and Fett, 2002, Ukuku et al., 2004, Danyluk et al., 2014). In those studies, both the tissue of the cantaloupe as well as the rind of fresh cut cantaloupes was examined. It was determined that minimally processed fresh cut fruit provided a good substrate for microbial survival at temperatures higher than 4°C (Ukuku and Fett, 2002). When stored at 4 and 20°C for 15 d, *L. monocytogenes* could survive but exhibited a 1 to 2 $\log_{10}$ CFU/cm^2 decline by the end of the trials (Ukuku and Fett, 2002). It was also

determined that sanitation of the cantaloupe surfaces with chlorine (1,000 ppm) and hydrogen peroxide (5 %) were effective in reducing *L. monocytogenes* counts by 2.0- to 3.5 logs as well as other microorganisms (Ukuku and Fett, 2002).

It 2013, a model was constructed for growth of *L. monocytogenes* on cut cantaloupe and other melons and compared to previous models for *Salmonella* and *Escherichia coli* O157:H7 (Danyluk et al, 2013). The model predicted about a 4 log CFU increase of *L. monocytogenes* on cantaloupe following 15 d at 5°C and a 1 log CFU increase after 6 d at 4°C (Danyluk et al., 2013). The growth kinetics of *L. monocytogenes* has also been modeled predicting that at all temperature ranges (4 to 43°C), the population of both *L. monocytogenes* and the background microorganisms would begin to increase almost immediately with little to no lag phase for most of the growth curves (Fang et al., 2013).

1.5.3 Inhibition of *L. monocytogenes* by Natural Microbiota

It has been suggested that the natural microflora on the surface of whole cantaloupe and fresh-cut pieces are antagonistic towards the growth and survival of *L. monocytogenes* (Ukuku et al., 2004). It was shown that reducing the native microflora by means of sterilization of the surface of whole melons led to greater survival of *L. monocytogenes* at both 5 and 25°C (Ukuku et al., 2004). The inhibitory effect of natural microflora was also examined by Al-Zeyara et al. (2011). It was determined that under non-selective conditions, an inoculum of 10 cells of *L. monocytogenes*/100 ml was able to reach final concentrations above 10^3 CFU/ml only when the number of competing microbes were initially below 10^3 CFU/ml, and were inhibited when competing microbe concentrations were above 4.5 log CFU/ml (Al-Zeyara et al., 2011). In an earlier study, it was determined that *E. coli* when in

co-cultures with *L. monocytogenes* in liquid cultures inhibited *L. monocytogenes* but the converse was not true for *L. monocytogenes* inhibiting *E. coli* (Mellefont et al., 2008).

The inhibitory effect of competitive-exclusion microorganisms in biofilms on *L. monocytogenes* was discussed in a previous study (Zhao et al., 2004). It was determined that at 37°C, two yeast isolates were weakly inhibitory to *L. monocytogenes*, reducing growth by 0.7-$\log_{10}$ CFU/ml, while nine bacterial isolates of *Enterococcus durans*, *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus plantarum*; were strongly inhibitory, resulting in a >5-$\log_{10}$ CFU/ml reduction within 24 h when compared to the positive control of *L. monocytogenes* alone (Zhao et al., 2004). It was also determined that at 4, 8 and 16°C at least four of the bacterial isolates were highly inhibitory at each of the temperatures. Six of the isolates were *Enterococcus durans*, two were *Lactococcus lactis* subsp. *lactis*, and one was *Lactobacillus plantarum* (Zhao et al., 2004).

1.5.4 Removal of Bacteria from Cantaloupe Surfaces

It was previously determined that the use of thermal processes is useful in the inactivation of pathogenic organisms on the surface of cantaloupe. One study looked at the inactivation of *Salmonella* on cantaloupe using hot water (Solomon et al., 2006). It was determined that whole melons that were treated with water at 85°C for 60 and 90 s resulted in reductions of up to 4.7 log CFU/cm^2 (Solomon et al., 2006). It was also noted that regardless of the temperature of the water used, the temperature of the flesh 10 mm from the surface of the rind remained at least 40°C cooler than the surface temperature of the melon (Solomon et al., 2006). This suggests that the hot water can penetrate the interior of the melons to only 10 mm, leaving the flesh of the melons relatively unchanged. These results are similar to the

finding of a study on the effect of hot water treatment (75°C for 1 min) on microbial and sensory quality of cantaloupe and transfer of *Escherichia coli* O157:H7 during cutting (Selma et al., 2006). Again, the use of hot water was both effective on reducing microbial growth as well as maintaining the sensory quality of the melon.

The effect of gaseous ozone was also examined on the microbial and sensory quality of cantaloupe during cutting (Selma et al., 2006). It was determined that at a concentration of 10,000 ppm for 30 min, gaseous ozone was effective at reducing mesophilic and psychrotrophic bacteria, molds and coliforms by 3.8, 5.1, 2.2 and 2.3 logs, respectively (Selma et al., 2006). A combination of gaseous ozone and hot water was effective in reducing total microbial populations.

1.6 Competitive Advantages of *L. monocytogenes*

1.6.1 Survival in Low Temperatures

L. monocytogenes has the ability to survive and grow over a wide range of temperatures, including refrigeration temperature (Gandhi et al., 2007). This ability to survive at such a board range, especially refrigerated temperatures, makes *L. monocytogenes* a persistent problem for food processors. When temperatures change, *L. monocytogenes* is able to respond in various ways. One of the responses is to increase the proportion of fatty acid chains ($C_{15:0}$ at the expense of $C_{17:0}$) when the temperature is below the optimum range (>30°C) (Gandhi et al., 2007). When grown at lower temperatures, there is an increase in the degree of unsaturated fatty acids (Gandhi et al. 2007). This helps to enhance the fluidity of the membrane.

When *L. monocytogenes* is exposed to lower temperatures, it produces cold shock proteins (Csps) in response to the temperature downshock and acclimation proteins (Caps) which are synthesized during balanced growth at low temperatures (Bayles et al., 1996, Gandhi et al., 2007). When subjected to cold shock, 12 Csp proteins were induced in cold-shock cultures, while about four Caps proteins were produced during balanced growth at 5°C (Bayles et al., 1996).

In all bacteria, survival in adverse conditions depends on the changes in the transcription of genes made possible by the association with alternative sigma factors with the core RNA polymerase. In *L. monocytogenes,* the sigma factor sigmaB (σ^B) is induced in response to temperature downshifts (Gandhi et al., 2007).

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CHAPTER 2

The Effect of the Inactivation of *hly* and *prfA* on Attachment, Growth and Survival of *Listeria monocytogenes* on the Surface of Cantaloupe

Abstract

In 2011, *Listeria monocytogenes* was implicated in a major multistate outbreak of listeriosis in the United States involving whole cantaloupe. To date, the genetic features of *L. monocytogenes* that allow adherence and growth on produce remain largely uncharacterized. In this study, we characterized two non-hemolytic mutants of one of the outbreak strains, 2011L-2858 (serotype 1/2b) for attachment, growth and survival on the surface of cantaloupe rind. One mutant, B2G6, harbored a single *mariner*-based transposon insertion in *hly*, encoding the hemolysin Listeriolysin O while the other, J2E3, harbored a single insertion in *prfA*, encoding a positive regulator for *hly* and numerous other virulence genes. Inactivation of either the *hly* or *prfA* gene did not negatively impact growth or survival of the bacteria on cantaloupe rind at either 25 or 37°C.When in mixed cultures with the wild-type parent strain, neither of the mutants exhibited a significant competitive disadvantage compared to the wild-type strain. The findings suggest that under the conditions of this study, these key virulence determinants of *L. monocytogenes* are not critical for the ability of this pathogen to grow on cantaloupe.

INTRODUCTION

Listeria monocytogenes is a facultative intracellular pathogen that causes a disease (listeriosis) associated with severe symptoms and high mortality in humans. Of the three major serotypes (1/2a, 1/2b, 4b) associated with human listeriosis, serotype 4b has been most commonly linked to listeriosis outbreaks, with serotypes 1/2a and 1/2b being less common (Gasanov et al, 2005, Kathariou, 2002, Lee et al., 2012). However, in 2011, a multistate listeriosis outbreak in the US involving whole cantaloupe was determined to be associated with strains of serotype 1/2a and 1/2b. This outbreak resulted in 147 reported cases of listeriosis, 33 deaths and 1 miscarriage (CDC, 2015). This listeriosis outbreak was the first involving cantaloupe and the largest reported to date (McCollum et al, 2013).

The genetic features of *L. monocytogenes* that allow it to adhere and grow on produce remain largely uncharacterized. One of the questions that remain to be addressed is the extent to which virulence determinants play a role in the ability of the pathogen to colonize, survive and grow on produce. The hemolysin, Listeriolysin O (LLO), and PrfA, a transcriptional activator for *hly* and numerous other virulence genes, are key virulence determinants of *L. monocytogenes*, extensively characterized for their roles in host-pathogen interactions (Hamon et al., 2012). However, their impact in environmental adaptations of *L. monocytogenes* outside of the host remains poorly understood, and no information is available on whether these virulence determinants impact the pathogen's ability to colonize fresh produce. The objective of the current study was to characterize the impact of these virulence determinants on the ability of the serotype 1/2b *L. monocytogenes* strain 2011L-

2858, associated with the 2011 cantaloupe outbreak, to adhere and grow on the surface of cantaloupe.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in Table 1. Mutant libraries were constructed in the serotype 1/2b strain 2011L-2858, isolated from cantaloupe implicated in the 2011 cantaloupe outbreak (CDC, 2015, McCollum et al. 2013) and hereafter referred to as strain 2858. *L. monocytogenes* was routinely grown in brain heart infusion (BHI) broth (Becton, Dickinson & Co., Sparks, MD). Agar media used were brain heart infusion agar (BHIA, BHI with 1.2% Bacto-agar, Becton, Dickinson & Co.). When needed, erythromycin (Em**,** MP Biomedicals, Solon, Ohio) was added at 5 µg/ml in BHI (BHI-Em5) or in BHIA (BHIA-Em5) and kanamycin (Km, (Fisher Scientific, Fair Lawn, NJ) was added at 10 µg/ml to BHI (BHI-Km10) and BHIA (BHIA-Km10). *L. monocytogenes* strains were grown in BHI at 37°C in standing cultures and preserved at -80°C in BHI with 20% glycerol.

Cantaloupe Rind Adherence and Growth Comparison

Overnight cultures of strain 2858 and the non-hemolytic mutants B2G6 and J2E3 were grown in 5 ml of BHI broth at 37°C for approx.16 h to a concentration of approx. 10^8 CFU/ml. The overnight cultures were then centrifuged for three min at 16,000 rpm. The supernatant was discarded and the bacterial pellet was suspended in 5 ml of sterile deionized water. This process was repeated twice to ensure that nutrient media used was washed off to

prevent the strains from surviving off of any residual nutrients. The inoculum was prepared by inoculating 5 ml of BHI with 50 µl of the washed cell suspension.

The cantaloupes used in this study were purchased from local grocery stores in Raleigh, NC. The majority of the cantaloupes purchased were imported from the Honduras or from Guatemala during winter months and from North Carolina, Georgia, and California during summer months. At retail cantaloupes were displayed at room temperature and were kept at room temperature during transportation to the laboratory where they were used within 2 h of purchase. Each cantaloupe was examined before use to ensure absence of external bruises, abrasions, cuts or mold growth before use. The cantaloupe rind was then removed from the flesh using an alcohol-sterilized knife and cut into 5x5x1cm fragments. The rind fragments were placed rind side up in groups of four into labeled, sterile petri dishes. The rind surface of each fragment was sprayed with sterile deionized water to allow the bacterial cultures to better spread over the fragments and prevent the bacterial cells from drying out on the fragments. Each fragment was spot inoculated in 10 evenly separated droplets of 10 µl each (total inoculum 100 µl, corresponding to 10^4 CFU/fragment); two fragments were inoculated for each time point, with several fragments similarly spotted with sterile water as control. The 10^4 CFU/fragment inoculation of the fragments was achieved by making a 1:100 dilution of the original washed cell suspension (50 µl original culture into 5 ml deionized water), corresponding to 10^6 CFU/ml, followed by spot inoculating 100 µl of the 10^6 CFU/ml inoculum. The petri dishes were sealed with parafilm and stored at either room temperature (25°C) or 37°C.

To assess growth, *Listeria* on the fragments were enumerated 30 min after inoculation, and at 24, 48, and 72 h for fragments stored at room temperature or at 30 min and 24 h for those incubated at 37°C. For each time point, two inoculated cantaloupe fragments per strain were individually placed in 50 ml Falcon™ conical centrifuge tubes (Corning Life Sciences) and washed in 10 ml of sterile deionized water for two min by vortexing at top speed. Dilutions of the rinse were then plated on BHIA (to determine total aerobic plate counts) as well as modified Oxford *Listeria* selective agar (MOX, Oxoid, Hampshire, UK) (to enumerate *Listeria*) and incubated at 37°C for 24 h and 48 h for BHIA and MOX, respectively. Each mutant/wild type comparison for adherence and growth was done in at least three independent trials.

Adherence assays were done by Victor Jayeola at the Kathariou laboratory, as described (Martinez et al., 2016). Briefly, cantaloupe was cut into 2x2x0.1 cm fragments and inoculated with approx. 10^5 CFU/ml of washed cells and harvested at stationary phase. The fragments were allowed to dry inside the biosafety cabinet for 1 h, placed inside petri-dishes, and then incubated at room temperature or 37°C for 1 h. Loosely associated cells were removed by rinsing and tightly adhered cells removed by vortexing. Three fragments per strain were processed for adherence in at least three independent trials. The *Listeria* populations from the fragments were enumerated on MOX.

Competitive Fitness Assessments

Mixed-strain inoculations were prepared by adding 25 µl of washed cells of the parental strain and 25 µl of washed cells of the mutant strain into 5ml of sterile deionized water. To enumerate the mutant CFUs from cantaloupe fragments inoculated with these 1:1

mixtures of the mutants and parental strains, approximately 50 colonies from the MOX plates were selected and streaked on blood agar (Remel, San Diego, CA). The plates were incubated at 37°C for 36 h and the ratio of hemolytic and non-hemolytic cultures was determined by visual observation for zones of hemolysis. Alternatively, colonies from the MOX plates were selected and either streaked directly onto BHIA-Em5 or inoculated into 96-well plates containing 200 µl of BHI and incubated for approx. 16 h. The 96-well cultures were then stamped using a sterile microplate replicator onto BHIA-Em5. The ratios of parental and mutant cultures were determined by visual observation for growth on the BHIA-Em5 plates. Each competitive fitness assay was done in at least three independent trials.

In Vitro Growth Comparison of Strain 2858 and Non-hemolytic Mutants

The parental strain 2858 and the two non-hemolytic mutants used in this study, B2G6 and J2E3, were grown in 5 ml of BHI at 37°C for approx. 16 h to a concentration of approx.10^8 CFU/ml. The cultures (4 µl) were inoculated into individual wells of a 96-well plate (200 µl of BHI / well), with each strain tested in triplicate. The 96-well plate was incubated in a BioTek microplate reader (BioTek Instrument, Inc., Winooski, VT) and at either 28°C or 37°C with optical density at 630nm (OD_{630}) measurements taken at 1 h intervals with 10 s of shaking before each measurement. Growth rates were reported relative to the optical density of sterile BHI broth, used as a control. Individual growth curves were processed in sequential sets of n data values, as previously described (Breidt et al, 1994).

Biofilm Formation of 2858, J2E3, and B2G6

Biofilm assays were contributed by Jeff Niedermeyer in the Kathariou lab. Overnight cultures were prepared by inoculating 5 ml of TSB broth with an individual colony of each of

the strains used in the assay followed by incubation at 37°C for approx. 18 h. The overnight cultures were then washed and resuspended in sterile deionized water. A 1:50 dilution of the washed cells was filled in a 96-well plate by filling the plate with 196 µl of TSB broth and 4 µl of each strain for a total volume of 200 µl per well. The 96-well plate was then incubated at 37°C for 48 h without agitation. After 48 h, the culture in each well was pipetted out and washed three times with 200 µl of deionized water and then allowed to air dry for approx. 45 min. Filter-sterilized crystal violet diluted to a concentration of 0.8 % with sterile deionized water was added to each culture. The cultures were then left at room temperature for 45 min followed by the removal of the crystal violet from the wells. The wells were then rinsed five times with 200 µl of sterile deionized water per wash. The plate was then tapped dry and followed by the addition of 200 µl of 95 % ethanol to the wells. The plate was incubated at room temperature for 15 m before transferring the ethanol from the wells into a new plate. Finally, the absorbance was measured at a wavelength of 590 nm.

Statistical Analysis

The optical densities of each of the strains were entered into a custom algorithm created by F. Breidt using Matlab software (The Mathworks, Inc., Natick, Mass.) to derive the relative growth rates and standard errors for each strain. The growth rates of the individual strains were fit into a General Linear Model (GLM) of SAS (SAS Institute, Cary, NC) to compute the analysis of variance. A General Linear Model was also fit to the values of the CFU/fragments for each of the strains inoculated individually on cantaloupe as well as the mixed culture ratios from the surface of the cantaloupe.

RESULTS

Screening of approximately 1900 transposon mutants of strain 2858 on blood agar plates revealed three that were non-hemolytic (C. Parsons and B. Costolo, unpublished findings). Two harbored a transposon insertion in *hly*, while in the third the transposon was localized in *prfA*. Mutants B2G6 and J2E6 harboring an insertion in *hly* (nt 225) and *prfA* (nt 65), respectively, were chosen for further work. Southern blot data confirmed that each of these mutants harbored a single insertion of the transposon (C. Parsons, unpublished findings; data not shown).

Inactivation of *hly* or *prfA* does not Impact Growth or Relative Fitness of *L. monocytogenes* on Cantaloupe Rind at 25°C or 37°C.

Un-inoculated cantaloupe fragments were negative for *Listeria* spp. throughout the study. Determinations of aerobic plate counts (APCs) on the inoculated and un-inoculated fragments indicated that each fragment harbored on the average 10^5 APC CFU prior to inoculation with a 4 log increase after 24 h at 25°C followed by further increases after 48 and 72 h (Fig. 2.1A). Similar APC increases were observed in inoculated fragments (Fig. 2.1A).

Initial *Listeria* spp. counts on MOX were approx. 10^4 CFU/fragment for each strain. After 24 h *Listeria* CFU/fragment increased by approx. 2 logs, with further increases noted after 48 and 72 h (Fig. 2.1B). Growth of the *hly* mutant B2G6 and the *prfA* mutant J2E6 was similar to that of the parental strain 2858 (Fig. 2.1B).

To determine potential impacts of the mutations on the relative fitness of the bacteria on cantaloupe, the rind was inoculated with 1:1 mixtures of the parental strain and each of the

mutants and incubated at 25°C. Screening of *Listeria* colonies from the MOX plates on blood agar plates revealed that for both mutants the ratio of parental strain to mutant remained around 50 % at all tested time points, suggesting that relative fitness of the mutant on the cantaloupe did not differ significantly from that of the parental strain (Fig. 2.2).

Initial *Listeria* spp. counts on MOX at 37°C were approx. 10^6 CFU/fragment for each of the strains individually as well as for the mixed cultures. After 24 h, *Listeria* CFUs/fragment increased by approx. 3 logs, with similar increases noted for the *hly* and *prfA* mutants and the parental strain (Fig. 2.3).

To determine potential impacts of the mutations on the relative fitness of the bacteria on cantaloupe at 37°C, the rind was inoculated with 1:1 mixtures of the parental strain and each of the mutants and incubated at 37°C. Screening of *Listeria* colonies from the MOX plates on blood agar plates revealed that for each mutant the ratio of parental to mutant remained around 50 % at the tested time points, suggesting that relative fitness of the mutant on cantaloupe did not differ significantly from that of the parental strain (Fig. 2.4).

Inactivation of *hly* Impacts Adherence of *L. monocytogenes* on Cantaloupe Rind at 25°C and 37°C, while *prfA* Inactivation has no Effect on Either Temperature

To determine potential impacts of the mutations on the initial adherence of *L. monocytogenes* on cantaloupe, the rind was inoculated with 10^5 CFU/ml of the parental strain as well as each of the mutants and incubated at both 25°C and 37°C. At 25°C, strain B2G6 had a statistically significantly difference from both the 2858 and J2E3 strain while there was no statistical difference between 2858 and J2E3 (Fig. 2.5A). Similar results were observed at 37°C, with strain B2G6 being significantly different from 2858 and J2E3 while 2858 and

J2E3 showed no difference (Fig. 2.5B). At 25°C, the average percentage of adherent cell per inoculated cells of B2G6 was approximately 37.8% while the percentages of 2858 and J2E3 were 24.2% and 26.1% respectively. This yielded a statistical difference of $p<0.0001$ between B2G6 and 2858 and J2E3. At 37°C, B2G6 percentage was 38.9% while 2858 and J2E3 were 22.1% and 27.0% respectively. The statistical analysis of the adherence show that there is significance difference ($p< 0.0001$ and 0.001 respectively) between B2G6 and 2858 and J2E3.

Inactivation of *hly* or *prfA* does not Impact Growth of *L. monocytogenes in vitro* at Either Temperature

Each of the mutants grew similarly to the parental strain in liquid laboratory media at 28 or 37°C (Fig. 2.6). At 28°C, average growth rates for 2858, B2G6 and J2E3 were 0.223, 0.226 and 0.222 h, respectively. As expected, growth rates were shorter at 37°C (0.136, 0.149 and 0.138 for 2858, B2G6 and J2E3, respectively), but were not significantly different from each other. At 37°C, the growth rates of all three strains were similar, while at 28°C, the B2G6 strain appeared to grow at a faster rate than the parental strain. Based on the statistical analysis of the growth rates at 37°C, there was no significant difference ($p> 0.05$) between the growth rates of the two hemolytic mutants and the parental strain. The statistical analysis of the mutant strains compared to the parental strain at 28°C show that there is significant difference ($p <0.0001$) between the growth rates of the B2G6 mutant compared to the parental strain and J2E3.

Inactivation of *hly* and *prfA* Significantly Impacts the Biofilm Formation of *L. monocytogenes*

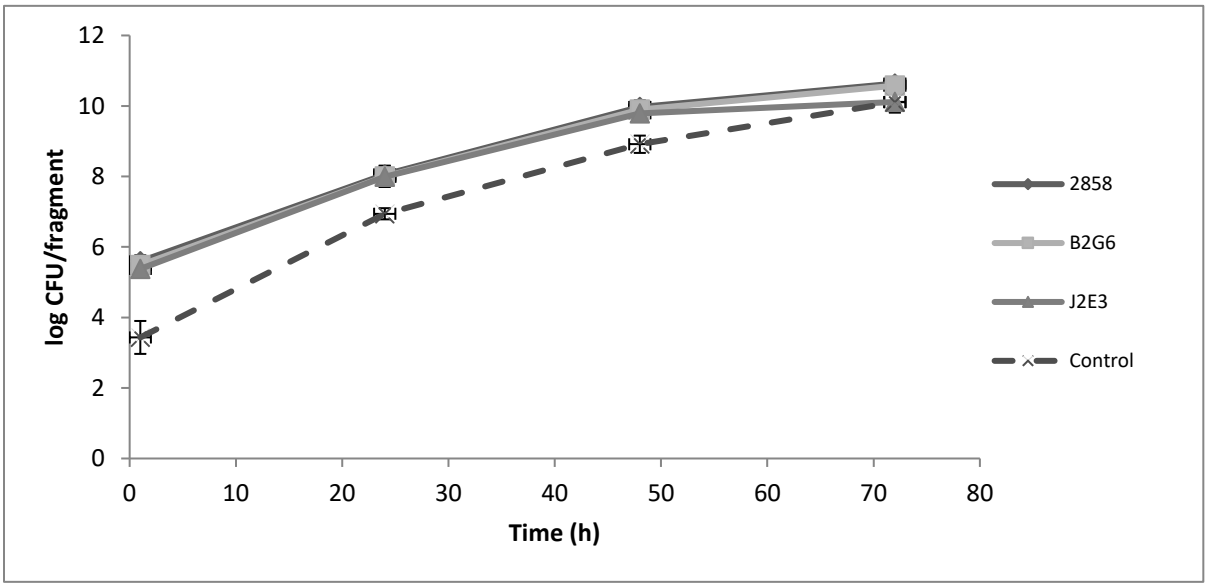
The parental strain showed statistical differences in regards to biofilm formation compared to the both the *hly* and *prfA* mutant (Fig. 2.7). The average absorbance at 590 nm for 2858 was 0.31, while the average absorbance for *hly* and *prfA* were 0.26 and 0.23, respectively. 2858 was significantly different from B2G6 and J2E3 with p-values of 0.013 and 0.0001, respectively.

Table 2.1: *L. monocytogenes* strains used in this study

Strains	**Genotype and Features**	**Transposon insertion**	**Phenotype**
2858	Serotype 1/2b strain from 2011 cantaloupe outbreak	-	Hemolytic, erythromycin susceptible (Em^S)
B2G6	Transposon mutant of 2858 in *hly*	*hly (*Listeriolysin O; nt 225)	Non-hemolytic, erythromycin resistant (Em^R)
J2E3	Transposon mutant of 2858 in *prfA*	*prfA* (nt 65)	Non-hemolytic, Em^R

Fig. 2.1: (**A**) APC CFU/fragment from BHIA plates after incubation at 25°C and (B) *Listeria* counts from MOX plates after incubation at 25°C. Data are averages from duplicate fragments, and from five independent trials.

(A)



(B)

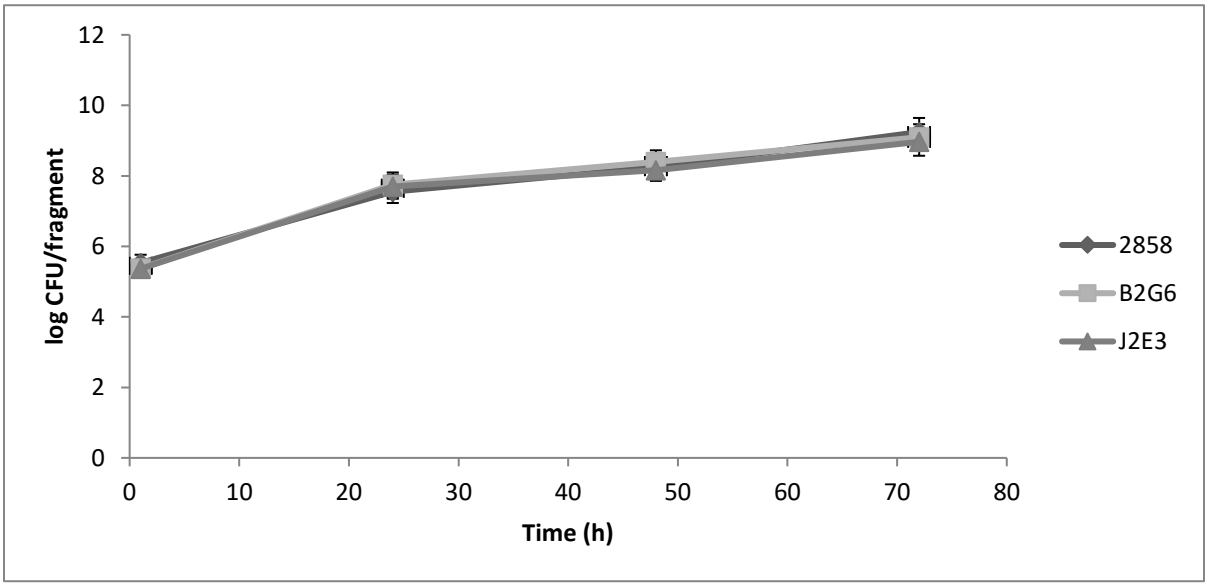


Fig. 2.2: Average of ratios from competitive fitness of 2858 parental strain vs. (A) *prfA* mutant J2E3 and (B) *hly* mutant B2G6 inoculated in 1:1 mixtures on cantaloupe fragments, in duplicate and from three independent trials, incubated at 25°C. Ratios were determined by sub-culturing individual colonies on plates with and without erythromycin, as described in Materials and Methods. Time '0' corresponds to 30 m after inoculation.

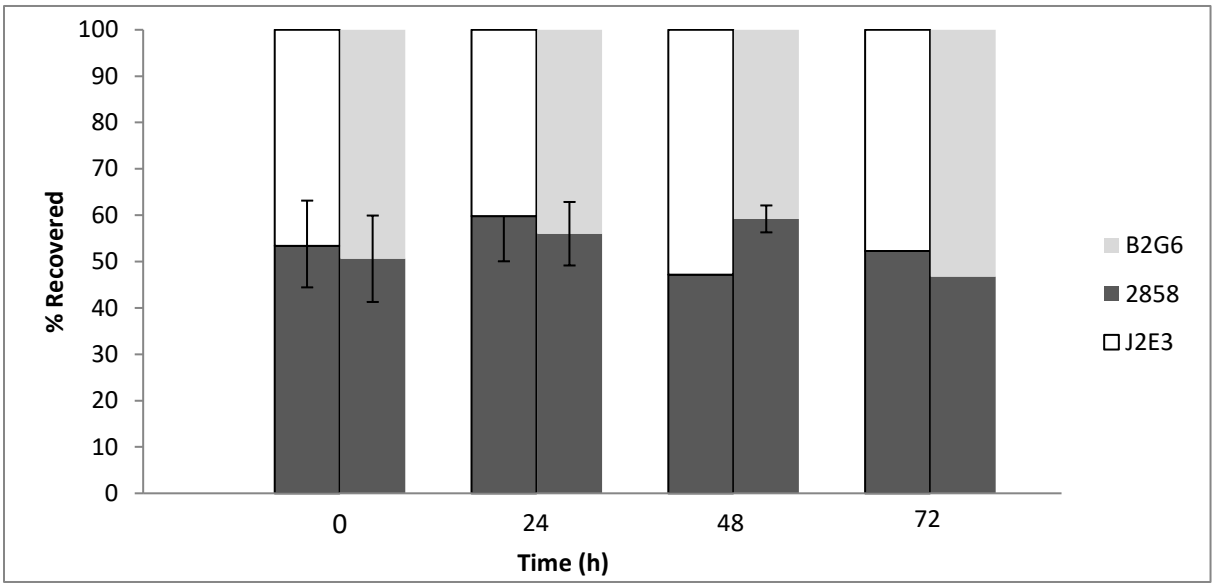


Fig. 2.3: Average of *Listeria* counts from MOX plates after incubation at 37°C.

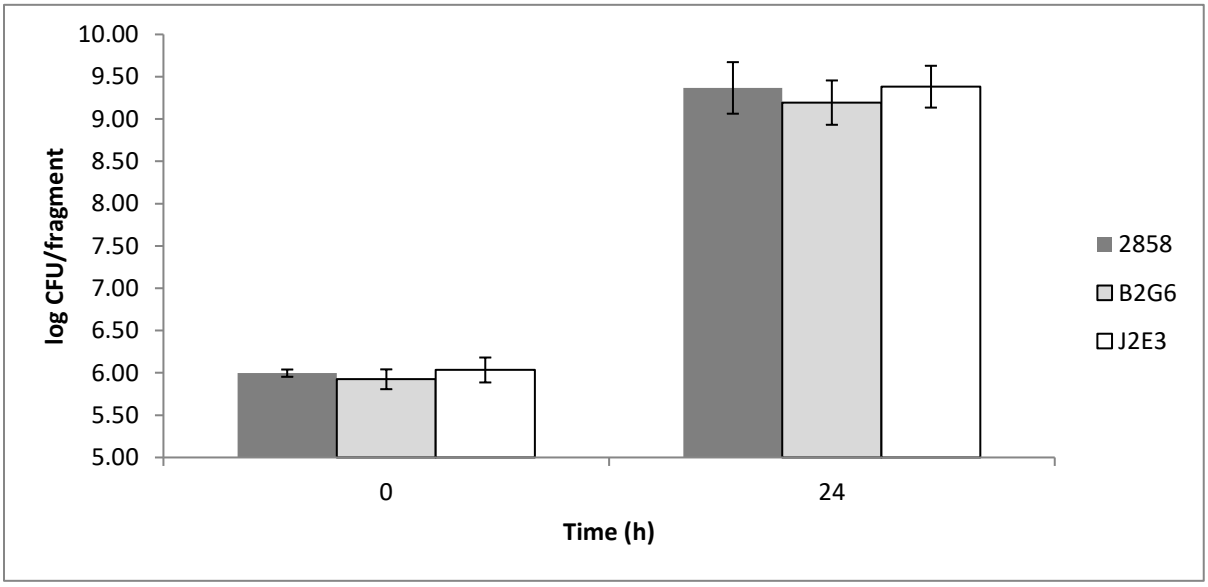


Fig. 2.4: Average of ratios from competitive fitness of 2858 parental strain vs non-hemolytic mutant (A) J2E3 and (B) B2G6 on cantaloupe fragments, in duplicate from three independent trials, incubated at 37°C over 24 h. Colonies from MOX plates were subculture in individual wells of 96-well plates which were then plated on media with and without erythromycin, as described in Materials and Methods.

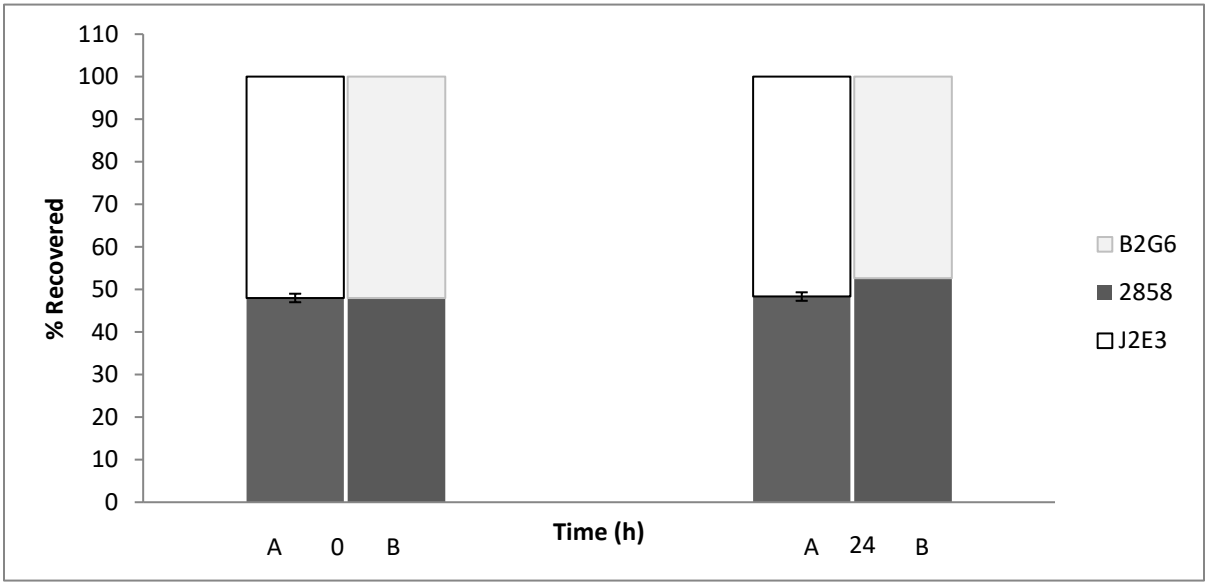
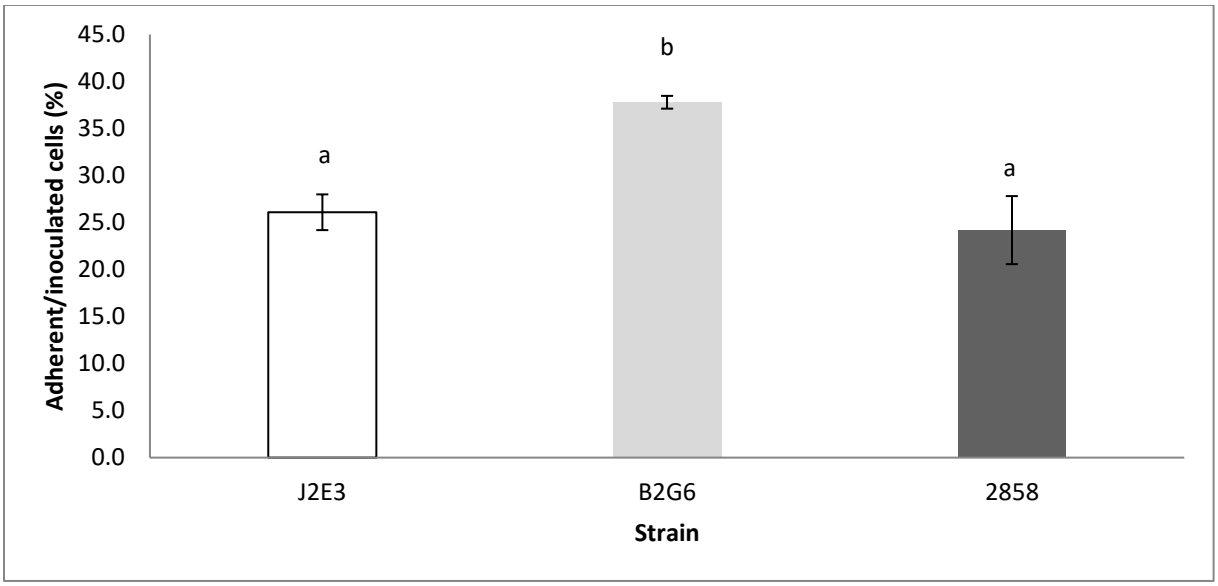


Fig. 2.5: Percentage of adherent/inoculated cells from MOX plates after incubation at (A) 25°C for 1 h and (B) 37°C for 1 h. Data is average from triplicate fragments from two independent trials. Lower case letters indicate statistically significant differences.

(A)



(B)

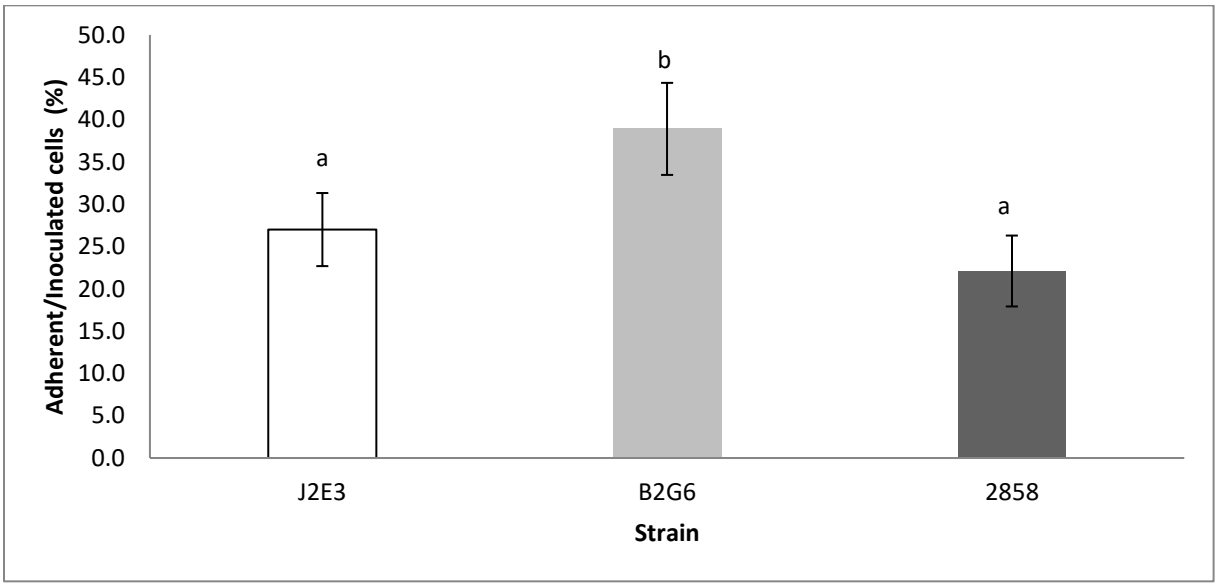
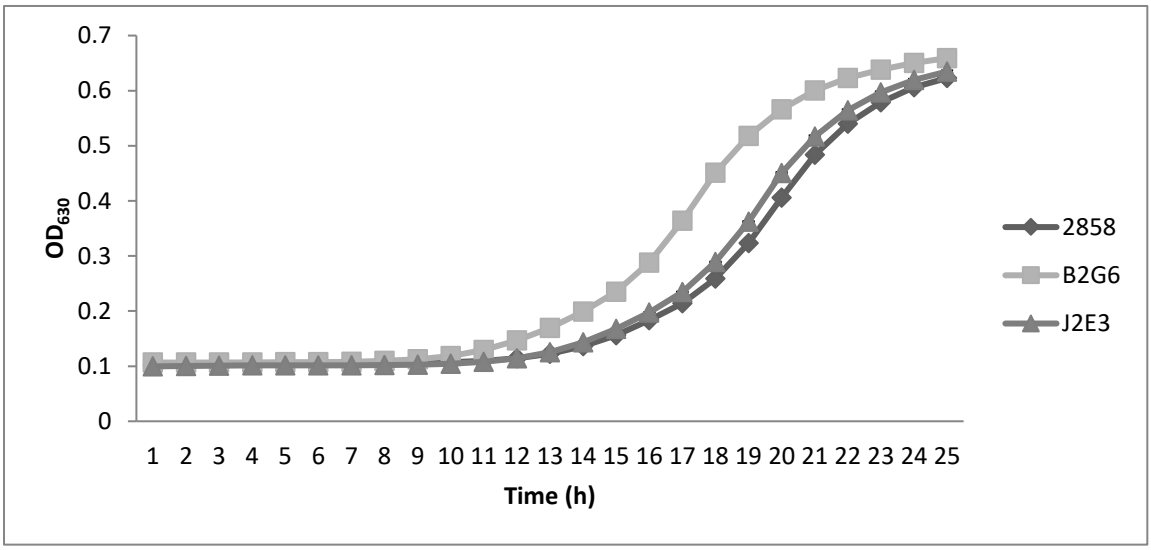


Fig 2.6: Growth of non-hemolytic mutants (B2G6 and J2E3) compared to parental strain (2858) in BHI grown at (A) 28°C and (B) 37°C. A_{630} was monitored at hourly intervals using a BioTek microplate reader, as described in Materials and Methods.

(A)



(B)

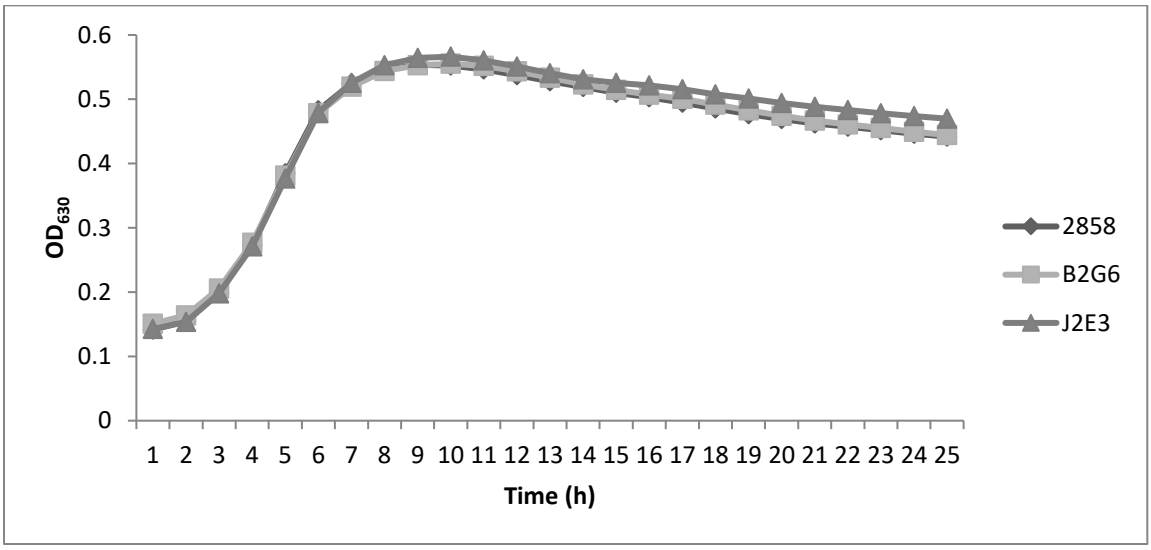
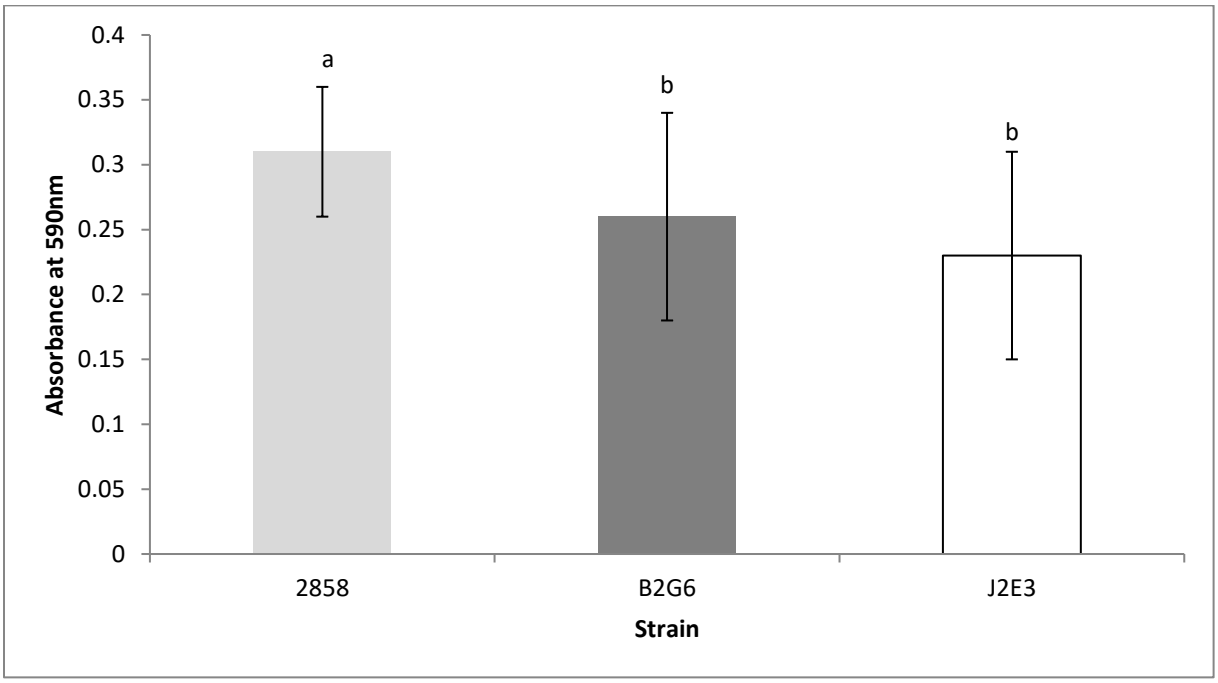


Fig. 2.7: Biofilm formation of mutants B2G6 and J2E3 compared to parental strain 2858. A_{590} was monitored using a BioTek microplate reader, as described in Materials and Methods. Lower case letters indicate statistically significant differences.



DISCUSSION

In this study, we show that inactivation of the major *L. monocytogenes* virulence determinants *hly* and *prfA* did not affect survival or growth of the *L. monocytogenes* cantaloupe outbreak strain 2858, on the surface of cantaloupe. Our initial experiments were performed at room temperature, since this is more likely to simulate storage conditions for whole cantaloupe at retail. However, both *hly* and *prfA* are thermoregulated genes, with expression induced at 37°C (Gray et al., 2006). To exclude that the lack of impact of the mutations at 25°C was due to the fact that the genes might not be expressed at this temperature, inoculated fragments were also incubated at 37°C, temperature permissive for

expression. At 37°C, the mutants were similar to the parental strain in their growth on the cantaloupe.

The adherence of the parental strain and the two non-hemolytic mutants at both 25°C and 37°C yielded similar results. At both temperatures, B2G6 produced statistically significantly higher adherence percentages of adherent cells per total inoculated cells compared to either 2858 or J2E3 while 2858 and J2E3 displayed no statistical difference. These data suggest that *prfA* had no significant impact on the adherence of *L. monocytogenes* on cantaloupe while there was an impact of the mutation of *hly* on adherence. However, since the inactivation of *hly* resulted in enhanced adherence, the adherence date suggests that *hly* is not necessary for *L. monocytogenes* adherence and may in fact suppress it.

The *in vitro* growth comparison of the parental strain and the two non-hemolytic mutants used in this study further supported the results of the cantaloupe assay. However, there is no significant difference ($p > 0.05$) between the parental strain and J23E mutant. This suggests that the B2G6 mutant grows at a faster rate in nutrient media at 28°C compared to the parental strain and J2E3 and that the inactivation of the *hly* gene has no negative effect on the growth of *L. monocytogenes*. These data are similar with the data obtained from the adherence assay in that B2G6 had better initial adherence to the cantaloupe at both 25 and 37°C than the parental strain or J2E3. This suggests that the lack of the *hly* gene improves the adherence and growth of *L. monocytogenes*, which could further emphasize that *hly*, is not responsible for cell adherence, growth or survival. The lack of significant differences between the parental strain and J2E3 also suggests that the inactivation of *prfA* has no detectable effect on growth of *L. monocytogenes*.

A previous study that examined the effect of the deletion of *prfA* on biofilm formation determined that the *prfA* mutant (NF- L1123) had decreased biofilm formation at room temperature, 30°C and 36°C compared to its parental strain (Lemon et al., 2010). It was also determined that the growth of the NF-L1123 in broth cultures were comparable to the growth of the wild type (Lemon et al., 2010). Similarly, the results in our study also suggest that the inactivation of *prfA* has minimal to no effect on the growth of *L. monocytogenes* in vitro. The *prfA* mutant of 2858 did appear to have decreased biofilm formation just as with the NF-L1123 mutant (Lemon et al., 2010). There were also significant decreases in biofilm formation by the *hly* mutant. It must be pointed out that while the *hly* mutant had decreased biofilm formation compared to the parental strain, there were increases in adherence of the cells compared to the parental strain. At this time, there is no explanation for this relationship as it is not seen in the *prfA* mutant as well. It can be suggested based on the results obtained from this study that any potential negative effects the inactivation of *hly* and *prfA* has on 2858's biofilm formation, those effects do not interfere with 2858's ability to survival and grow on cantaloupes.

It can be suggested that the presence and growth of the background microbes on cantaloupe rind had similar impact on the growth of the mutants and the parental strain, while, the growth of *L. monocytogenes* had no detectable impact on the increase in APCs. However, possible impacts on background microbes that were not assessed via APC determinations remain unknown. In the current study, relatively high numbers of *L. monocytogenes* were employed as inoculums. However, even at lower inoculum levels, (10^2 and 10^3 CFU/fragments) LM showed comparable increases (Appendix figure C.1), further

suggesting that the native microbes on the surface of cantaloupe did not limit growth of *L. monocytogenes* on cantaloupe rind.

The absence of any significant differences between 2858 and its *hly* or *prfA* mutants suggest that *hly* or *prfA* did not play a major role in the growth and survival of this particular outbreak strain of *L. monocytogenes* on the surface of cantaloupe rind. Based on this, it is necessary to identify other *L. monocytogenes* genes that may have contributed to the 2011 outbreak.

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CHAPTER 3

Adherence, Growth and Survivability of Motility-Impaired *Listeria monocytogenes* Mutants on Cantaloupe Rind

Abstract

Listeria monocytogenes is a food borne pathogen implicated in a 2011 multistate outbreak associated with cantaloupe. This was the largest listeriosis outbreak to date and the first to involve cantaloupe (McCollum et al., 2013). To date, the genetic features of *L. monocytogenes* that allow adherence and growth on produce remain largely uncharacterized. In this study, two non-motile transposon mutants of one of the outbreak strains, 2011L-2858 (serotype 1/2b), were characterized for attachment, growth and survival on the surface of cantaloupe rind. One of the mutants, L1E4, harbored an insertion in the DEAD-box RNA helicase gene (*lmo0866* homolog), while the other, M1A5, harbored an insertion in a gene from a flagellum biosynthesis and chemotaxis gene cluster. When inoculated alone, neither mutant was significantly impaired in growth or survival on the cantaloupe at either 25 or 37°C. However, when co-inoculated with the wildtype parental strain, the RNA helicase mutant L1E4 had a clear competitive disadvantage relative to the parental strain while the relative fitness of M1A5 was not noticeably impacted. Genetic complementation of L1E4 with the intact RNA helicase gene restored relative fitness on cantaloupe. The findings suggest that the DEAD Box RNA helicase encoded by the *lmo0866* homolog is critical for relative fitness of *L. monocytogenes* on cantaloupe.

INTRODUCTION

Listeria monocytogenes is a facultative intracellular microorganism that causes the disease listeriosis, which is associated with severe symptoms and high mortality in humans. Of the three serotypes (1/2a, 1/2b, 4b) associated with the majority of cases of listeriosis, serotype 4b is most commonly linked to outbreaks, with serotypes 1/2a and 1/2b less commonly implicated. However, in 2011, a major multistate listeriosis outbreak in the United States associated with whole cantaloupe was found to be associated with strains of serotype 1/2a and 1/2b. This was largest listeriosis outbreak to date and the first to involve cantaloupe (McCollum et al., 2013).

To date, the genetic features of *L. monocytogenes* that allow this organism to adhere and grow on the surface of produce remain largely uncharacterized. One feature examined in this study was the potential impact of motility. In *L. monocytogenes*, there are four to six flagella per cell with production of flagella being temperature-dependent. At 37°C, most *L. monocytogenes* strains do not produce flagella and are non-motile due to MogR repression of flagellin gene transcription, while at 30°C and below, *L. monocytogenes* is motile due to the inhibition of MogR by GmaR (Lemon et al., 2007).

The impact of motility on the ability of L. *monocytogenes* to adhere to surfaces and form biofilms remains poorly understood. What is known is that *L. monocytogenes* is able to easily contaminate food during processing because of its ability to adhere and persist on food contact surfaces (Farber and Peterkin, 1991, Chae and Schraft, 2000). It is also suggested that

the ability of *L. monocytogenes* to produce high levels of extracellular carbohydrates may contribute to biofilm formation (Chae et al., 2006). Limited information is currently available on the potential role of motility in colonization of fresh produce. Currently, studies have shown that minimally processed fresh-cut fruit provided a good substrate for microbial growth at temperatures higher than 4°C (Ukuku and Fett, 2002). When stored at 4 and 20°C for 15 d, *L. monocytogenes* was able to survive but exhibited a 1 to 2 $\log_{10}$ CFU/cm^2 decline by the end of the trials (Ukuku and Fett, 2002). The objective of this study was to characterize two *mariner*-based non-motile mutants of L. *monocytogenes* 2011L-2858, a serotype 1/2b strain from the 2011 cantaloupe outbreak, regarding their colonization potential and fitness on cantaloupe rind.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in Table 1. Mutant libraries were constructed in the serotype 1/2b strain 2011L-2858 (hereafter designated as strain 2858), isolated from cantaloupe implicated in the 2011 cantaloupe outbreak (CDC, 2012, McCollum et al., 2013). *L. monocytogenes* was routinely grown in brain heart infusion broth (BHI) (Becton, Dickinson & Co., Sparks, MD). Agar media used were brain heart infusion agar (BHIA, BHI with 1.2 % Bacto-agar, Becton, Dickinson & Co.). When needed, erythromycin (Em**,** MP Biomedicals, Solon, Ohio) was added at 5 µg/ml in BHI (BHI-Em5) or in BHIA (BHIA-Em5) and kanamycin (Km, Fisher Scientific, Fair Lawn, NJ) was added at 10 µg/ml to BHI (BHI-Km10) and BHIA (BHIA-Km10). *L. monocytogenes* strains were routinely grown in BHI at 37°C in standing cultures and preserved at -80°C in BHI with 20 % glycerol.

Cantaloupe Adherence, Growth and Survival Assay

Strain 2858 and the motility impaired mutants M1A5 and L1E4 were grown in 5 ml of BHI at 37°C for approx. 16 h to a concentration of approx. 10^8 CFU/ml. The overnight cultures were then centrifuged for three min at 16,000 rpm. The supernatant was discarded and the bacteria were washed twice in 5 ml of sterile deionized water. The inoculum was prepared by inoculating 5 ml of BHI with 50 µl of the washed cell suspension, resulting in a suspension with approx. 10^6 CFU/ml.

Cantaloupes used in this study were purchased from local grocery stores in Raleigh, NC. The majority of the cantaloupes purchased was imported from the Honduras or from Guatemala during the winter months and were from North Carolina, Georgia, and California during summer months. At retail, cantaloupes were displayed at room temperature and were kept at room temperature during transportation to the laboratory where they were used within 2 h of purchase. Each cantaloupe was examined to exclude fruit with external bruises, abrasion, cuts or mold growth. The cantaloupe rind was then removed from the flesh using an alcohol-sterilized knife and cut into 5x5x1cm fragments. The rind fragments were placed rind side up in groups of four into labeled, sterile plastic petri dishes (100 mm diameter, Fisher Scientific, Pittsburgh, PA). The rind surface of each fragment was sprayed with sterile deionized water to allow the bacterial cultures to better spread over the fragments and prevent the bacterial cells from drying out on the fragments. Each fragment was spot-inoculated in 10 evenly separated droplets of 10 µl each (total inoculum 100 µl, corresponding to approx. 10^5 CFU/fragment); two fragments were inoculated for each time point, with several fragments similarly spotted with sterile water as controls. The 10^5

CFU/fragment inoculation of the fragments was achieved by making a 1:100 dilution of the original washed cell suspension (50 µl original culture: 5 ml deionized water), corresponding to 10^6 CFU/ml, followed by spot inoculating 100 µl of the 10^6 CFU/ml inoculum. The petri dishes were sealed with parafilm and stored at either room temperature (25°C) or 37°C.

Bacteria on the fragments were enumerated at 30 min after inoculation, and at 24, 48, and 72 h for fragments stored at room temperature or at 30 min and 24 h after inoculation for those incubated at 37°C. For each time point, two inoculated cantaloupe fragments per strain were individually placed in 50 ml Falcon™ conical centrifuge tubes (Corning Life Sciences, Durham, NC) and vortexed at top speed (Vortex Genie 2, Diagger, Vernon Hills, Ill) in 10 ml of sterile deionized water for two min. Dilutions of the rinse were then plated on BHIA (to determine total aerobic plate counts [ACPs]) as well as on modified Oxford *Listeria* selective agar (MOX, Oxoid, Hampshire, UK) to enumerate *Listeria* and incubated at 37°C for 24 h and 48 h for BHIA and MOX, respectively. Each mutant/wild type comparison for adherence and growth was done in at least three independent trials.

Competitive Fitness Assessments

Mixed strain inoculations were prepared by inoculating 25 µl of strain 2858 and 25 µl of each mutant strain into 5 ml of sterile deionized water. To enumerate the mutant populations from cantaloupe fragments inoculated with these 1:1 mixtures, approx. 50 colonies from the MOX plates were selected and streaked on BHIA-Em5. The plates were incubated at 37°C for 36 h and the ratio of erythromycin-resistant and erythromycin-susceptible cultures were determined by visual observation. Alternatively, colonies from the MOX plates were inoculated into 96-well plates containing 200 µl of BHI and incubated for

approx. 16 h. The 96-well cultures were then stamped using a sterile microplate replicator onto BHIA-Em5 which was incubated at 37˚C for 48 h. The ratios of parental and mutant cultures were determined by visual observation for growth on the BHIA-Em5 plates. Each competitive fitness assay was done in at least three independent trials.

Genetic Complementation of L1E4

Genetic complementation was performed by Cameron Parsons. Genetic complementation of L1E4 was carried out utilizing *Listeria* shuttle vector pPL2, as previously described (Azizoglu 2010, Lauer 2002). A 2012 bp fragment was PCR amplified using primers L1E4compF (5'-CTAAGGTACCTTC GATAATAGCAAGGAA AGAT-3') and L1E4compR (5'-GTAACCCGGGCCACACTCCCGTATCCTTAA-3'), and purified using QiaQuick PCR Clean Up kit (Qiagen, Valencia CA). This region began 341 bp upstream of the start codon of LMOf2365_0884, and included putative promoter sequence identified by Softberry BPROM software. Primers were designed with restriction sites for endonucleases KpnI and XmaI respectively. The purified fragment was then digested with KpnI and XmaI and ligated into similarly digested vector pPL2 using T4 DNA ligase (Promega Madison, WI). The recombinant plasmid was electroporated into *E. coli* strain S17-1, which enabled its conjugative transfer into mutant L1E4. Chromosomal insertion was confirmed using primers NC16 (5'-GTCAAAACATACGCTCTTATC-3'), located on the chromosome of *L. monocytogenes* in the tRNA ^{Arg} gene, and catR (5'-GACAATTGGAAGAGAAAAGAG-3'), located in the chloramphenicol resistance gene on the backbone of the vector. The same methodology was used for insertion and confirmation of the empty vector pPL2 into L1E4.

In vitro Growth Comparison of 2858 Strain and Motility Impaired Mutants

Overnight cultures of the parental strain 2858 and the two motility mutants used in this study, L1E4 and M1A5, were grown in 5 ml of BHI at 37°C for approx. 16 h to a concentration of approx.10^8 CFU/ml. A 96-well plate (Corning Inc., Corning, NY) was filled with 200 µl of BHI in each well. The wells were then inoculated with 4 µl each strain (each strain tested in triplicate). The plate was then placed into a BioTek microplate reader (BioTek Instrument, Inc., Winooski, VT) and incubated for 24 h at either 28°C or 37°C with optical density at 630 nm (OD_{630}) measurements taken at 1 h intervals with 10 s of shaking before each measurement. Growth rates for each of the motility mutants and the parental strain 2858 were reported relative to the optical density of sterile BHI broth, used as a control. To determine growth rates, individual growth curves were processed in sequential sets of n data values, as previously described by Breidt et al (1994).

Statistical Analysis

The optical densities of each of the strains were entered in to a custom algorithm created by F. Breidt using Matlab software (The Mathworks, Inc., Natick, Mass.) to derive the relative growth rates and standard errors for each strain. The growth rates of the individual strains were fit into a General Linear Model (GLM) of SAS (SAS Institute, Cary, NC) to compute the analysis of variance.

A general linear model was also fit to the values of the CFU/fragments for each of the individual strains inoculated on cantaloupe as well as the mixed culture ratios from the surface of the cantaloupe.

RESULTS

Screening of approx.1900 transposon mutants on soft agar revealed 9 that appeared to be motility-impaired. Two of these mutants, M1A5 and L1E3, were found to harbor single copies of the transposon in different locations on the chromosome and were chosen for assessment of potential impacts on colonization of cantaloupe. M1A5 harbored an insertion in the lmo0694 homolog (nt 161), a member of a motility and chemotaxis gene cluster, while L1E4 harbored an insertion in the DEAD-box RNA helicase gene, *lmo0866* homolog (nt 981). Under phase contrast microscopy, the parental strain had tumbling motility characteristic of *L. monocytogenes* in comparison to both of the mutant strains that showed a lack of tumbling motility. While both strains showed a lack of motility, there was greater lack of motility in the L1E4 mutant than the M1A5 mutant (Fig 3.1). At 37°C, the L1E4 mutant appeared to be less hemolytic on blood agar plates than the parent strain or the M1A5 mutant. At both 25°C and 37°C, the colonies of L1E4 on BHIA were smaller than those of the parental strain or M1A5. L1E4 colonies also had a slight concaved appearance on agar.

Inactivation of *lmo0694* does not Impact Growth of *L. monocytogenes* on Cantaloupe Rind at 25°C or 37°C

Uninoculated control fragments were negative for *Listeria* spp. throughout the study. Determinations of APCs on uninoculated cantaloupe fragments indicated on average 10^5 APC/fragment prior to inoculation with a 4 log increase after 24 h at 25°C followed by further increases after 48 and 72 h (Fig.3.2A). Similar APC increases were observed in inoculated fragment (Fig 3.2A).

Initial *Listeria* spp. counts on MOX were approx. 10^6 CFU/fragment for each of the strains individually as well as for the mixed cultures. The DEAD-box RNA helicase and flagella biosynthesis mutant strains grew similarly to the parental strain 2858. After 24 h, *Listeria* CFU/fragments increased by approx. 2 logs, with further increases after 48 and 72 h (Fig 3.2B).

At 37°C, the initial *Listeria* spp. counts on MOX were approx. 10^6 CFU/fragment for each of the strains individually. After 24 h, *Listeria* CFU/fragment increased by approx. 3 logs (Fig 3.3). To determine potential impacts of the mutations on the competitive fitness of the bacteria on cantaloupe, the rind was inoculated with 1:1 mixtures of the parental strain and each of the mutants and incubated at 25°C. Screening of *L. monocytogenes* colonies from the MOX plates that were streaked on BHI-Em5 plates revealed that for M1A5 the ratio of parental strain to mutant remained around 50% at all tested time points, suggesting that relative fitness of the mutant did not differ significantly from that of the wild-type parental strain (Fig.3.4A). However, L1E4 was impaired ($p<0.0001$) in its survival after 24 h when in combination with the wild-type parental strain (Fig.3.4B). After 24 h, the percent of L1E4 fell below 5% of the total and remained below 20% for the remaining time points ($p>0.6177$) (Fig.3.4B).

Fitness assessments were also done at 37°C. The findings were similar to those obtained at 25°C. After 24 h, the L1E4 mutant was significantly less likely to be recovered from the cantaloupe fragments than its wildtype parental strain ($p<0.3634$) (Fig.3.5).

Genetic Complementation of Mutant L1E4 Confirms Impact of the DEAD-box RNA Helicase in Competitive Fitness on Cantaloupe

Mixed-culture testing of L1E4-C, a genetically complemented derivative of L1E4 harboring an intact copy of the DEAD-box RNA helicase gene revealed restoration of competitive fitness on cantaloupe to levels similar to those of the wild type parental strain. The complemented derivative of the L1E4 mutant showed no significant difference in competitive fitness and remained at or above 50 % throughout the study when inoculated in a 1:1 mixture with the wildtype strain (Fig.3.6). In contrast, L1E4-E which harbors the empty vector pPL4 was like L1E4 in such mixed-culture assays, being markedly less likely ($p<0.1136$) to be recovered from the inoculated fragments than the parental strain (Fig.3.6).

Growth of the Mutants in Laboratory Media at 28°C and 37°C

Each of the mutants grew similarly to the parental strain in liquid laboratory media at 28°C or 37°C (Fig.3.7). At 28°C, average growth rates for 2858 and L1E4 were 0.241 and 0.246, respectively. As expected, at 37°C the growth rates were shorter (0.103 and 0.109, respectively). The growth rate for the complementation clones of L1E4 grown at 37°C produced similar results to the two original strains (Fig.3.7). The growth rates of 2858 and L1E4 were compared to those of F2365 and its helicase mutant (ROA4) as well. At both 28 and 37°C, both helicase mutants showed similar average growth rates to their parental strains. However, in the presence of 3.5% ethanol the helicase mutants had decreased growth rates at 37°C (Appendix E).

Table 3.1: *L. monocytogenes* strains used in this study

Strains	Genotype and Features	Phenotype
2858	Wild-type strain from 2011 cantaloupe outbreak	Em^S (Erythromycin susceptible)
L1E4	Transposon mutant of 2858 DEAD-box RNA helicase in *lmo0866* homolog (nt 981)	Non-motile, cold sensitive, Em^R (Erythromycin resistant)
M1A5	Transposon mutant of 2858 in *lmo0694* homolog (nt 161) (flagella biosynthesis and chemotaxis gene cluster)	Non-motile, Em^R
L1E4-C	Complemented L1E4 mutant	Motile, cold growth proficient; $Em^{R,}$ Cm^R (Chloramphenicol resistant)
L1E4-E	L1E4 mutant with empty vector (pPL2)	Non-motile, cold-sensitive; $Em^{R,}$ Cm^R

Fig 3.1: Swarming of 2858 and motility-impaired mutants grown on TSA soft agar plate. Colonies on plate were obtained by placing 100 µl of inoculum on TSA soft agar plate as described in Materials and Methods and incubating at 37˚C for 24 h. Duplicate spots of 2858, M1A5, and L1E4 are enclosed in rectangles. Spot plating was performed by C. Parsons in the Kathariou Laboratory.

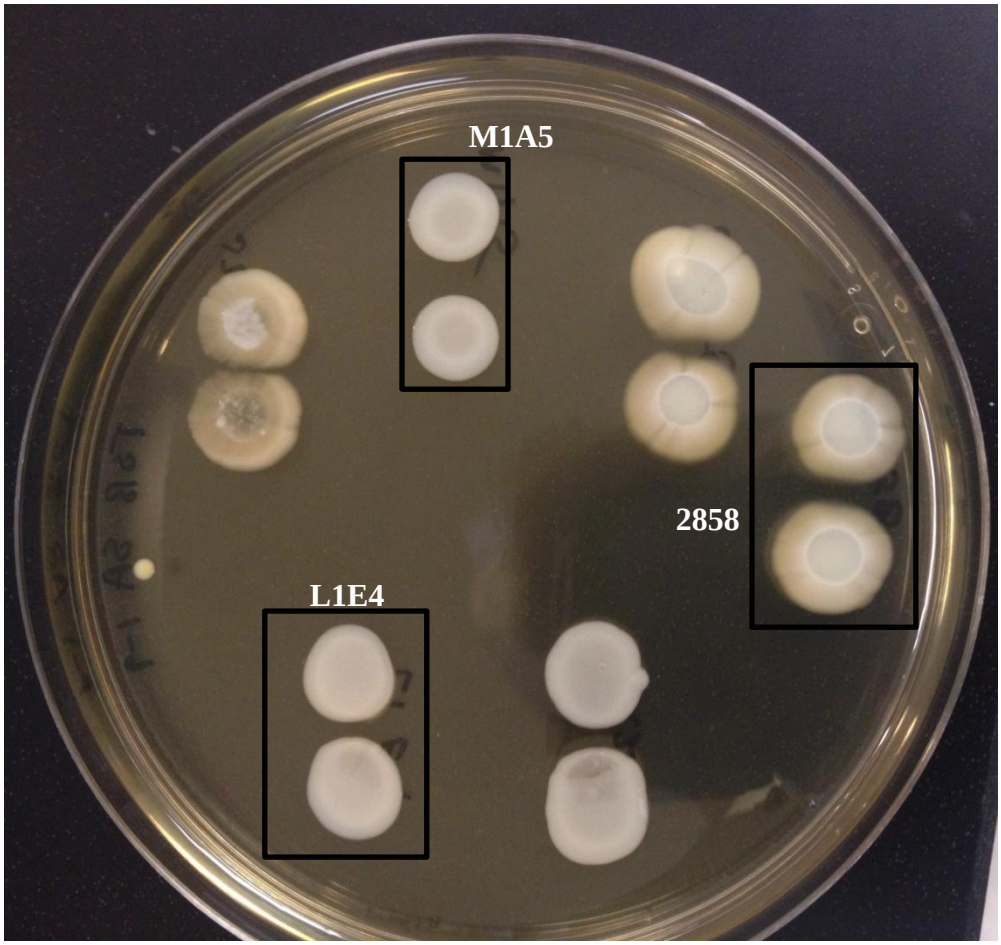
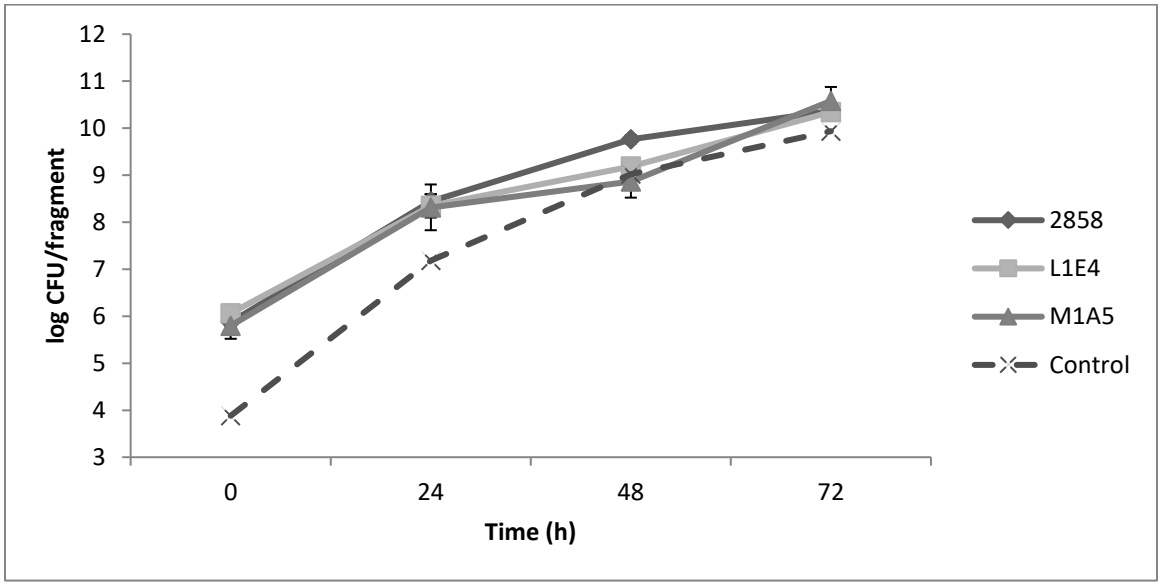


Fig. 3.2: (**A**) APCs CFU/fragment from BHIA plates after incubation at 25°C and **(B)** *Listeria* counts from MOX plates after incubation at 25°C. Data are averages from duplicate fragments, over three independent trials, done as described in Materials and Methods. Controls indicate uninoculated fragments.

(A)



(B)

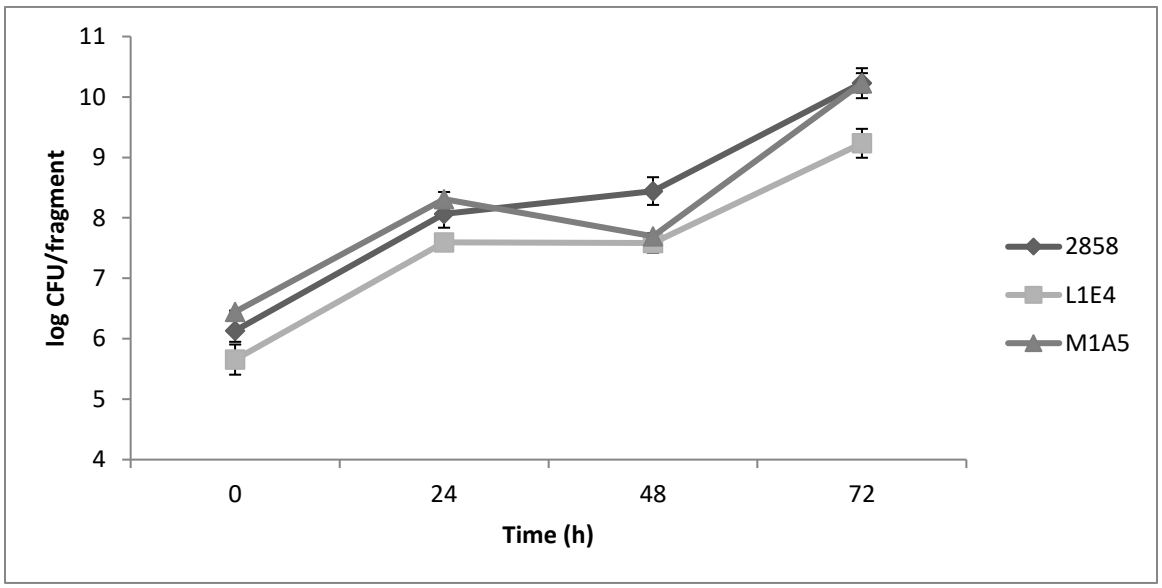


Fig. 3.3: Average of *Listeria* counts from MOX plates after incubation at 37°C.

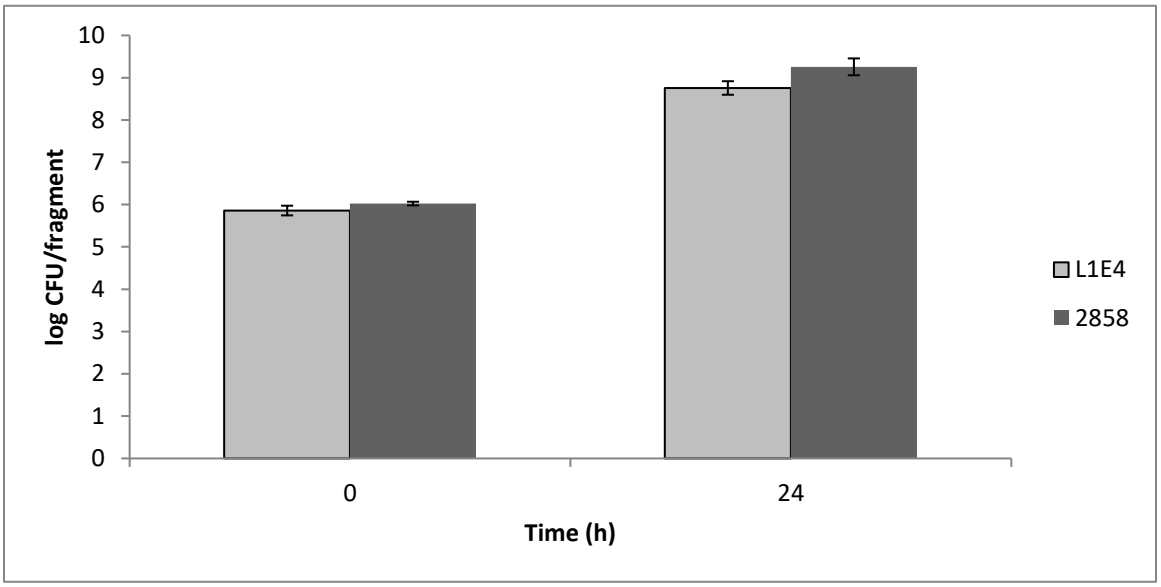
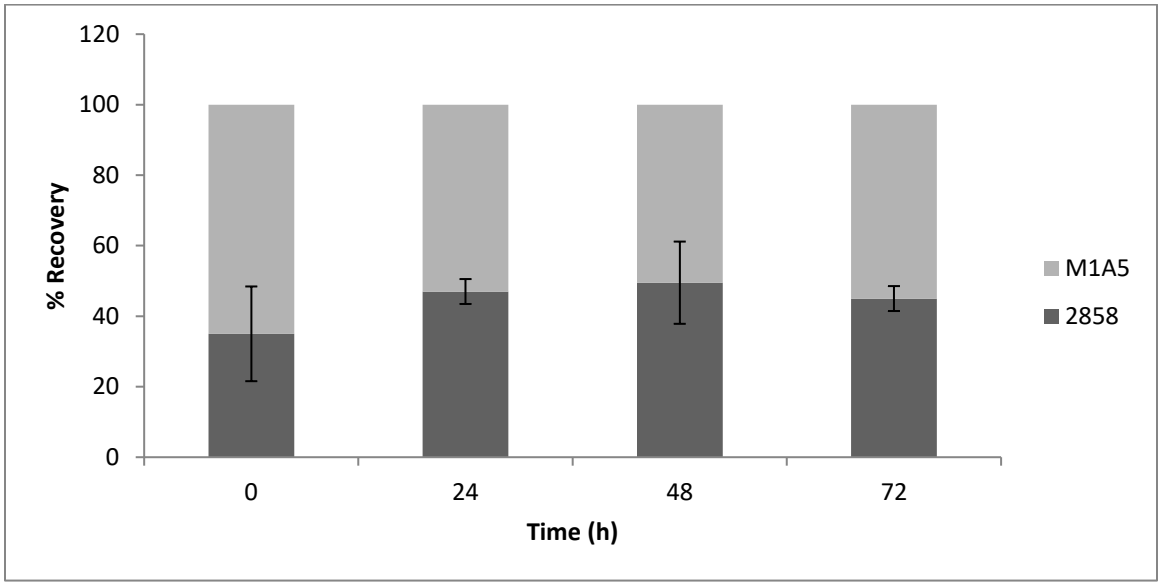


Fig.3.4: Average of ratios from competitive fitness of 2858 parental strain vs. non-motile mutant **(A)** M1A5 and **(B)** L1E4 on cantaloupe fragments incubated at 25°C. Ratios were determined by sub-culturing individual colonies on plates with and without erythromycin, as described in Materials and Methods.

(A)



(B)

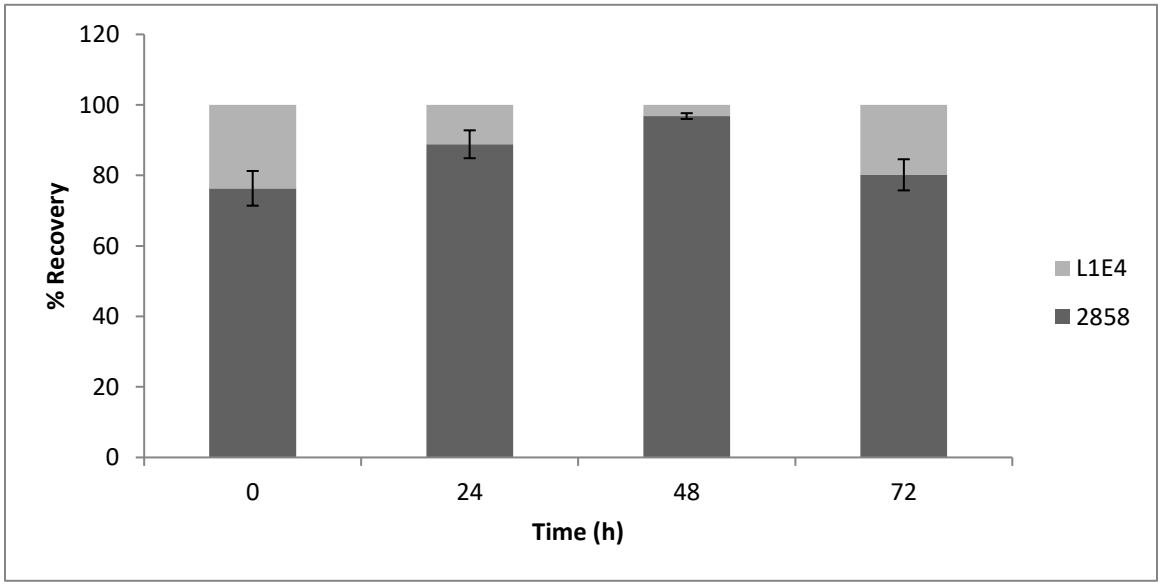


Fig. 3.5: Average of ratios from competitive fitness of 2858 parental strain vs. helicase mutant L1E4 on cantaloupe fragments incubated at 37°C over 24 h. Colonies from MOX plates were subculture in individual wells of 96-well plates which were then plated on media with and without erythromycin, as described in Materials and Methods.

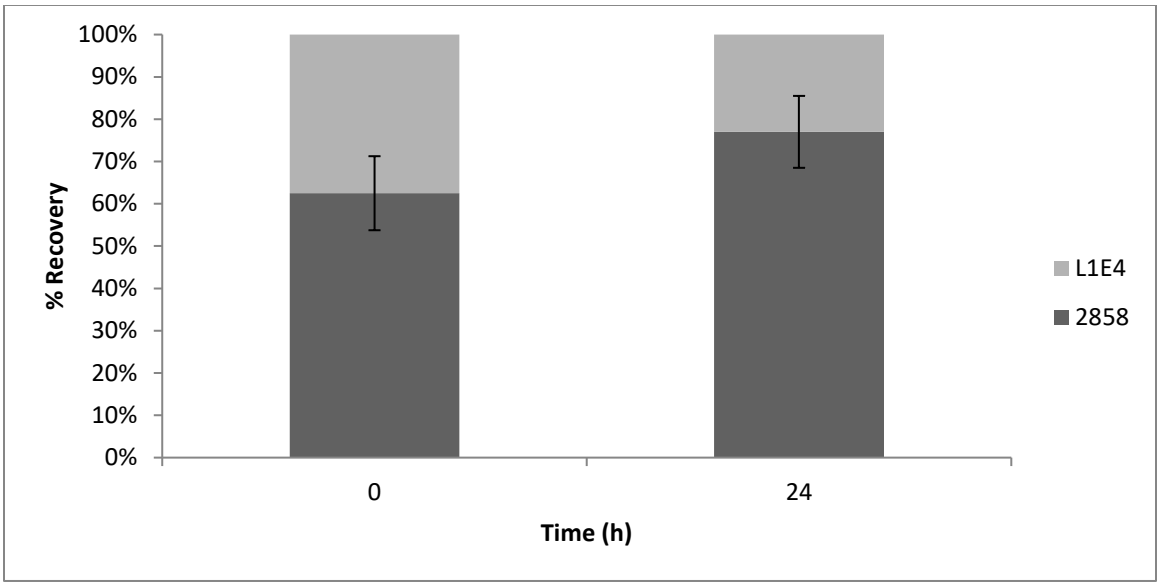


Fig. 3.6: Restoration of competitive fitness of L1E4 following genetic complementation. Shown are averages of ratios between 2858 parental strain (gray) vs. L1E4 with empty vector alone (L1E4-E; diagonal lines) and the complemented L1E4 (L1E4-C; white) on cantaloupe fragments incubated at 25°C over 72 h. Colonies from MOX plates were subcultured in individual wells of 96-well plates which were then plated on media with and without erythromycin,as described in Materials and Methods.

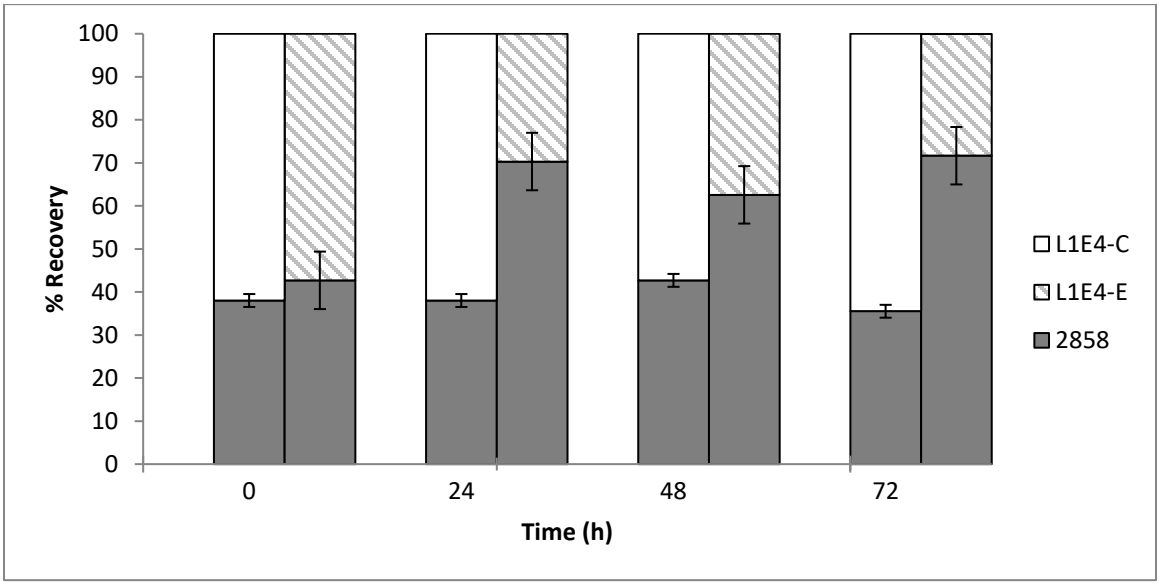
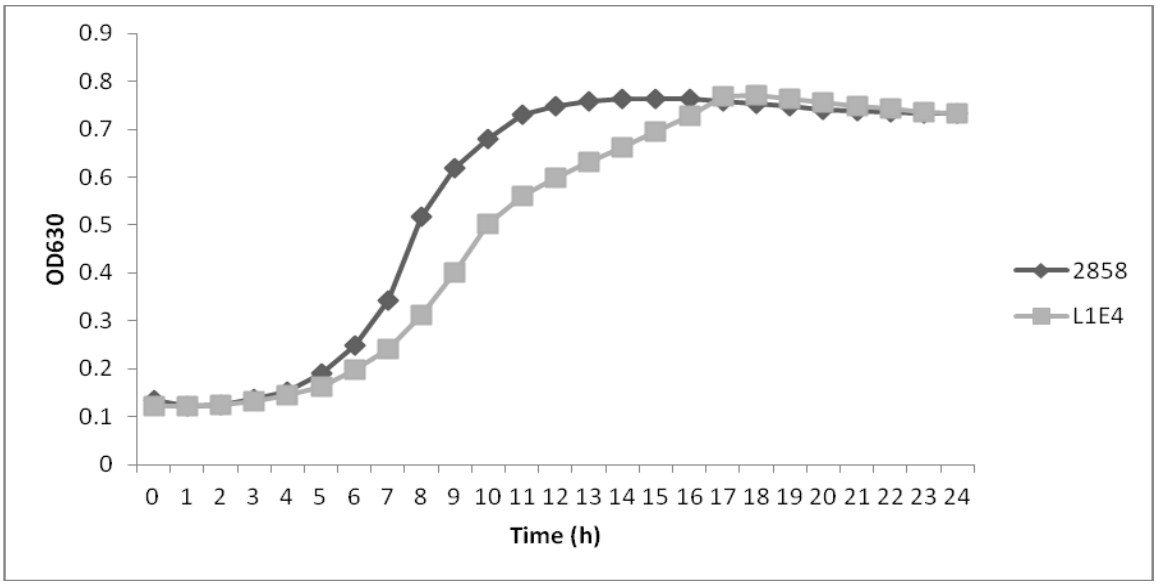


Fig 3.7: Growth of L1E4 compared to parental strain 2858 in BHI at (A) 28°C and (B) 37°C and (C) growth of 2858, L1E4, L1E4-C, and L1E4-E at 37°C. A_{630} was monitored at hourly intervals using a BioTek microplate reader, as described in Materials and Methods.

(A)



(B)

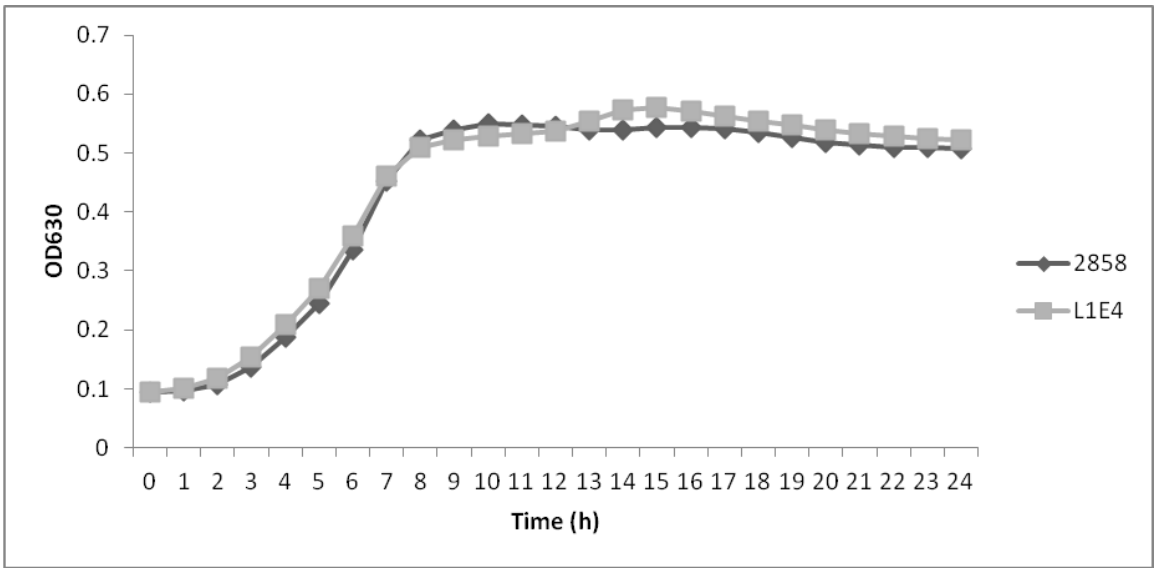
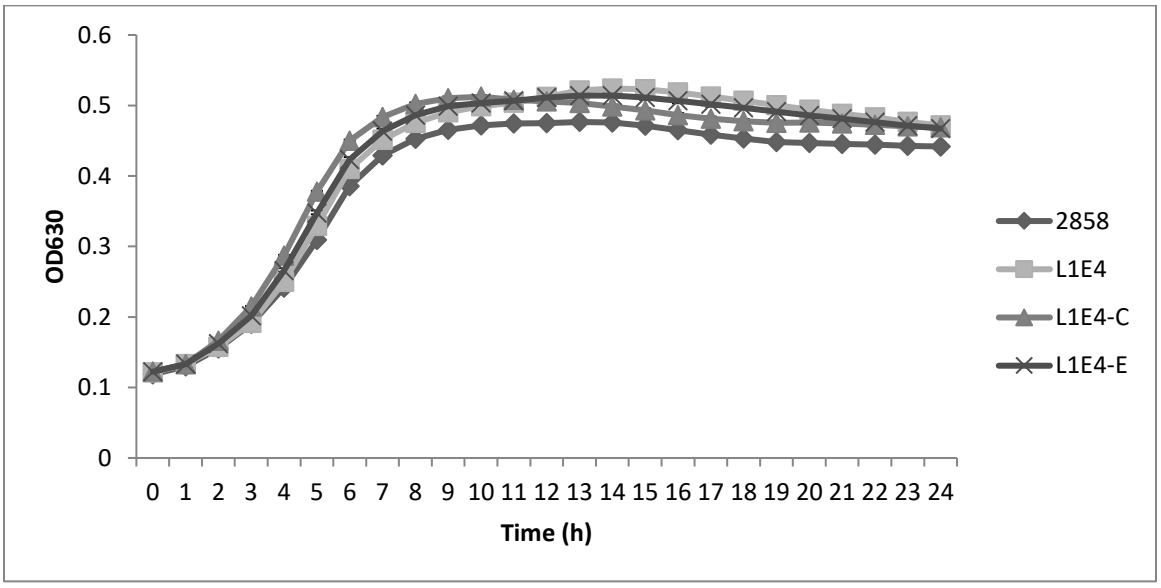


Fig 3.7 continued

(C)



DISCUSSION

DEAD-box proteins get their name from the shared amino acids, Aspartic acid (D)-Glutamic acid (E)-Alanine (A)-Aspartic acid (D), abbreviated DEAD for the single letter code of each amino acid. Four predicted DEAD-box RNA helicase genes (*lmo0866*, *lmo1246*, *lmo1450*, *lmo1722*) have been identified in *L. monocytogenes* EGD-e (Glaser et al., 2001, Markkula et al., 2012). It was previously determined that the deletion of these DEAD-box RNA helicase genes in *L. monocytogenes* EGD-e resulted in cold sensitivity, reduced tolerance to a variety of stresses as well as impaired motility (Markkula et al., 2012 a,b). Our hypothesis was that the motility-impaired phenotype and possibly other alterations in the pleiotropic RNA helicase mutant L1E4 might affect the bacterium's ability to adhere to and grow on produce.

Similarly, the motility-impaired phenotype of the M1A5 mutant might also be associated with altered produce colonization potential.

In the case of mutant L1E4, the mutation appeared to have no significant impact when the mutant was inoculated alone onto cantaloupe rind but it significantly reduced competitive fitness relatively to the wild type strain on cantaloupe rind. Our findings consistently suggested that the mutant strain was being outcompeted by the parental strain, with the number of L1E4 colonies falling below 15 % of the total after the first 24 h. This trend was consistent for competitive fitness assessments at both 25 and 37°C. Genetic complementation of L1E4 restored competitive fitness, confirming that the impaired fitness was due to the specific inactivated gene encoding a DEAD-box RNA helicase and not to other, unidentified mutation(s) potentially harbored by L1E4.

It is of interest that L1E4 was markedly reduced in relative fitness when tested together with the wild type parental strain but exhibited normal growth on the cantaloupe when inoculated alone. The latter findings suggest that L1E4 was not reduced in relative fitness relative to the native microbiota on the cantaloupe rind and imply a role of the DEAD-box RNA helicase in relative fitness specifically in regard to other *Listeria*e. Even though the presence of native microflora was found to inhibit the growth of *L. monocytogenes* (Ukuku and Fett, 2002, Ukuku et al., 2004), the extent to which this takes place with the strains employed in this study was not determined. If inhibition indeed takes place, it appears to be similar for the wildtype parental strain and the mutants examined here, including the RNA helicase mutant L1E4.

When incubated at lower temperatures (4°C and 10°C), the growth of L1E4 was inhibited. The growth rates of the parental strain and mutants at both 28°C and 37°C were similar to the results reported by (Markkula et al., 2012 a, b). At 28°C, the growth rates and OD_{630} in liquid cultures of the L1E4 mutant compared to the parental strain was decreased just as seen by the majority of the DEAD-box RNA helicase deletion mutants studied by (Markkula et al., 2012 a,b). Just as with the previous study, at 37°C, there was little difference in the growth rates of the parental strain and L1E4. The lack of swarming seen by L1E4 was similar to the reduction in motility seen by *lmo0866, lmo1450* and *lmo1722* deletion mutants studied by Markkula et al., 2012 with a reduction in the diameter in the growth zone. The growth of both mutants compared to 2858 on TSA-YE was determined as well (Appendix A). At 25°C, both M1A5 and L1E4 exhibited similar colony morphology and growth as 2858. However, at temperature below 10˚C, there was a noticeable decrease in the size and growth of L1E4, while M1A5 continued to show similar cell morphology to 2858. This further shows that the inactivation of the DEAD-box RNA helicase genes contribute to *L. monocytogenes* cold tolerance. Again, these results were similar to the results shown by (Markkula et al., 2012 a, b) where the deletion of DEAD-box RNA helicase genes resulted in the *Listeria* strains becoming cold sensitive.

These findings were similar to those seen in Azizoglu et al. (2010) in which the insertion inactivation of catalase gene, *kat* resulted in decreases in colony sizes at 10, 25 and 37°C. There were differences between the catalase mutant from Azizoglu et al. and the L1E4 mutant when it came to growth in liquid cultures. Growth was impaired in the catalase mutant at 4°C, but not at 10, 25, or 37°C; while the L1E4 mutant had impaired growth at

both 25 and 37°C. It was also shown that genetic complementation of *kat* into ROA3 resulted in normal colony size and growth of ROA3 similar to the results seen in with the genetic complementation of L1E4 with the DEAD-box RNA helicase gene. Growth curves of 2858 and its helicase mutant (L1E4) and F2365 and its helicase mutant (ROA4) in 3.5 % ethanol at 37°C and at 28 and 37°C were also performed in this study (Appendix E). In all three of the growth curve studies, L1E4 had similar growth trends with ROA4 in comparison to their parental strains. In 3.5 % ethanol, both L1E4 and ROA4 had impaired growth compared to the parental strains even though L1E4 outgrow ROA4. These results were similar to those seen by (Markkula et al., 2012 a) where the deletion of DEAD-box helicase genes resulted in decreased growth in the presence of ethanol. This further suggests that the absence or interference of the DEAD-box RNA helicase gene cluster impacts the survival and growth of *L. monocytogenes* on cantaloupe.

It must be mentioned that the results of this study show that the inactivation of the selected motility related genes resulted in differing results in regards to the impact on colonization on cantaloupe. While L1E4 was shown to be impaired on cantaloupe at both 25 and 37°C; M1A5 showed no significant impairment. This suggests that not all motility-impaired mutants have similar impacts on the colonization of *L. monocytogenes* on cantaloupe.

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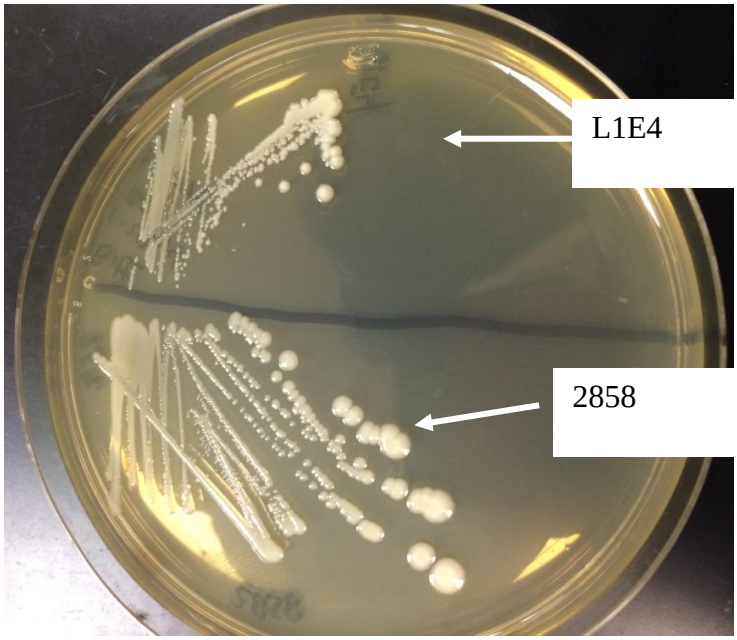
APPENDICES

Appendix A- Images of 2858 and Motility Mutants Grown at Various Temperatures

(A)



(B)

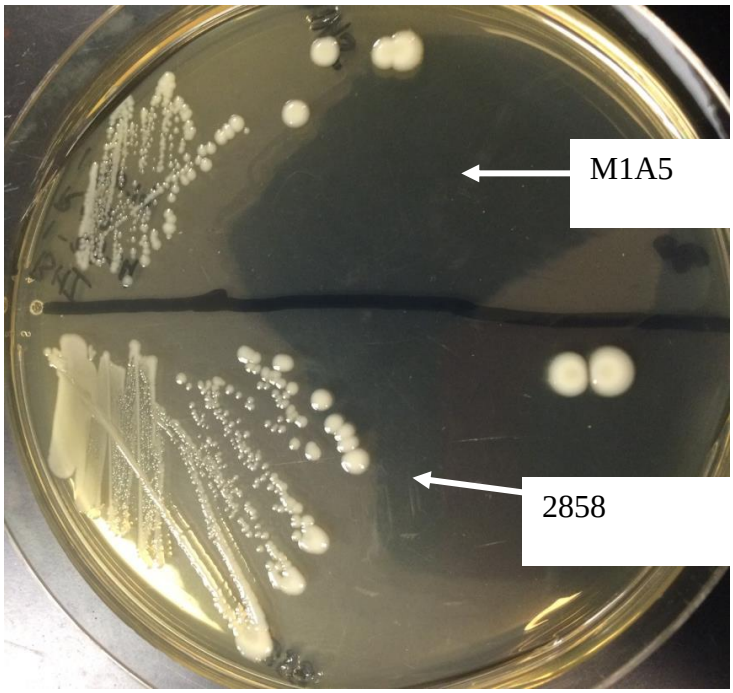
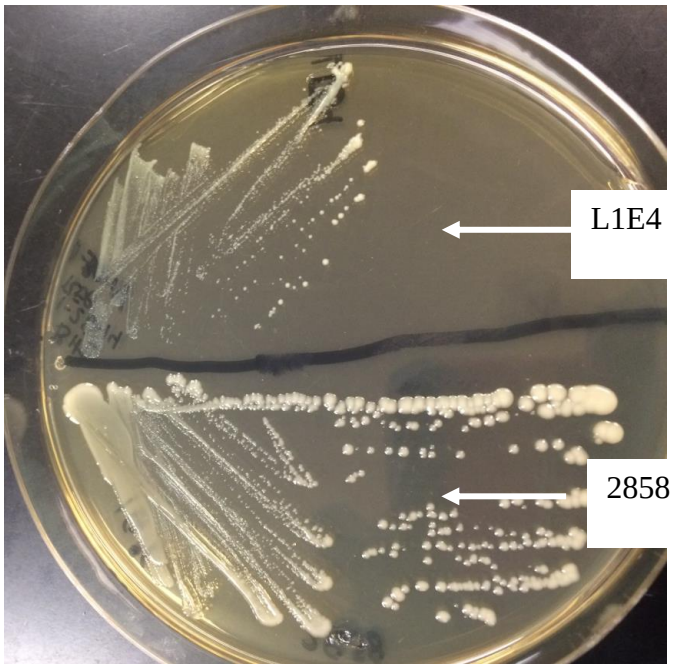


Figure A.1 2858 and (A) L1E4 (B) M1A5 mutants grown at 25°C on TSA-YE

(A)



(B)

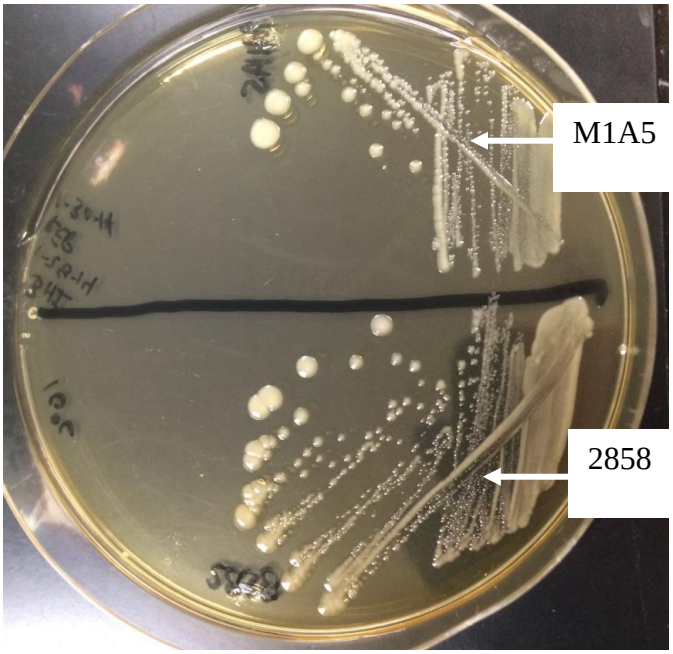
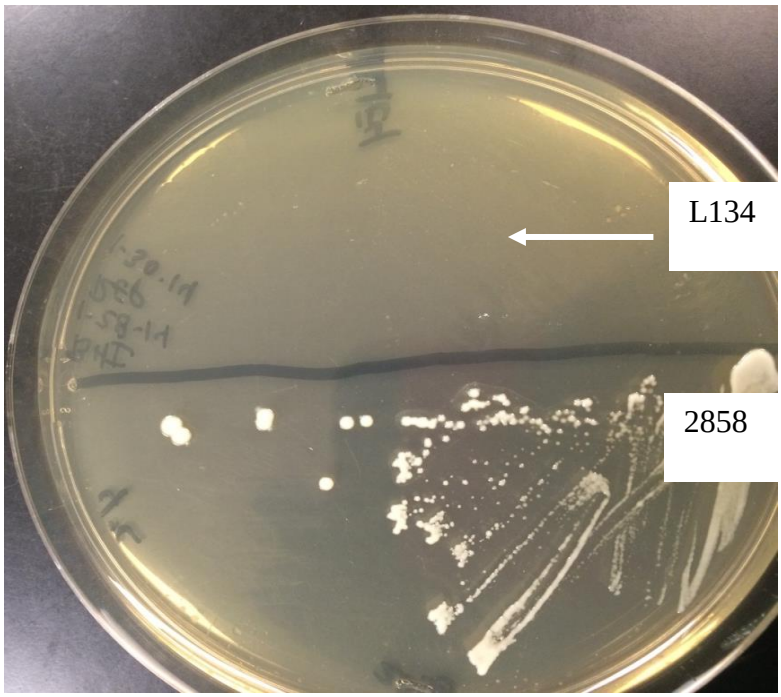


Figure A.2 2858 and (A) L1E4 (B) M1A5 mutants grown at 10°C on TSA-YE

(A)



(B)

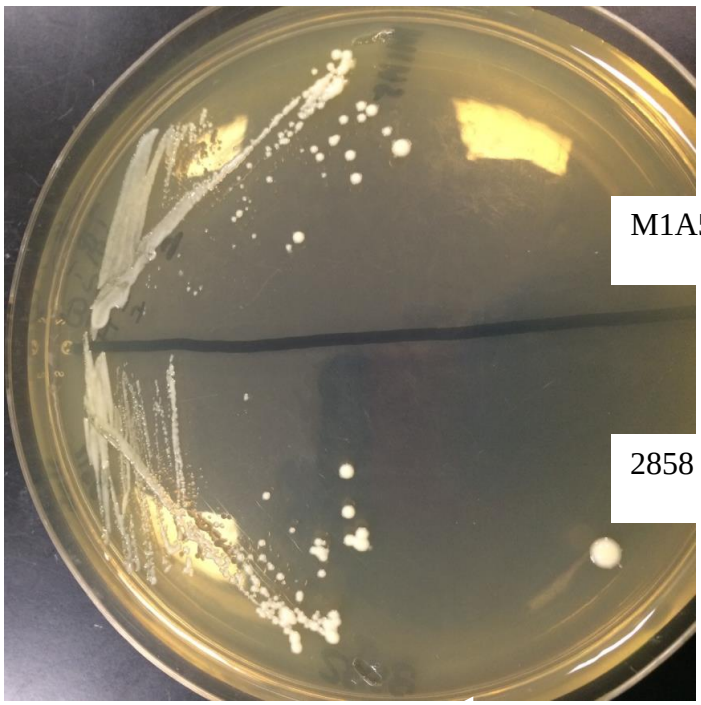
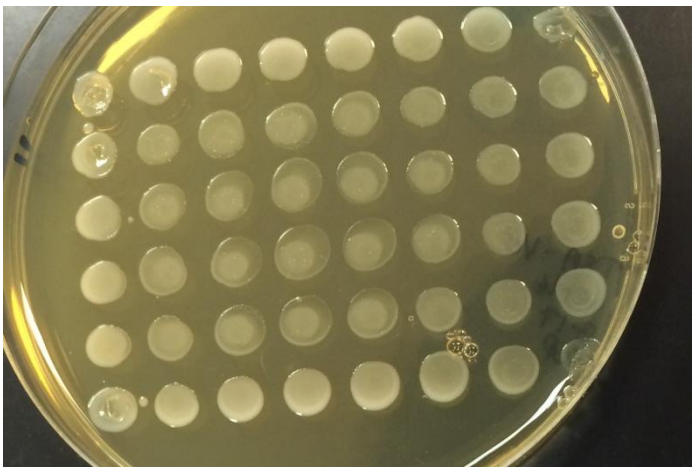


Figure A.3 2858 and (A) L1E4 (B) M1A5 mutants grown at 4°C on TSA-YE

Appendix B- Competitive fitness of 2858 and Mutant Strains Grown on TSA-YE with and Without Erythromycin

(A)



(B)

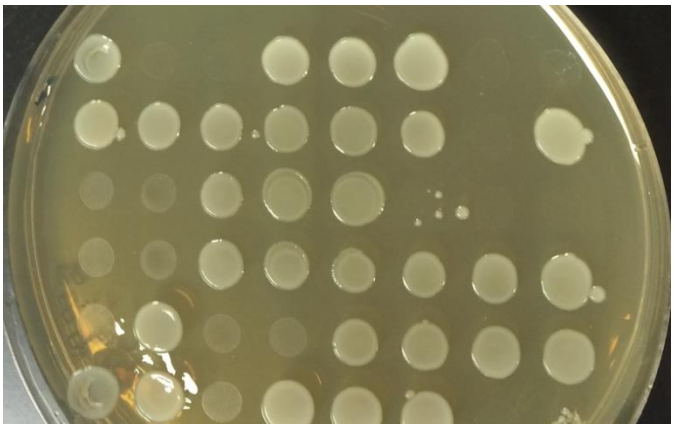
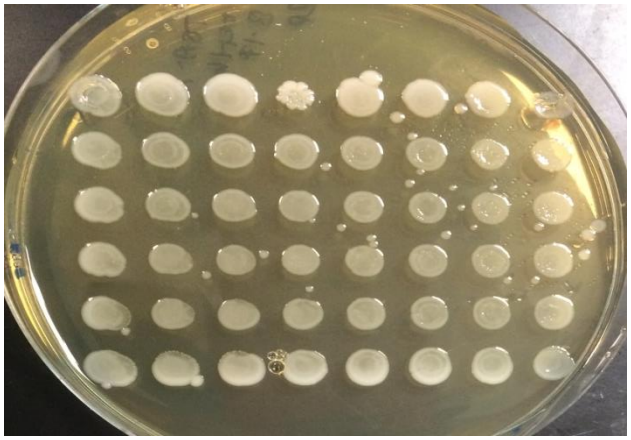


Figure B.1 Spot inoculation of mixed culture of 2858 and B2G6 strain incubated on (A) TSA-YE and (B) TSA-YE with erythromycin at 37°C for 24 h.

(A)



(B)

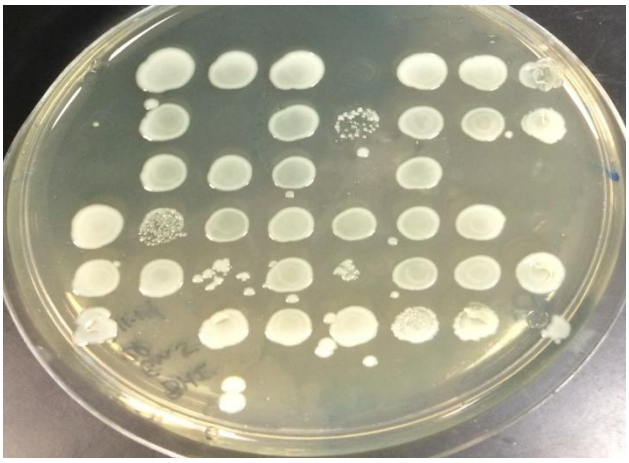


Figure B.2 Spot inoculation of mixed culture of 2858 and B2G6 strain incubated on (A) TSA-YE and (B) TSA-YE with erythromycin at 37°C for 24 h.

Appendix C- 2858 Growth with a Lower Inoculation Concentration

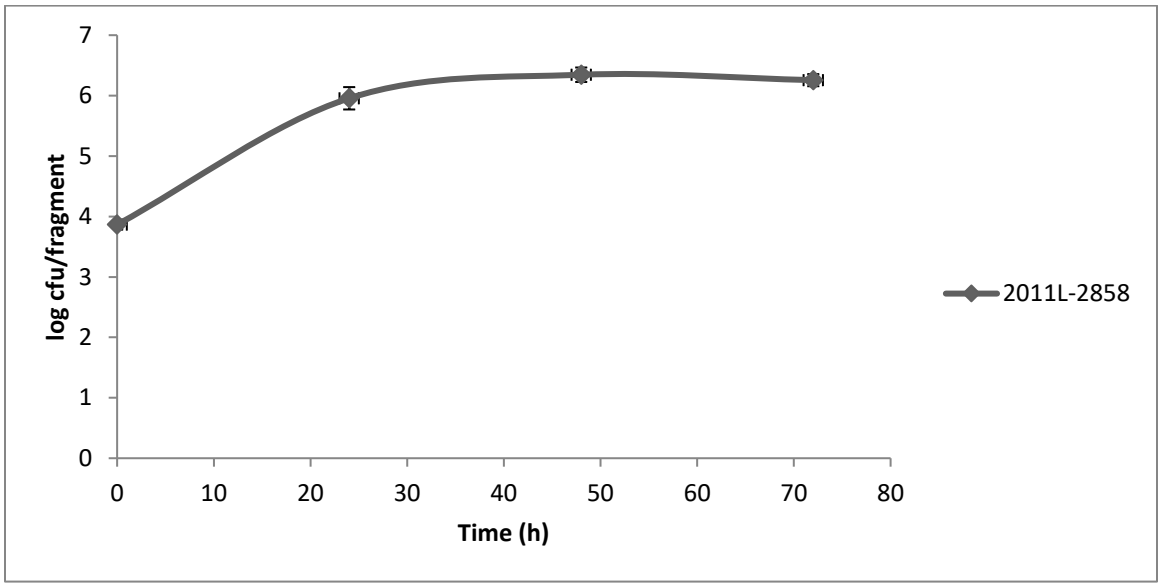


Figure C.1 Growth of 2858 on cantaloupes incubated at 25°C with an initial concentration of approx. 10^3 CFU/ml of inoculum. Cells were grown on MOX agar and incubated at 25°C for 24 h. Time 0 corresponds to 30 min after inoculation.

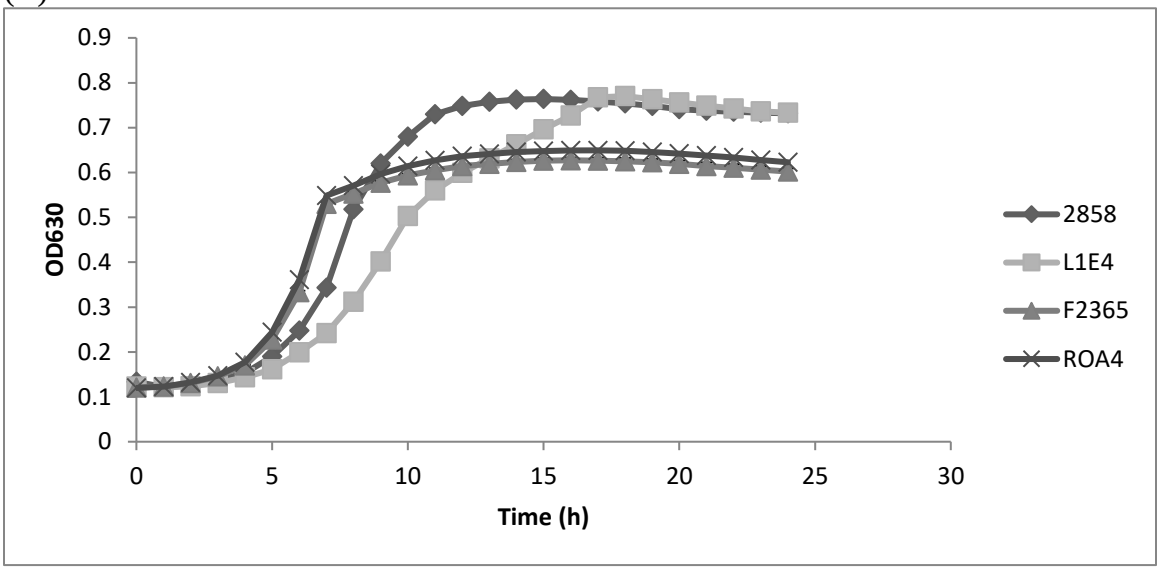
Appendix D- Nalidixic Acid Susceptible Testing of *L. Monocytogenes* on MOX Media

ID	**dilution**	**MOX**	**MOX-NA 20**
2858	$10^{-5}/10^{-6}$	158/70	67/88
F2365	$10^{-5}/10^{-6}$	800/81	850/55
B2G6	$10^{-5}/10^{-6}$	963/108	887/102

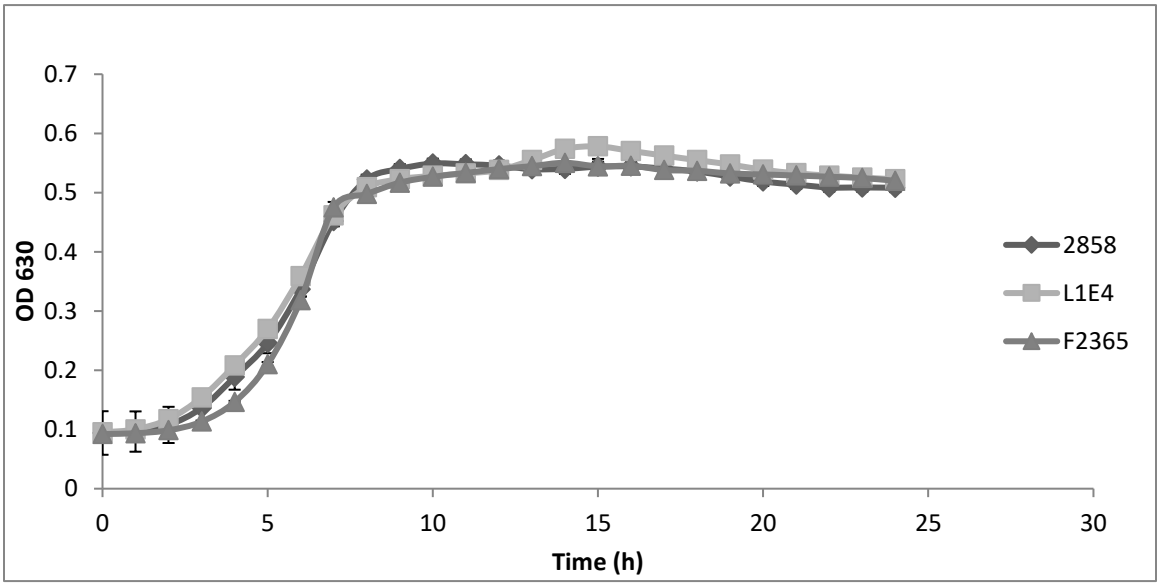
Table D.1 Table of cell counts of various *L. monocytogenes* strains and their susceptibility to 20 µg/ml nalidixic acid. Cultures were plated on MOX agar with 20 µg/ml nalidixic acid added to them and incubated at 37°C for 24 h. Counts represent 10^{-5} and 10^{-6} dilutions respectively. This experiment was done in the course of efforts to identify alternative media that would allow recovery of *Listeria* from cantaloupe in the absence of selective media such as MOX.

Appendix E- Growth curves of 2858 and L1E4 Mutant Compared to F2365 and ROA4 Mutant at Various Conditions

(A)



(B)



Appendix E cont.

(C)

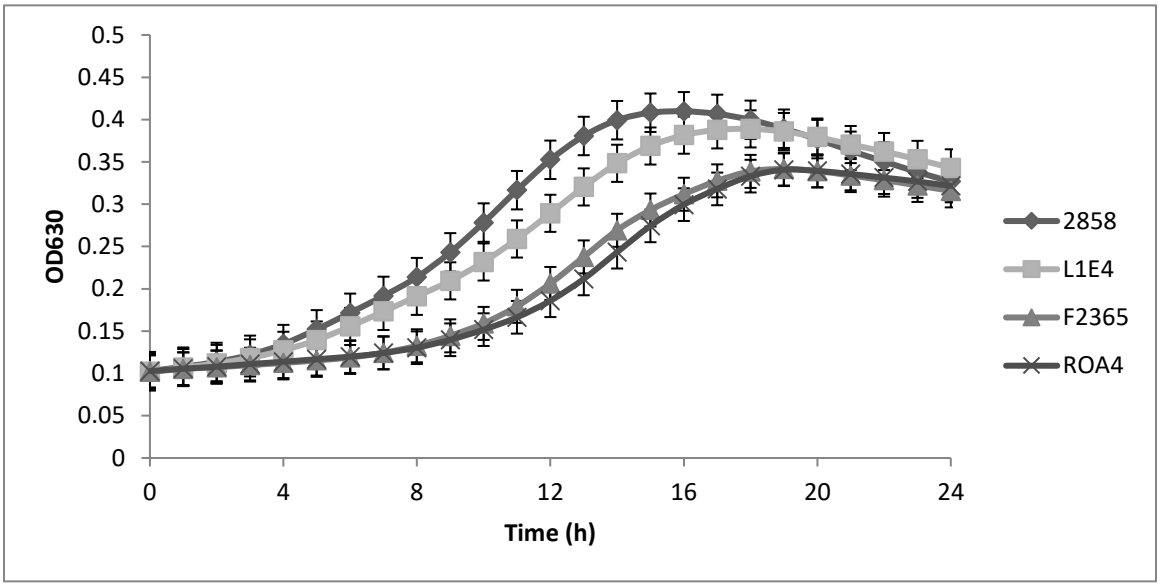
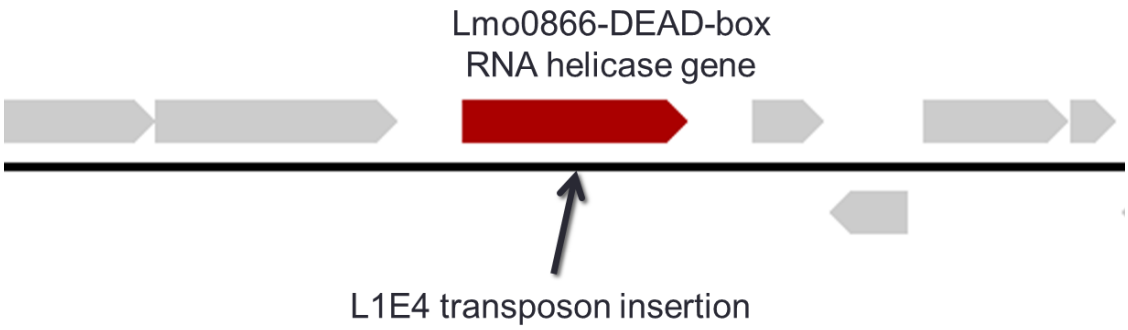


Figure E.1 Growth of non-motile mutants L1E4 and 2858 compared to F2365 and its helicase mutant ROA4 in BHI grown at (A) 28°C and (B) 37°C and (C) in 3.5% ethanol at 37°C.

Appendix F- Regions of Transposon Insertion of 2858 Motility Mutants

(A)



(B)

Figure F.1-Regions of the transposon insertion in 2858 used in the creation of the motility impaired mutants (A) M1A5 and (B) L1E4

Appendix G- Regions Of Transposon Insertion of 2858 Hemolytic Mutants

(A)

(B)

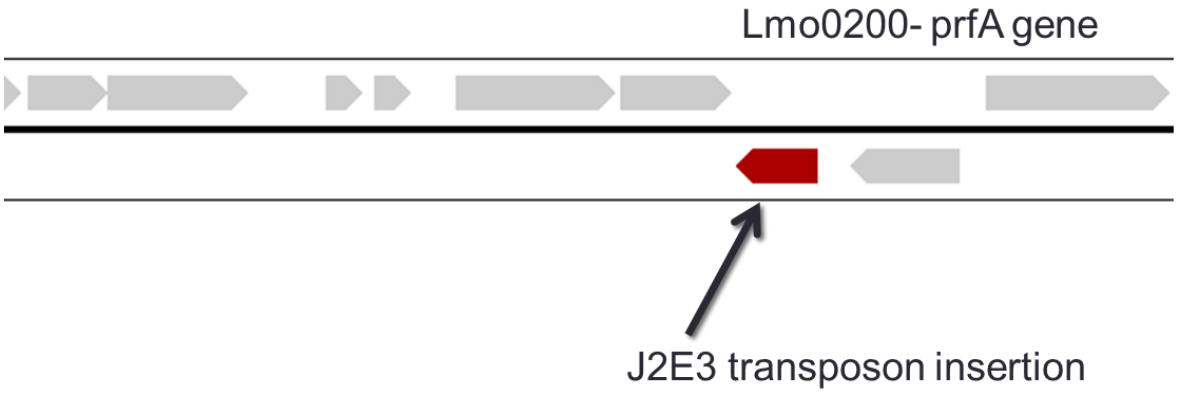


Figure G.1-Regions of *L. monocytogenes* 2858 harboring the transposon insertion in (A) B2G6 and (B) J2E3

Appendix H

Construction of 2858 Non-hemolytic Mutants B2G6 and J2E3

Construction of a mutant library and identification of non-hemolytic mutants was done by C. Parsons. Plasmid pMC38 carrying a *mariner*-based transposon system was used to construct a mutant library of strain 2858 (Cao et al. 2007). DNA of pMC38 was first transformed into *Escherichia coli* DH5α by electroporation followed by the selection of a transformant from Luria Broth (LB) agar plates supplemented with kanamycin (10 µg/ml) to start a culture for plasmid purification. The transformant was incubated at 37°C overnight. Following incubation, the plasmid was purified by using a plasmid extraction kit (Qiagen, Valencia, CA). The transposon selective marker used was erythromycin resistance while the plasmid contained a gene conferring resistance to kanamycin. The plasmid was introduced into *L. monocytogenes* 2858 by electroporation as previously described (Cao et al. 2007). The culture was then incubated on BHI-Em5 plates and incubated at 30°C for 3-4 d. Plasmid retention was confirmed by screening the colonies individually on BHIA-Em5 and BHIA-Km10. Retention of the plasmid was indicated by growth on both media.

A colony that demonstrated plasmid retention was inoculated in BHI-Em5 and the culture was incubated overnight at 30°C. The culture was then grown at 40°C overnight. Individual colonies were streaked in duplicate on BHIA-Em5 and BHIA-Km-10 and screened for growth. Putative mutants that grew on BHIA-Em5 but not on BHIA-Km10 were selected, inoculated in 250 µl BHI in 96-well microtiter plates (Costar, Corning, NY) and grown overnight at 37°C. The mutants were screened as described below.

To identify non-hemolytic mutants, the mutants from the 96-well plates were spotted on blood agar (Remel Inc., TSA supplemented with 5 % sheep blood) using a sterile 48-pin stainless steel replicator. The plates were incubated at 37°C for 36 h and examined for hemolysis zones typical of *L. monocytogenes*. The number of transposon insertions in each mutant was determined by Southern blot. A polymerase chain reaction (PCR) obtained using pMC38 DNA as a template with primers Maq254 and Maq206 (Cao et al., 2007). The probe was labeled with digoxigenin (Genius kit, Roche, Indianapolis, IN). Labeling was done by adding sterile water to 10 ng-1 µg DNA to have a final volume of 15 µl. The DNA was denatured by boiling in hot water for 10 m, followed immediately being placed on ice. Next, 2 µl of 10x Hexanucleotide Mix, 2 µl 10x DIG DNA labeling mix and 1 µl Klenow (5 U/µl) were mixed with the denatured DNA and incubated at 37°C overnight. The genomic DNA of selected mutants were isolated by using a DNeasy kit (Qiagen, Valencia, CA) and digested using the restriction enzyme, EcoRI (New England Biolabs, Waverly, MA). The digested genomic DNAs were electroporated in TBE buffer with 0.8% buffer at 85V for 2.5 h. The DNA fragments were then transferred onto nylon membranes in 10x SSC buffer by capillary action.

To identify the sites of transposon insertion, arbitrary PCR was performed to amplify the DNA sequences flanking the transposon as described (Cao et al., 2007). The first round of PCR was performed using primers Marq207/255 and Marq207/269. For the second round of PCR, 5 µl of a 1/25 dilution from the first round of PCR was used in a 20-µl reaction. The PCR products were sequenced using primers Marq208/256 and Marq208/270.

Appendix I

Construction of Non-Motile Mutants M1A5 and L1E4

Construction of a *mariner*-based mutant library and identification of motility impaired mutants was done by C. Parsons. Plasmid pMC38 carrying a *mariner*-based transposon system was used to construct a mutant library of strain 2858 (Cao et al. 2007). DNA of pMC38 was first transformed into *Escherichia coli* DH5α by electroporation followed by the selection of a transformant from Luria Broth (LB) agar plates supplemented with kanamycin (10 µg/ml) to start a culture for plasmid purification. The transformant was incubated at 37°C overnight and the plasmid was purified by using a plasmid extraction kit (Qiagen, Valencia, CA). The transposon selective marker used was erythromycin resistance while the plasmid backbone contained a gene conferring resistance to kanamycin. The plasmid was introduced into *L. monocytogenes* strain 2858 by electroporation as previously described (Cao et al. 2007). The culture was then incubated on BHI-Em5 plates and incubated at 30°C for 3-4 d. Plasmid retention was confirmed by screening the colonies individually on BHIA-Em5 and BHIA-Km10. Retention of the plasmid were indicated by growth on both media.

A colony that demonstrated plasmid retention was inoculated in BHI-Em5 and the culture was incubated overnight at 30°C. The culture was then grown at 40°C overnight. Individual colonies were streaked in duplicate on BHIA-Em5 and BHIA-Km10 and screened for growth. Putative mutants that grew on BHIA-Em5 but not on BHIA-Km10 were selected, inoculated in 250 µl BHI in 96-well microtiter plates (Costar, Corning, NY) and grown overnight at 37°C.

To identify non-motile mutants, the mutants from the 96-well plates were spotted on soft BHI agar (BHI with 4 % agar) using a sterile 48-pin stainless steel replicator. The plates were incubated at 30°C for at least 48 h and examined for migration of the bacterial cells underneath the surface of the agar. Mutants unable to swarm in the soft agar were chosen for further confirmation on soft agar and were examined for motility of the cultures using phase contrast microscopy. The number of transposon insertions in each mutant was determined by Southern blot using the *mariner*-based transposon *ermC* as probe. The *ermC* probe was amplified with polymerase chain reaction (PCR) with pMC38 DNA as template and primers Maq254 and Maq206 (Cao et al., 2007), and labeled with digoxigenin (Genius kit, Roche, Indianapolis, IN). Labeling was done by adding sterile water to 10 ng-1 µg DNA to have a final volume of 15 µl. The DNA was denatured by boiling in hot water for 10 m, followed immediately being placed on ice. Next, 2 µl of 10x Hexanucleotide Mix, 2 µl 10x DIG DNA labeling mix and 1 µl Klenow (5 U/µl) were mixed with the denatured DNA and incubated at 37°C overnight. The genomic DNA of selected mutants were isolated by using a DNeasy kit (Qiagen, Valencia, CA) and digested using the restriction enzyme, EcoRI (New England Biolabs, Waverly, MA). The digested genomic DNAs were electrophoresed in TBE buffer with 0.8 % buffer at 85V for 2.5 h. The DNA fragments were then transferred onto nylon membranes in 10x SSC buffer by capillary action.

To identify the sites of transposon insertion, arbitrary PCR was performed to amplify the DNA sequences flanking the transposon as described (Cao et al., 2007). The first round of PCR was performed using primers Marq207/255 and Marq207/269. For the second round of PCR, 5 µl of a 1/25 dilution from the first round of PCR was used in a 20-µl reaction. The PCR products were sequenced (Genewiz Inc., South Plainfield, NJ, USA) using primers Marq208/256 and Marq208/270.