DESICCATION TOLERANCE IN *LISTERIA MONOCYTOGENES*: MECHANISMS AND IMPORTANCE FOR FOOD SAFETY

by

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ABSTRACT

This study examined some of the environmental, physiological, and genetic factors or mechanisms which contribute to *L. monocytogenes'* desiccation survival under food processing conditions. Desiccation experiments were carried out on stainless steel coupons stored at 43% RH, 15°C. The level of initial contamination had no impact ($p>0.05$), whereas the presence of a mature biofilm, prior osmoadaptation, and the presence of salt (5%) and lard (20-60%) on the SS coupons significantly ($p<0.05$) increased the bacterium's desiccation survival. An Lm568 transposon mutant library was constructed to screen for novel genes involved in desiccation survival. Fifteen tolerant and 16 sensitive desiccation mutants were sequenced. Interrupted genes involved in motility and FA membrane modification were the most common in tolerant mutants whereas energy and membrane transport related genes were the most recognized in sensitive mutants. Lastly, a spontaneous desiccation resistant Lm568 variant was isolated, emphasizing the importance of understanding desiccation tolerance for food safety.

LIST OF ABBREVIATIONS AND SYMBOLS USED

A	Absorbance
ABC	ATP-binding cassette
AdeC	Adenine deaminase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATR	Acid tolerance response
BCFA	Branched chain fatty acid
BHI	Brain heart infusion
BKD	Branched chain α-keto acid dehydrogenase
B. japonicum	*Bradyrhizobium japonicum*
bp	Base pairs
B. subtilis	*Bacillus subtilis*
Buk	Butyrate kinase
C	Carbon
CA	Canada
Caps	Cold acclimation proteins
cbiD	Cobalt-precorrin-6A synthase
CFU	Colony forming units
CoA	Coenzyme A
CsbA	General stress proteins controlled by σ^B
Csp	Cold shock proteins
C15:0	Saturated 15 carbon fatty acids
delta	Time to first log reduction (Weibull model)
dH_2O	Distilled water
DK	Denmark
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
D-value	Decimal time reduction
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
EGD-e	*L. monocytogenes* strain EGD-e
EPS	Exopolymeric substances
erm	Erythromycin
EutBC	Ethanolamine ammonia lyase
FA	Fatty acid
FabH	β-ketoacyl carrier protein synthase III
FlgD	Flagellar basal body rod modification protein
FlgL	Flagellar hook associated protein
FliM, FliY	Flagellar motor switch proteins
Flip, FlhB	Flagellar biosynthesis proteins
g	Relative centrifugal force
Glu-tRNA	Glutamate tRNA ligase
GntR	Family of transcription factors

GTP	Guanosine triphosphate
H^+	Hydrogen
Himar1	*Himar1* transposon
His	Imidazole glycerol phosphate synthase
HK	Histidine kinase
HMDS	Hexamethyldisilazane
HPP	High pressure processing
Hsps	Heat shock proteins
InlC	Internalin C
K^+	Potassium
kan	Kanamycin
KCl	Potassium chloride
K_2CO_3	Potassium carbonate
KdpFABC	Multi-subunit ATP-driven potassium pump
L	Left
lmo	Gene designation for *L. monocytogenes* EGD-e sequenced genome
L. monocytogenes	*Listeria monocytogenes*
Lm568	*Listeria monocytogenes* strain 568
Lpd	Dihydrolipoamide dehydrogenase
MANOVA	Multivariate analyses of variance
$MgCl_2$	Magnesiuym chloride
$MgSO_4$	Magnesium sulphate
MotB	Motor rotation protein
MSE	Mean standard error
n	Number of samples
N	Nitrogen
NaCl	Sodium chloride
O-Ag	O-antigen
ORF	Open reading frame
OsO_4	Osmium tetroxide
p	Shape parameter (Weibull model) Not to be confused with p when referring to statistical probabilities
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PE	Phosphatodylethanolamine
PEG	Polyethylene glycol
PG	Phosphatidylglycerol
pKa	Acid dissociation constant
pMC39	Thermosensitive DNA shuttle vector carrying the mariner transposon
(p)ppGpp	Guanosine pentaphosphate
P. putida	*Pseudomonas putida*
PrfA	Positive regulator factor A
PS	Peptone saline

Ptb	Phosphate acetyl/butyryltransferase
PTS	Phosphotransferase system
QS	Quorum sensing
R	Right
RE	Restriction enzyme
RH	Relative humidity
RNA	Ribonucleic acid
Rpo	RNA polymerase
RR	Response regulator
RTE	Ready-to-eat
S. aureus	*Staphylococcus aureus*
SCFA	Saturated chain fatty acid
SDS	Sodium-dodecyl-sulphate
SEM	Scanning electron microscopy
S. enterica	*Salmonella enterica*
SOC	Super optimal broth with catabolite repression
Spp.	Species
SS	Stainless steel
TAE	Tris-acetate EDTA
Tafi	Thin aggregative fimbriae
TCA cycle	Tricarboxylic acid cycle
TCS	Two-component system
T_m	Phase transition temperature
tRNA	Transfer RNA
TSB	Tryptic soy broth
TSB-glu	Tryptic soy broth + 1% glucose (v/v)
UFA	Unsaturated fatty acid
US	United States
v/v	Volume/volume concentration
w/v	Weight/volume concentration
σ^B (sigB)	Alternative sigma factor B

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CHAPTER 1 INTRODUCTION

In recent years, the foodborne bacterial pathogen *Listeria monocytogenes* has become a significant issue in the Canadian food supply, causing repeated recalls and outbreaks of foodborne illness. The bacterium which was first discovered in rodents in 1926 (Murray *et al.,* 1926), was not recognized as a human pathogen of concern until the 1980's which saw a considerable rise in the number of human and animal cases in both Europe and North America (Fleming *et al.,* 1985; Schwartz *et al.,* 1989; McLauchlin, 1996). Notably was the first confirmed foodborne outbreak in 1981 where consumption of contaminated coleslaw in Halifax Nova Scotia led to 41 cases of listeriosis and 18 deaths in pregnant women and neonates (Schlech *et al.,* 2000). Since then, *L. monocytogenes* has been widely acknowledged as a very serious, invasive pathogen often causing life-threatening foodborne disease in susceptible persons including infants, pregnant women, and elderly and immunocompromised individuals (Lorber, 2005).

The majority of foods implicated in *L. monocytogenes* outbreaks are ready-to-eat (RTE) products such as soft cheeses, milk, vegetables, and processed meats. In 2008, 22 people died and 57 fell ill after the consumption of *L. monocytogenes* contaminated deli meats produced by a Maple Leaf Foods® plant in Toronto, Ontario (Weatherill *et al.,* 2009). It wasn't long after in 2011 when the consumption of contaminated cantaloupe from a Colorado farm led to 33 deaths and 147 cases of listeriosis, making it the largest food-borne outbreak in the United States since 1924 (Centers for Disease Control and Protection, 2012). These episodes impose a great economic burden on both public health services and the food industry. Increasing consumer demand for fresh, minimally

processed ready-to-eat foods necessitates the need for improved control of *L. monocytogenes* in food production environments.

L. monocytogenes is almost ubiquitous in nature and has been isolated from freshwater, wastewater, mud, soil, and animal feces (Gelbíčová and Karpíšková, 2012). Animals act as carriers of *L. monocytogenes* without appearing ill (Kalender, 2002). Not surprisingly, *L. monocytogenes* is most often isolated from food-animal environments and fresh produce grown in these areas may become contaminated prior to being harvested. However, contamination of retail foods tends to occur primarily in food production environments (Tompkin *et al.,* 1999). Here *L. monocytogenes* has been found to survive in drains, standing water, residues, food-contact surfaces and floors for extended periods of time (in some cases over two years) with the same genotype being re-isolated for years despite cleaning efforts and periods with inactivity (Wulff *et al.* 2006; Tompkin *et al.,* 1999). The widespread occurrence of this organism and its ability to survive the harsh conditions employed by the food industry to suppress bacterial growth (low pH, moderate to high salt levels, and refrigeration) make *L. monocytogenes* difficult to control in the food supply chain (O'Driscoll *et al.,* 1996; Glass and Doyle, 1989; Shahamat *et al.,* 1980). Once introduced, ineffective sanitation may permit the establishment and subsequent distribution of persistent strains throughout processing facilities (Lunden *et al.,* 2003).

The capacity of *L. monocytogenes* to adhere to stainless steel, glass, polypropylene, rubber, and other processing surfaces has been well documented (Mafu *et al.,* 1990). Survival of bacterial pathogens on food contact surfaces promotes the subsequent cross-contamination of foods (Midelet *et al.,* 2006; Rodriguez *et al.,* 2007; Keskinen *et al.,* 2008). Recently it was shown that *L. monocytogenes* can survive desiccation for three months on stainless steel surfaces in a simulated food processing environment, suggesting

that the bacterium's persistence in food plants is partly related to its desiccation tolerance (Vogel *et al.,* 2010). In addition, like several other bacteria, *L. monocytogenes* can form biofilms on solid surfaces (Blackman *et al.,* 1996), further enhancing its ability to survive under dry conditions (Truelstrup Hansen and Vogel, 2011) and resist disinfection and removal (Pan *et al.,* 2006).

Despite the food safety concerns that persistent strains of *L. monocytogenes* impose on the food industry, the mechanisms used by this bacterium to adapt and survive in low water content environments is largely understudied. To date, the effects of salt, biofilm, osmolytes, organic material, and some food soils on *L. monocytogenes* desiccation survival have been evaluated (Takahashi *et al.,* 2011; Vogel *et al.,* 2010; Truelstrup Hansen and Vogel, 2011; Dreux *et al.,* 2008; Dreux *et al.,* 2007). However, more knowledge on the adaptation responses that take place during desiccation stress are needed in order to eliminate this pathogen from the food supply chain.

1.1. Thesis Objectives and Hypotheses

The overall aim of this study was to examine some of the factors or mechanisms, which may cause or alter *L. monocytogenes'* extended desiccation survival under food processing conditions including environmental, physiological, and genetic factors. To accomplish this, the research for this thesis was divided into three sections. Part 1 investigates the impact of commonly occurring environmental conditions (food soils, variations in contamination levels, biofilm formation) on *L. monocytogenes* desiccation survival on food-grade stainless steel. Part 2 involved the screening of a large transposon insertion mutant library for novel genes contributing to *L. monocytogenes*' desiccation tolerance. Lastly, Part 3 focused on the isolation of stably desiccation resistant

spontaneous variants of *L. monocytogenes* from the survivor tail of desiccation curves. As a whole this research will contribute to an improved understanding of the mechanisms of desiccation tolerance and survival in *L. monocytogenes* that in turn will aid in the development of new or more effective intervention strategies.

The specific objectives for this work were as follows:

Part 1:

1) Determine if *L. monocytogenes'* desiccation survival kinetics are independent or dependent on the initial level of surface contamination.
2) Evaluate the impact of varying stages of *L. monocytogenes* biofilm formation on subsequent desiccation survival.
3) Investigate how the individual components of foods (salt, lipids) commonly associated with listeriosis outbreaks impact *L. monocytogenes*' desiccation survival on stainless steel processing surfaces.

Part 2:

1) Identify novel genes contributing to *L. monocytogenes'* desiccation tolerance through the screening of a transposon insertion mutant library.
2) Determine if the genes which impact desiccation survival play a similar role in adaptation to osmotic stress.

Part 3:

1) Investigate whether the persistence of *L. monocytogenes* in the food industry is due to the spontaneous breeding of desiccation resistant variants.

CHAPTER 2 LITERATURE REVIEW

2.1. Overview of *L. monocytogenes*

Listeria monocytogenes is Gram-positive, rod shaped, non-spore forming and facultative anaerobic bacteria which can cause listeriosis in immunosuppressed and elderly individuals as well as pregnant women, unborn and newly delivered babies (Wagner and McLauchlin, 2008). Symptoms are generally flu-like including chills, headache, and fatigue but can evolve into meningitis or sepsis and cause abortions in more serious cases (Schuchat *et al.,* 1991). The disease can be treated successfully with antibiotics, however, the human mortality rate remains high (20-40%) despite these treatment options (Ryser and Marth, 1991). There are 13 serovars of *L. monocytogenes,* all of which can potentially cause disease, however, 98% of human isolates belong to serovars 4b, 1/2a, and 1/2b (Barbour *et al.,* 2001), with the two largest recent outbreaks in Canada (Gilmour *et al.,* 2010) and the US (Laksanalamai *et al.,* 2012) belonging to mainly 1/2a strains. Consumption of contaminated foods is believed to be the principle route of infection but transmission by either direct contact with the environment, infected animals, or by cross-infection between patients is also possible (Farber and Peterkin, 1991). The minimal infectious dose for listeriosis is unclear and most likely varies between individuals, however, it is generally accepted that levels below 100 colony-forming units (CFUs) per gram of food are very unlikely to cause disease (Health Canada, 2011).

L. monocytogenes is particularly a problem in many Ready-to-Eat (RTE) foods where the products are not further processed (cooked) before consumed. The precautions often imposed (refrigeration, 2-5% NaCl, low pH) in RTE foods to reduce the risk of food-borne illness, pose little threat to *L. monocytogenes*. More than 60% of all food recalls in

the United States between 1996 and 2000 were a result of detecting *L. monocytogenes* contamination (Wong *et al.,* 2000). Foods, which have been implicated in these outbreaks, include soft cheeses, hot dogs and other processed meats, seafood, cantaloupe, radishes, and cabbage to name a few (Farber and Peterkin, 1991).

L. monocytogenes can grow between pH 4.3 and 11 (Skandamis *et al.,* 2008; Vasseur *et al.,* 2001), in salt levels up to 14% (Shabala *et al.,* 2008), and between 0 and 45°C (Walker *et al.,* 1990; Le Marc *et al.,* 2002) with optimal growth at 37°C (Le Marc *et al.,* 2002). Perhaps due to its versatility, the bacteria has been isolated from a diverse range of environments including soil, water, foods, wildlife, domestic animals, and humans (Gelbíčová and Karpíšková, 2012; Piffaretti *et al.,* 1989). Contamination of food can occur before harvest, especially fresh produce since soil or irrigation water can become contaminated by feces from animals such as birds, mammals, fish and invertebrates, which have been reported to carry the bacteria in their feces without any apparent disease (Nightingale *et al.,* 2004). After harvest, food materials can become contaminated in processing facilities, retail establishments, or consumer homes although early evidence pointed toward the majority of outbreaks resulting from contamination within food processing facilities (Tompkin *et al.,* 1999).

Once introduced into a processing plant, *L. monocytogenes* can persist for years, despite the harsh environmental conditions associated with food processing and preservation, and sanitary efforts (Lunden *et al.,* 2000). Recently it was shown that *L. monocytogenes* can survive desiccation for three months on stainless steel surfaces in a simulated food processing environment, suggesting a link between persistence and desiccation tolerance (Vogel *et al.,* 2010). In addition, persistent strains have been shown to adhere to food contact surfaces at significantly higher levels compared to non-persistent

strains (Lunden *et al.,* 2000; Norwood and Gilmour, 1999). Given that attachment plays a large role in initiating biofilm formation, it is not surprising that persistent strains also show enhanced biofilm forming abilities (Borucki *et al.,* 2003; Møretrø and Langsrud, 2004). Furthermore, the presence of a biofilm protects cells from desiccation, UV light, and treatment with antimicrobial and sanitizing agents, and subsequently promotes the cross-contamination of foods (Truelstrup Hansen and Vogel, 2011; Pozos *et al.,* 2004; Pan *et al.,* 2006). The combined ability of *L. monocytogenes* to adhere to and form biofilms on solid surfaces, together with its desiccation tolerance, make this pathogen difficult to defeat in the food industry.

2.2. *L. monocytogenes* Biofilm Formation in the Food Industry

Biofilms are commonly defined as communities of microorganisms attached to a surface or interface that produce an extracellular matrix of polymeric substances (EPS) in which cells are embedded (Costerton *et al.,* 1995; Stoodley *et al.,* 2002). They can consist of single or multiple microbial species with the latter being predominant in most environments. In the food industry, the attachment and subsequent biofilm formation of pathogenic bacteria on food contact surfaces is of great concern since cells can detach from these communities and cross-contaminate foods (Brooks and Flint, 2008). *L. monocytogenes* and other natural microflora are capable of forming biofilms in "sanitation-dead" corners of food-processing plants such as air or liquid filtration systems, stainless steel surfaces, rubber or Teflon seals, and machine joints (Kumar *et al.,* 1998; Lee Wong, 1998). Where there are metallic surfaces, biofilms can cause biocorrosion resulting in costly damage to pipes and other surfaces (Beech and Sunner, 2004). Furthermore, biofilm bacteria may develop other properties including increased resistance

to UV light, increased rate of genetic exchange, altered biodegradative capabilities, and increased secondary metabolite production (Goodman *et al.,* 1994; Møller *et al.,* 1998; Wolfaardt *et al.,* 1994; Gilbert *et al.,* 2002; Mah and O'Toole, 2001). Understanding the conditions which promote or induce biofilm formation and the mechanisms involved in EPS production and cell organization, are key to locating and eliminating biofilms in areas of food production.

2.2.1. Initial Attachment to Surfaces

Bacteria have the ability to attach to both biotic and abiotic surfaces, however, biofilm formation by *L. monocytogenes* and other food-borne pathogens is most commonly a problem on abiotic processing surfaces. *L. monocytogenes* has been shown to attach to stainless steel, aluminum, rubber, silicone, glass, polyvinyl chloride, polyurethane, and polycarbonate surfaces to name a few (Beresford *et al.,* 2001; Chae *et al.,* 2006; Mafu *et al.,* 1990). Not a lot is known about the mechanisms governing this adhesion but several studies have shown that it partly depends on the nature of the surface (roughness, hydrophobicity, charge) and the surface properties of the cell (Carballo *et al.,* 1992; Hood and Zottola 1995).

The first step in the attachment of bacteria to solid surfaces involves the bacteria being transported close enough to allow initial attachment to take place (Palmer *et al.,* 2007). This includes overcoming van der Waals forces, electrostatic forces, and hydrophobic interactions (Carpentier and Cerf, 1993; Gilbert *et al.,* 1991; Van Loosdrecht *et al.,* 1987). During this initial attachment bacteria can be easily removed by fluid shear forces such as rinsing (Marshall *et al.,* 1971). The next step is the irreversible attachment of cells to the surface and may involve the production of exo-polysaccharides or specific

ligands such as pili or fimbriae (Dunne, 2002). At this point, much stronger physical or chemical forces are required to remove the bacteria from the surface (Palmer *et al.,* 2007). It is believed that the transition from reversible attachment to irreversible involves the use of covalent and hydrogen bonding as well as hydrophobic interactions (Kumar and Anand, 1998). These forces occur as a result of cells either donating or accepting electrons from the surface (Palmer *et al.,* 2007).

2.2.1.1. Cell Surface Charges

Bacterial cells generally have a net negative charge on their cell wall at neutral pH (Rijnaarts *et al.,* 1995). The magnitude of this charge is referred to as the zeta-potential of a cell and this varies from species to species and is most likely influenced by culture conditions (Gilbert *et al.,* 1991; Kim and Frank, 1994), age of the culture (Walker *et al.,* 2005), ionic strength (Dan, 2003), and pH (Husmark and Rönner, 1990). A bacterial strain of *Stenotrophomonas maltophilia* possessing an unusual positive zeta-potential, demonstrated high levels of attachment on glass and Teflon, both of which have a negative surface charge (Juker *et al.,* 1996). However, as the ionic strength of the suspending medium was increased, attachment efficiency decreased and the zeta potential became negative, suggesting that high ionic strength suppresses or overwhelms the natural surface charge of bacteria. Mafu *et al.,* (1990) also concluded that high ionic strengths suppressed electrostatic interactions between *L. monocytogenes* and various inert surfaces. One explanation for this phenomenon is that high ionic strength medium has more ions available to shield and neutralize the surface charges of cells (Palmer *et al.,* 2007).

Cell surface charge can also be influenced by the pH of the suspending medium. Husmark and Rönner (1990) demonstrated that *Bacillus cereus* spores maximally attached

to both hydrophobic and hydrophilic glass when the pH of the suspending medium was equal to the isoelectric point of the *Bacillus* spores (pH 3) making the cell surface neutral. Above a pH of 4, attachment to the hydrophilic glass decreased greatly as a result of electrostatic repulsion between the spore surface and the glass which were both negatively charged. This is supported by Smoot *et al.* (1998) who compared the attachment of *L. monocytogenes* to both stainless steel and Buna-N rubber at pH values ranging between 4 and 9 and reported decreasing attachment under alkaline conditions. Similarly, Briandet *et al.* (1999) found that pre-culturing *L. monocytogenes* cells in lactic acid supplemented media increased the bacterium's attachment to stainless steel. Mafu *et al.* (1991) reported that when the pH of the medium was decreased, the hydrophobicity of *L. monocytogenes* Scott A increased.

Hydrophobic interactions have been widely suggested to play a role in cell adherence to surfaces (Hood and Zottola, 1995). Reid *et al.* (1999) and Millsap *et al.* (1997) determined that cells with high surface hydrophobicity tended to have higher nitrogen/carbon ratios whereas cells with hydrophilic surfaces had higher oxygen/carbon ratios. Therefore, it appears that the presence of surface proteins increases cell surface hydrophobicity. In addition, Chavant *et al.* (2002) showed that stationary phase cultures and cultures grown at higher temperatures generally expressed increased surface hydrophobicity. This may explain why Smoot *et al.* (1998) observed increased attachment of *L. monocytogenes* to stainless steel and Buna-N rubber at 45°C compared to 10°C. On the other hand, Briandet *et al.* (1999) and Giovannacci *et al.* (2000) reported that *L. monocytogenes* incubated at lower temperatures (< 8°C) showed a weaker negative charge compared to cells incubated at 15, 20, or 37°C at neutral pH. Several authors have mentioned that *L. monocytogenes* lacks an isoelectric point over the pH range 2-7

(Briandet *et al.,* 1999; Giovannacci *et al.,* 2000). Rijnaarts and colleagues (1995) hypothesized that this may be linked to the presence of phosphate groups with a very low pKa (< 2.1) in the phosphodiester bridges of cell wall teichoic acids. Chae *et al.* (2006) measured the surface hydrophobicity of 21 *L. monocytogenes* strains and found that the most strains were either moderately hydrophilic or hydrophobic with a few strains showing strong hydrophilic or hydrophobic surfaces. However, the zeta potentials of all strains remained negative. This suggests that *L. monocytogenes* has a dynamic cell surface that constantly changes in response to the environment and no one factor may be responsible for the adherence of *L. monocytogenes* to inert surfaces.

2.2.1.2. Influence of Surface Type and Roughness

Just as the bacterial surface charges impact cellular adherence to materials, so does the charge of the material surface. Exercising the laws of attraction, bacteria with an overall negative surface charge should attach more to positively charged surfaces, intermediately to hydrophobic surfaces, and poorly to negatively charged surfaces. In general, stainless steel and glass are considered to be naturally negatively charged (hydrophilic) whereas rubber and plastic are considered to be hydrophobic (Bower *et al.,* 1996). However, research shows that the hydrophilic nature of stainless steel differs between types (e.g. 316 is more hydrophilic than 314) and can also possess regions of hydrophobicity (Bower *et al.,* 1996) which may aid in explaining some of the contradicting results from similar attachment studies.

Sinde and Carballo (2000) found that *L. monocytogenes* strains adhered in higher numbers to more hydrophobic materials and attached the least to stainless steel surfaces. Similarly, Rodriguez and colleagues (2008) reported that *L. monocytogenes* biofilms

adhered more strongly to hydrophobic surfaces than hydrophilic surfaces (increased attachment to polyethylene compared to glass). These results confirm the general hypothesis that stainless steel is the best material for food contact. However, Helke *et al.* (1993) found that *L. monocytogenes* showed similar attachment to both stainless steel and rubber surfaces. Furthermore, when Beresford and colleagues (2001) investigated the attachment of *L. monocytogenes* to 17 different food-use approved materials representing metal, rubber and polymers, they found attachment to be greatest on stainless steel 405 and the least on polypropylene. Smoot *et al.* (1998) also reported that *L. monocytogenes* attached more quickly to stainless steel than Buna-N rubber but interestingly, cell detachment from Buna-N rubber was significantly lower than from stainless steel. Blackman and Frank (1996) formed *L. monocytogenes* biofilms at 21°C in TSB on stainless steel, Teflon, nylon, and polyester floor sealant and found that biofilm formation was greatest on the polyester floor sealant (negative/neutral) and least on the nylon (positively charged). Again these results are the opposite of what would be expected for a negatively charged cell but the TSB medium most likely created a conditioning film that interfered with charges on both the cell and material surfaces.

In addition to the hydrophilic/hydrophobic nature of a surface, the roughness is also important to consider in attachment studies. Stainless steel for example, may appear smooth but under SEM examination many cracks and crevices can be observed, which can provide a greater area for cell attachment and protection from cleaning chemicals and fluid forces (Palmer *et al.,* 2007). Verran and Whitehead (2006) reported that surfaces with scratches and crevices similar in size to bacterial cells retained higher numbers of cells than materials with much larger surface depressions. This was demonstrated by Marsh *et al.* (2003) who found that the most structurally complex biofilms formed by *L.*

monocytogenes were in the crevices of a stainless steel surface. Greater cell attachment has also been observed on surfaces with increased roughness (Leclercq-Perlat and Lalande, 1994; Pedersen, 1990). Arnold and Bailey (2000) found that electro-polished stainless steel showed significantly less bacterial attachment and slower subsequent biofilm formation.

2.2.1.3. Impact of Conditioning Films

When there is a bulk flow of organic and/or inorganic molecules over a surface, the molecules are carried toward the surface by either diffusion or turbulent flow and form what is known as a conditioning film (Kumar and Anand, 1998). These attached molecules may alter the physical-chemical properties of the surface including free energy, hydrophobicity and electrostatic charges that may subsequently impact bacterial attachment (Dickson and Koohmaraie, 1989). Skim milk has been found to reduce the attachment of *Staphylococcus aureus*, *L. monocytogenes*, *Serratia marcescens*, and thermophilic bacilli to stainless steel (Parkar *et al.,* 2001; Barnes *et al.,* 1999). Similarly, *L. monocytogenes* and *Salmonella* Typhimurium exhibited lower attachment to stainless steel and Buna-N in the presence of skim, 2% whole, 2% chocolate, and whole milk as well as in the presence of the milk components casein, alpha-lactalbumin, and beta-lactoglobulin, compared to phosphate buffered saline (PBS) (Helke *et al.,* 1993). Similar results were seen when surfaces were pretreated with milk and milk components for 1 h prior to attachment in PBS and when cells were pretreated with skim milk or beta-lactoglobulin prior to attachment in PBS. Kim and Frank (1994) found that *L. monocytogenes'* attachment to stainless steel was enhanced in the presence of chemically defined minimal media compared to in TSB. Organic materials may compete with bacteria

for binding sites, thus reducing overall cell attachment to surfaces. In contrast, whey proteins or lactose on stainless steel or rubber surfaces has been reported to increase the attachment of milk associated microorganisms (Speer and Gilmour, 1985). Similar results were seen by Verran and Whitehead (2006) who reported that proteinaceous material such as bovine serum albumin on inert surfaces increased the number of retained cells after a cleaning cycle in comparison to cells being retained on a clean surface. These contradicting results are most likely due to the impact of the culture media on cell surface charges, the surfaces used, and the different organic molecules used to create the conditioning films.

2.2.1.4. Role of Flagella and Extracellular Fibrils in Adhesion to Surfaces

Several studies have investigated the role of flagella and fimbriae in bacterial attachment to surfaces. While a number of these studies suggest that flagellum-mediated motility is required for bacterial attachment, an equal number have concluded that this may not be true. Lemon *et al.* (2007) observed that both a non-motile and a non-flagellated *L. monocytogenes* mutant attached at significantly lower levels to glass coverslips than the parent strain. Pratt and Kolter (1998) on the other hand demonstrated that motility but not chemotaxis, was critical for *E. coli* attachment and subsequent biofilm formation on abiotic surfaces. Vatanyoopaisarn *et al.* (1999) observed that at 22°C a flagellin *L. monocytogenes* mutant attached to stainless steel at levels 10-fold lower than the parent strain even under conditions preventing active motility (growth in nutrient limited PBS). At 37°C when flagella were not produced, attachment of both strains was identical. The authors concluded that flagella, independent of cell motility, act as adhesive structures during early stages of attachment under static conditions. However, they also found that the flagellin

mutant attached significantly less at 37°C than at 22°C, indicating that surface structures other than flagella contribute to attachment.

Herald and Zottola (1988) found that *L. monocytogenes* produced extracellular fibrils when attached to stainless steel at 21°C but not at 10 or 35°C. Mafu *et al.* (1990) also noted the production of extracellular materials by *L. monocytogenes* on surfaces (especially glass and polypropylene) when incubated at 4 and 20°C for 1 h. Chae *et al.* (2006) reported that *L. monocytogenes* cells attached to glass coverslips produced significantly more extracellular carbohydrates than planktonic cells after a 3 h period and that these high levels also promoted the formation of a biofilm at 37°C.

2.2.2. General Characteristics of Biofilms

There are four main steps in biofilm formation; (1) conditioning of the surface by macromolecules, (2) initial adherence, (3) physical irreversible adherence that involves the production of exopolymers, and (4) growth of the microorganisms and formation of EPS within microcolonies leading to a mature biofilm structure (Chavant *et al.,* 2002). Such structures are dynamic systems in which cells grow, die, and/or are released (Costerton *et al.,* 1987), allowing cross contamination of other areas including other surfaces, liquids, and the air. The initiation step triggering biofilm development is usually in response to a specific environmental cue such as nutrient availability; however, signals may vary among organisms (Kolter *et al.,* 1993). Bacteria undergo a transition from free, planktonic cells to sessile, surface-attached cells when ample nutrients are provided. Biofilms will continue to develop as long as fresh nutrients are continuously supplied (Kolter *et al.,* 1993). Once the nutrient source has become depleted, cells will detach and return to a planktonic mode of growth, presumably to search for a fresh source of nutrients (Kolter *et al.,* 1993).

Therefore it is thought that the starvation response pathway plays an important role in the biofilm developmental cycle.

The components of EPS differ between bacterial species but it is generally accepted that the main component is extrapolysaccharides but may also include proteins, lipids, nucleic acids, and other biopolymers (Flemming and Wingender, 2010). Mature biofilms can have very complex architectural features that can be studied using scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM). In addition, Fourier-transform infrared (FT-IR) spectroscopy and Raman microscopy are two common techniques used to identify microorganisms and other substances inside physiological intact microbial communities (Ngo-Thi *et al.,* 2003; Pätzold *et al.,* 2006). To form these structures, microorganisms must integrate external and internal signals, be aware of their neighbours, and coordinate a timed series of multicellular behaviours (O'Toole *et al.,* 2000). This cell-to-cell signaling is known as quorum sensing (QS) (Xavier and Bassler, 2003) and will be discussed in more detail below.

2.2.3. Biofilms Formed by L. monocytogenes

L. monocytogenes strains vary in their ability to adhere and form biofilms on surfaces (Boruki *et al.,* 2003; Kalmokoff *et al.,* 2001; Chae and Schraft, 2000; Pan *et al.,* 2010). Borucki and colleagues (2003) found that *L. monocytogenes* serotypes 1/2a and 1/2c as well as persistent strains from milk samples, showed increased biofilm formation under static conditions in comparison to other serotypes and non-persistent strains, respectively. Similarly, Pan *et al.* (2010) also concluded that serotype 1/2a strains generally formed higher density static biofilms than 4b strains. The opposite was reported by Djordjevic *et al.* (2002) where lineage I strains (1/2b and 4b), which contained a large

number of human listeriosis isolates, produced more biofilm than lineage II (1/2a, 1/2c) and III strains (4c, 4a), suggesting a correlation between the ability to form a biofilm and virulence. Di Bonaventura *et al.* (2008), however, found no differences in the static biofilms formed by 44 strains of *L. monocytogenes* with regard to lineage, source (environmental vs. food), and origin (fish vs. meat). On the other hand, some authors have reported that *L. monocytogenes* does not form a biofilm but merely adheres as single cells to surfaces via extracellular fibers (Kalmokoff *et al.,* 2001; Hood and Zottola, 1997a). These differences are most likely due to the experimental conditions used in the studies including time, temperature, inoculation level, surface type, strains tested, and the media used.

Marsh *et al.* (2003) described a three-tiered approach to differentiate the attachment and subsequent biofilm forming abilities under static conditions of various outbreak related strains of *L. monocytogenes* on stainless steel surfaces via SEM imagery. The first stage was described as the formation of microcolonies on the surface. The second stage is the organization of microcolonies into a net-like pattern which in time develops into a honeycomb-like structure containing individual *L. monocytogenes* cells surrounded by EPS. In the last stage the biofilms thicken resulting in a complex three-dimensional structure. The authors noted that biofilm complexity differed between replicates, possibly due to the randomness of the initial event which triggers biofilm formation. Similarly, biofilms were also not uniform across a single surface in the same experiment. Some areas showed mature biofilm coverage whereas others were still developing. The latter may be a result of cells detaching during SEM preparation leaving behind residual EPS (Marsh *et al.,* 2003). The EPS observed in the biofilms was very thread-like, and connected cells to each other as well as to the stainless steel surface. The honeycomb structure was composed

of cells stacked upon each other around and between holes with the stringy EPS anchoring cells in place. This structure has also been reported by Thar and Kuhl (2002) and is thought to provide strength against shear forces, limit the energy costs of individual cells, and maximize the surface area for nutrient absorption (Schaudinn *et al.,* 2007). The holes in the honeycomb-like architecture likely serve as empty channels for which water, nutrients, and waste can be transported. This biofilm architecture is much different from the traditional "mushroom" structure which has been described in *Staphylococcus spp.* where cells are completely embedded in thick, gummy, EPS (Götz, 2002).

The structure of *L. monocytogenes* biofilms formed under dynamic conditions (flow cells) differ from those formed under static conditions. Unlike the honeycomb described structure under static conditions, biofilms formed under dynamic conditions exhibit dense, ball shaped microcolonies separated by poorly colonized zones and composed of short rod chains of cells that form a knitted network (Rieu *et al.,* 2008b). Overall, dynamic biofilms also show greater volume and thickness (Rieu *et al.,* 2008a; 2008b).

2.2.3.1. Extracellular Polymeric Substances

The extracellular polymeric substances (EPS) synthesized by microbial cells vary greatly in their composition and hence their chemical and physical properties. EPS is also commonly referred to as extrapolysaccharides based on the production of alginate, colonic acid, and other carbohydrates by many Gram-negative biofilm forming bacteria (Lux *et al.,* 2004; Tielen *et al.,* 2005; Danese *et al.,* 2000). Less is known about the components of Gram-positive EPS; however, Hussain *et al.* (1993) found that biofilm EPS formed by coagulase-negative *Staphylococcus epidermidis* in a chemically defined media was a

mixture of 80% (w/w) teichoic acid and 20% protein. Since EPS traps both cells and cell products in the biofilm matrix, other molecular species such as nucleic acids, glycoproteins, and phospholipids may also be present (Sutherland, 2001). The amount of EPS synthesized within a biofilm will depend greatly on the availability of carbon substrates both inside and outside the cell (Sutherland, 2001).

Very little is known about the components of *L. monocytogenes* biofilm EPS. Borucki *et al.* (2003) and Zameer *et al.* (2010) have both used ruthenium red staining to reveal the presence of extracellular carbohydrates surrounding *L. monocytogenes* biofilm cells. However, since the dye may also bind to carbohydrates of the *Listeria* cell wall, these results are not conclusive. When *L. monocytogenes* adhered to stainless steel and synthetic rubber was treated with trypsin, attachment was reduced by 99% (Smoot and Pierson, 1998). Furthermore, *L. monocytogenes* biofilm detachment occurred following treatment with endopeptidase K (Franciosa *et al.,* 2009).

Recently, Harmsen *et al.* (2010) showed that the biofilm matrix of *L. monocytogenes* contains extracellular DNA (eDNA) that plays an important role in initial adhesion and in the early stages of biofilm formation. The presence of eDNA could be a result of either cell lysis (Qin *et al.,* 2007; Rice *et al.,* 2007) or vesicle release (Whitchurch *et al.,* 2002), whereas active transport is a more speculative explanation. The addition of DNaseI to surface attached *L. monocytogenes* cells significantly reduced cellular attachment and subsequent biofilm formation, whereas enzymatic removal of both RNA and proteins had no impact on cell adhesion (Harmsen *et al.,* 2010). The addition of high molecular weight (MW) eDNA did not restore attachment however, when combined with culture supernatant treated with DNaseI and proteinase K, high MW eDNA could. The addition of peptidoglycan to DNaseI treated bacteria also restored initial attachment. When

low but not high molecular weight eDNA was added alongside peptidoglycan to the DNaseI treated cells, attachment was decreased. This suggests that short DNA segments may act as inhibitors of components involved in adhesion (Harmsen *et al.,* 2010). The strong reliance of *L. monocytogenes* on eDNA for adhesion and further biofilm development could be a consequence of cells lacking the mechanisms to produce structural components such as extracellular polysaccharides (Harmsen *et al.,* 2010). The authors also observed a decrease in the efficiency of DNaseI over time, possibly indicating that additional structural component are produced during the later stages of biofilm formation. Furthermore, an *L. monocytogenes* putative DNA translocase mutant (lmo1386) showed reduced biofilm formation compared to the wildtype (Chang *et al.,* 2012).

Lastly, a transcriptome study by Hefford *et al.* (2005) comparing *L. monocytogenes* (Lm568) biofilm-grown and planktonic cells, identified protein and sugar metabolism genes that are more prevalent in biofilm-grown cells. A number of these genes encode glycolytic enzymes (fructose-1,6-bisphosphate aldolase, triose phosphate isomerase, enolase, phosphoglycerate mutase, phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase), as well as alternative energy generating mechanisms such as the B chain of an H^+ transporting ATP synthase and the A chain of flavocytochrome *c* fumarate reductase, which allows the use of fumarate as an alternative electron acceptor under conditions of oxygen limitation (Hefford *et al.,* 2005). Other proteins were related to active growth including FtsZ required for formation of the Z-ring during septation (Jacobs and Shapiro 1999), MreB required for maintaining the rod shape (Ayako *et al.,* 1992), and tetrahydrodipicolinate succinylase required for synthesis of the pentapeptide bridge of peptidoglycan (Beaman *et al.* 1997).

2.2.3.2. Quorum Sensing

Cell-to-cell signaling during biofilm formation is known as quorum sensing (QS) and is defined as "a process of bacterial cell-to-cell communication involving the production and detection of extracellular signaling molecules called autoinducers" (Xavier and Bassler, 2003). In order for this to occur there is a threshold cell density necessary such that a sufficient quantity of the signaling molecule will be produced (Bassler, 2002). The autoinducer produced by Gram-positive bacteria is typically an oligopeptide and signaling between cells is via a two-component phosphorelay system which has also been shown to regulate virulence in *Staphylococcus aureus* and sporulation in *Bacillus subtilis* (Bassler, 2002). Ermolaeva *et al.* (2004) discussed the presence of a diffusible, low molecular weight, hydrophobic autorepressor that restricts the expression of the PrfA virulence regulon in *L. monocytogenes* via a QS mechanism. *L. monocytogenes* has also been shown to possess a QS system showing high similarity to the *agr* system in *S. aureus* (Autret *et al.,* 2003).

The four-gene *agr* operon encodes four proteins; AgrB is responsible for export and proteolytic processing of the QS peptide AgrD, while AgrC (histidine kinase) and AgrA (response regulator) comprise a two-component signal transduction system (Novick, 2003). ArgD signaling has also been found to regulate extracellular proteases necessary for biofilm dissolution (Boles and Horswill, 2008). Mutants of *agrA* and *agrD* in *L. monocytogenes* showed decreased biofilm forming abilities in both dilute and nutrient-rich media (Rieu *et al.,* 2007). In addition, exponential but not stationary phase cells of the *agrD* mutant showed significantly reduced invasion of Caco-2 cells. The invasion of Caco-2 cells is mediated by internalin A (InlA) (Mengaud *et al.,* 1996) and the *agrD* mutant showed decreased expression of *inlA* during growth and reduced levels of InlA in the cell

wall. InlA expression is maximal during exponential growth and decreases upon entry into stationary phase (Dramsi *et al.,* 1993), which explains why exponential phase cells of the *agrD* mutant demonstrated decreased virulence

Another interspecies communication signal molecule found in *L. monocytogenes* is autoinducer 2 (AI-2), which is produced by the enzyme LuxS. LuxS has been associated with biofilm formation, pathogenicity, and motility in a number of Gram-negative and -positive pathogenic bacteria (Daines *et al.,* 2005; McNab *et al.,* 2003; Prouty *et al.,* 2002; Tannock *et al.,* 2005; Wen and Burne, 2002; Xu *et al.,* 2006; Tian *et al.,* 2008). The *luxS* gene encodes S-ribosylhomocysteinase, an enzyme which catalyzes the hydrolysis of S-ribosylhomocysteine (SRH) to homocysteine and 4,5-dihydroxy-2,3-pentadione (DPD), which serves as a precursor for AI-1 (Rajan *et al.,* 2005). Both Sela *et al.* (2006) and Belval *et al.* (2006) concluded that *L. monocytogenes luxS* deficient mutants produced much denser biofilms than the parent strain. It appears that an intact *luxS* gene represses components required for attachment and biofilm formation. Belval *et al.* (2006) found that the *luxS* mutant contained larger quantities of the AI-1 precursors and that SRH in particular could increase the number of attached cells and may be the reason for the increase in biofilm formation.

2.2.4. Effect of Environmental and Physiological Factors on Biofilm Formation

Any study of biofilms must appreciate that biofilms develop in an enormous number of environments and the structure of any one biofilm formed under a specific set of parameters, is usually unique to that single environment and microflora (Sutherland, 2001). As so, many environmental factors have varying effects on *L. monocytogenes* biofilm formation in addition to those known to affect initial cell attachment.

The availability of nutrients has been described as a determining factor for whether or not cells will take on a surface attached lifestyle. Significantly denser biofilms were reported to be formed by *L. monocytogenes* in low nutrient broths (1/10 BHI, Welshimer's broth) compared to those containing high levels of nutrients (BHI, TSB) (Riedel *et al.,* 2009; Moltz, 2005). Similar results have been seen for *S. enterica* and *Salmonella* spp. (Kostaki *et al.,* 2012; Stepanovic *et al.,* 2004). However, indifferent and opposite results where high nutrients (BHI>TSA>meat broth) were more beneficial for *L. monocytogenes* biofilm development have also been reported (Kostaki *et al.,* 2012; Stepanović *et al.,* 2004).

To further determine the specific nutrient needs of biofilm growing cells, studies have focused on individual molecules which may impact biofilm development. Pan *et al.* (2010) found that all *L. monocytogenes* strains tested (serotypes 1/2a and 4b) showed enhanced biofilm formation in TSB when the salt concentration was increased from 0.5% to 7.0% at 22.5 and 30°C but not at 37°C. Optimal salt concentrations were 5% at 22.5°C and 2% at 30 and 37°C. The majority of strains formed denser biofilms at all three temperatures with the addition of glucose in a range from 1-10%. Furthermore, the combination of salt and glucose resulted in the densest biofilms. Ethanol was also found to enhance biofilm formation in some strains at 22.5°C whereas at 37°C it appeared to have an inhibitory effect (Pan *et al.,* 2010). Similarly, Gravesen *et al.* (2005) reported that sublethal concentrations of ethanol or isopropanol increased *L. monocytogenes'* attachment to polystyrene at 10, 20, and 30°C but not at 37°C. Kim and Frank (1995) concluded that both mannose and trehalose enhanced *L. monocytogenes* biofilm development at 21°C on glass slides however, in contrast to the previous study 1-20 g/l of glucose did not have any effect. Lastly, Sandasi *et al.* (2008) found that the addition (1

mg/ml) of five common antimicrobial essential oil components (α-pinene, 1,8-cineole, (+)-limonene, linalool and geranyl acetate) individually to *L. monocytogenes* 6 h preformed biofilm on PVC significantly enhanced biofilm growth. Conversely, the presence of these antimicrobial oils caused a reduction in the metabolic activity of the biofilm after 6 h of incubation. On the other hand, Oliveira *et al.* (2010) found that treatment of a 240 h formed biofilm for 60 min with a combination of *Cymbopogon nardus*, and *C. citratus* essential oils was capable of reducing 100% of the biofilm cells, suggesting that the type of oil and the contact time are important determinants for the efficiency of antimicrobial plant oils.

Determining the impact of temperature on biofilm formation can be difficult due to the corresponding change in growth rates of cells at these temperatures. Although several authors have concluded that growth rate is not directly correlated with biofilm formation (Chae and Schraft, 2000; Djordjevic *et al.,* 2002; Pan *et al.* 2010), it is inevitable that most cells will multiply faster at warmer temperatures (e.g. 32-37°C). At 8°C Chavant *et al.* (2002) concluded that *L. monocytogenes* does not form a biofilm but merely attaches to surfaces. Other studies refer to surface growth at low temperatures as biofilms but in fact they are at most single layers of cell coverage showing the beginning stages of fibril formation (Di Bonaventura *et al.,* 2007). As mentioned earlier, temperature also impacts bacterial surface properties that can impact attachment and subsequent biofilm formation on certain materials. Di Bonaventura *et al.* (2008) found that at 4, 12, and 22 °C *L. monocytogenes* formed higher levels of biofilm on glass compared with polystyrene and stainless steel; however, at 37°C higher levels of biofilm formed on glass and stainless steel compared to polystyrene. Interestingly, cell hydrophobicity was also found to be significantly higher at 37°C than at 4, 12, and 22°C. Chae *et al.* (2006) however, found that attachment at 37°C on glass was independent of hydrophobicity level but nonetheless

cell surface hydrophobicity appeared to increase with temperature. Chavant *et al.* (2002) confirmed this trend, noting that *L. monocytogenes* was strongly hydrophilic at low temperatures, suggesting that modifications in the cell wall composition had occurred. It is well known that bacteria can maintain membrane fluidity at low temperatures by modifying cell wall lipid composition to include those with lower melting points, whereas the opposite is seen when bacteria are exposed to higher temperatures (Hébraud and Potier, 1999; Russell, 2002; Püttmann *et al.,* 1993; Rilfors *et al.,* 1978).

As previously discussed, some authors propose that flagella might act in biofilm formation as both a surface adhesive and as a provider of force-generating motility. A study by Lemon and colleagues (2007) concluded that *L. monocytogenes* flagellin and *motB* mutants defective in flagella biosynthesis and motility respectively, were defective in both biofilm formation and initial attachment compared to the parent strain. On the other hand, a recent study has shown that non-motile but flagellated and non-flagellated *L. monocytogenes* mutants are both capable of forming hyperbiofilms under dynamic but not static conditions (Todhanakasem and Young, 2008). These mutants originally displayed reduced initial surface attachment but within 1 h of medium flowing through the cell chamber, bacteria progressively colonized the surface, surpassing the wildtype by 24 h. The exact reason that hyperbiofilms are formed by these mutants remains unknown.

2.3. Stress Response of *L. monocytogenes* to Adverse Environmental Conditions

L. monocytogenes encounters several different stressful environmental conditions as it is transferred along the food supply chain. The cells depend on various stress-sensing mechanisms to detect situations which may be detrimental to their survival. A number of responses to environmental conditions including cold, heat, osmotic, acid, alkaline, and

oxidative stress, share similar adaptation methods. A brief review of some of these mechanisms in response to general and specific stresses is provided below.

2.3.1. General Stress Response Mechanisms

2.3.1.1. Two-Component Systems (TCS)

To efficiently adapt to environmental changes, bacteria receive and transmit appropriate stimuli using signal transduction systems. Of these systems, the two-component signal transduction systems (TCS) are the most widespread in bacteria and can sense changes in the environment including low pH, and osmotic, oxidative, and ethanol stress (Bourret, 1995; Kallipolitis and Ingmer, 2001). TCSs consist of a membrane-associated histidine kinase (HK), and a response regulator (RR) that enables the cell to respond by altering gene expression (Cotter *et al.,* 1999). When exposed to a specific environmental stimulus, a histidine residue in the HK becomes autophosphorylated and the phosphoryl group is then transferred to an aspartic acid residue in the RR causing changes in the transcriptional levels of target genes (Kallipolitis and Ingmer, 2001). Two well-known TCSs in *L. monocytogenes* are LisRK and KdpE. LisRK consists of *lisR* and *lisK* which encode the RR and HK, respectively. This system has been shown to respond to stresses such as low pH and regulate virulence gene expression (Cotter *et al.,* 1999). KdpE on the other hand is an uptake system involved in the inward transport of potassium (K^+) in response to osmotic stress (Kallipolitis and Ingmer, 2001). Another two-component regulatory system CheA/Y is involved in transducting chemotaxis signals (Dons *et al.,* 2004).

2.3.1.2. Global Regulatory Proteins

Global regulatory proteins regulate the genome-wide expression of genes encoding enzymes and transport proteins in response to changes in their environment. Alternative sigma factors are bacterial transcription initiation factors that enable specific binding of RNA polymerase to gene promoters. Every molecule of RNA polymerase holoenzyme contains exactly one sigma factor subunit which allows it to correctly identify initiation sites. In the *L. monocytogenes* EDGe strain four alternative sigma factors have been identified (σ^B, σ^C, σ^H, σ^L) (Glaser *et al.,* 2001). Sigma B, encoded by *sigB,* is the most studied alternative sigma factor and has been shown to regulate more than 150 genes (Raengpradub *et al.,* 2008). Sigma B is a well-known regulator of general stress responses in Gram-positive bacteria such as *Listeria*, *Staphylococcus*, and *Bacillus spp.* (Becker *et al.,* 1998; Haldenwang, 1995). In *L. monocytogenes* σ^B responds to acid stress, oxidative and osmotic stress, carbon starvation, and growth at low temperatures (Becker *et al.,* 1998; Ferreira *et al.,* 2003; Wiedmann *et al.,* 1998). More specifically it has been shown to be involved in transcriptional regulation, cell transport, envelope modifications, metabolism, protein synthesis and modification, virulence-associated functions, and osmolyte transport (Raengpradub *et al.,* 2008). The alternative sigma factor σ^L also known as σ^{54} and encoded by the *rpoN* gene, is known to regulate the expression of 77 genes involved in carbon and nitrogen metabolism, flagella biosynthesis, and virulence (Studholme and Buck, 2000; Arous *et al.,* 2004). As so, it has also been associated with cold, acid, and osmotic stress responses (Raimann *et al.,* 2009). Sigma C is an extracytoplasmic function sigma factor which is activated upon heat stress (Zhang *et al.,* 2005) whereas the remaining sigma factor σ^H may play roles in alkaline and starvation stress responses (Rea *et al.,* 2004).

CodY is a GTP-binding protein that regulates more than 100 genes, which are generally involved in adaptation to poor growing conditions and expression of stationary phase genes (Slack *et al.,* 1993; Slack *et al.,* 1995; Molle *et al.,* 2003). These genes are typically repressed during exponential phase growth and are induced when cells encounter nutrient limitation and experience reduced growth rates. They include genes involved in extracellular degradative enzymes, transport systems, catabolic pathways, genetic competence, and branched-chain amino acid and flagellin biosynthesis (Slack *et al.,* 1993; Slack *et al.,* 1995; Molle *et al.,* 2003; Fisher *et al.,* 1996; Serror and Sonenshein, 1996; Shivers and Sonenshein, 2004; Bergara *et al.,* 2003). Many of the genes under CodY control are induced by the activation of RelA, a ribosome-bound enzyme that converts GTP to the small nucleotide (p)ppGpp which is an alarmone that mediates the stringent response to nutitional stress by interacting with RNA polymerase to upregulate genes involved in amino acid biosynthesis and stress survival (Abranches *et al.,* 2009).

Another response regulator DegU, encodes a pleiotropic regulator involved in the expression of both motility at low temperatures and *in vivo* virulence in mice (Knudsen *et al.,* 2004; Williams *et al.,* 2005). The transcriptional repressor MogR also controls motility by repressing flagella motility genes at high temperature (37°C) (Gründling *et al.,* 2004). Other global regulatory proteins include PrfA and VirR which regulate a wide range of stress response and virulence gene expression (Mandin *et al.,* 2005; Chakraborty *et al.,* 1992). Hfq, an RNA-binding protein, has been implicated in *L. monocytogenes*' ability to withstand osmotic and ethanol stress and contributes to long-term survival under amino acid-limiting conditions (Christiansen *et al.,* 2004). Lastly, the transcriptional repressors CtsR and HrcA have been shown to negatively regulate heat shock genes (Hanawa *et al.,* 2000; Nair *et al.,* 2000).

2.3.2. Intracellular Stress and Regulation of Virulence

L. monocytogenes virulence potential and ability to survive harsh environmental conditions are highly linked as the bacterium has most likely acquired these mechanisms to survive intracellular stresses and cause disease. Once *L. monocytogenes* has been consumed it is subjected to an increase in temperature and must also survive the low pH of the stomach. Next, the organism is faced with harsh conditions of the intestine including high osmolarity, low oxygen, bile salts, weak acids, nutrient starvation, and cationic peptides of the innate immune system (Liu, 2008). Once cells travel to the phagocytic cells of the spleen, only a small portion will survive the strong oxidative stress imposed by the phagocytic vacuole and reach the cytoplasm where they will proliferate (Portnoy *et al.,* 1988; Tilney and Portnoy, 1989). The stress responses elicited to survive some of these conditions will be discussed below.

The virulence regulon of *L. monocytogenes* is controlled by the regulator PrfA that is activated in response to environmental cues including high temperature (37°C) (Leimeister-Wächter *et al.,* 1992), stress conditions (Sokolovic *et al.,* 1990), nutrient starvation (Ripio *et al.,* 1996), contact with host cells (Renzoni *et al.,* 1999), and changes in the cytoplasmic environment (Renzoni *et al.,* 1999; Moores *et al.,* 1999). PrfA, along with σ^B modulate the expression of several genes of the internalin family (*inl*) including *inlA* and *inlB* which are critically involved in the internalization of *L. monocytogenes* into nonphagocytic mammalian cells (Gaillard *et al.,* 1991; Lecuit *et al.,* 1997; Braun *et al.,* 1998).

2.3.3. Cold Stress Response

L. monocytogenes has the ability to grow over a wide range of temperatures (0-45°C) and its survival and growth at refrigeration temperatures (2-4°C) make the control of this pathogen difficult in foods. In order for *L. monocytogenes* cells to survive exposure to cold stress, they must overcome hardships such as increased membrane rigidity, reduced protein and enzyme activity, slow transport and nutrient uptake processes, interrupted gene expression processes, and protein damage and alteration (Soni *et al.,* 2011). The results from cold exposure transcriptome analysis studies (Chan *et al.,* 2007; Cacace *et al.,* 2010) reveal that cold stress adaptation responses in *L. monocytogenes* regulate the transcription of genes involved in 1) specific and general stress protection; 2) membrane fluidity and function; 3) resumption of gene expression activity; 4) protein folding and degradation; 5) uptake of carbon sources and cold protective nutrients; 6) oxidative stress protection; 7) energy production, and 8) specific amino acid and lipid biosynthesis pathways.

Following a downshift in the environmental temperature *L. monocytogenes* produces cold shock proteins (Csp) and cold acclimation proteins (Caps) that continue to be synthesized during balanced growth at low temperatures (Bayles *et al.,* 1996). These proteins have also showed roles in osmotic stress (Schmid *et al.,* 2009). Csp are a family of small, highly conserved, nucleic acid binding proteins that can act as RNA chaperones and minimize secondary folding (Graumann *et al.,* 1996; Graumann *et al.,* 1997), or they may act as transcriptional activators or antiterminators (Bae *et al.,* 2000; Brandi *et al.,* 1994; La Teana *et al.,* 1991). Three Csp family proteins (CspA, CspB, and CspD) have been classified in *L. monocytogenes* (Schmid *et al.,* 2009). σ^B is also activated in response to cold stress and stimulates the accumulation of compatible solutes including glycine betaine and carnitine (Angelidis and Smith, 2003). Lastly, in response to low temperatures,

L. monocytogenes changes the composition of its cell membrane to include higher levels of $C_{15:0}$ (at the expense of $C_{17:0}$) and unsaturated fatty acids (FAs), and a switch from iso to anteiso-branched chain FAs to help enhance membrane fluidity (Beales, 2004; Annous *et al.,* 1997).

2.3.4. Heat Stress Response

When bacteria are exposed to elevated temperatures denaturation of proteins occurs causing aggregation and loss of functionality and enzymatic activity. To overcome these effects the organisms must regulate cellular mechanisms to restore membrane and nucleic acid functions, remove heat degraded proteins, and produce new replacement proteins to restore metabolic functions (Liu, 2008). Genome-wide transcriptome analysis has revealed that heat stress responses in *L. monocytogenes* focus on the upregulation of genes involved in heat shock and SOS global responses to DNA damage, as well as cell division and cell wall synthesis (Hu *et al.,* 2007a, 2007b; van der Veen *et al.,* 2007). Heat shock proteins (Hsps) belong to one of two classes: molecular chaperones or adenosine triphosphate (ATP)- dependent proteases (ATPases) that aid in refolding and breaking down damaged proteins, respectively (Hu *et al.,* 2007a). The first group is controlled through HrcA and CstR transcription repression mechanisms whereas the second group is positively regulated by σ^B (Liu, 2008). To date, a number of Hsps have been identified in *L. monocytogenes*, including GroES, GroEL, DnaK, DnaJ, HtrA, and Clp proteins (Liu, 2008). Heat stress exposure also increases the production of SOS response DNA repair genes, PrfA-controlled virulence genes, and the TCS's *kpdE*, and *lisR* (Soni *et al.,* 2011). Down-regulated are genes involved in cell division and cell wall synthesis which often results in elongated cells under heat stress (van der Veen *et al.,* 2007).

Lastly, similar to cold stress, *L. monocytogenes* reacts to increases in temperature by modifying its membrane lipid composition. Decreases in the amounts of branched and medium chain FAs and increases in long chain FAs have all been associated with *L. monocytogenes* growth at increasing temperatures in attempt to raise the melting point of the lipid membrane and subsequently control membrane fluidity (Juneja and Davidson, 1993).

2.3.5. Acid Tolerance Response

L. monocytogenes encounters low pH environments both in foods and during gastric passage and in the phagosome of the macrophage (Cotter and Hill, 2003). Exposure to mild acidity (pH 5.5) is enough to induce the acid tolerance response (ATR) (O'Driscoll *et al.,* 1996). The ATR involves the synthesis of acid shock proteins (ASPs) and the involvement of other resistance mechanisms such as glutamate decarboxylase, arginine deiminase, and F_0F_1-ATPase systems (Liu, 2008). In *L. monocytogenes* the enzyme F_0F_1-ATPase uses ATP to translocate protons across the cell membrane to maintain the intracellular pH (Cotter *et al.,* 2000). Similarly, glutamate decarboxylase and arginine deiminase also function to remove amino acids from the cytoplasm (Small and Waterman, 1998).

Like the previous stresses, membrane lipid composition is altered in response to pH changes. Increases in the straight chain fatty acids C14:0 and C16:0 and decreases in levels of C18:0 have been reported in acid-adapted *L. monocytogenes* cells (van Schaik *et al.,* 1999). Other changes include significantly higher levels of anteiso-FAs and lower levels of iso-FAs (Giotis *et al.,* 2007). Similar results were reported for cells exposed to alkaline stress (pH 8 or 8.5) leading to these changes in the FA composition having been

suggested to increase membrane fluidity (Russell *et al.,* 1995) and limit alkali and detergent damage (Nielsen *et al.,* 2005; Mendonca *et al.,* 1994).

2.3.6. Osmotolerance

When bacteria are faced with high extracellular osmotic pressure the result is rapid water loss and subsequent increases in intracellular solute concentrations. Such actions can lead to the disruption of various biological processes. The accumulation of osmolytes or compatible solutes is one of the main osmotic stress adaptation strategies in *L. monocytogenes*. Compatible solutes are molecules that can be accumulated to high concentrations within cells without adversely affecting vital cellular processes (Csonka, 1989). In general, they are small, soluble molecules carrying no charge at physiological pH and function to stabilize protein structure and function while aiding to maintain cell volume during exposure to elevated osmolarity (Kempf and Bremer, 1998; Sleator and Hill, 2002). This osmoadaptation response occurs in two steps, first is the intracellular accumulation of K^+ via KdpE, followed by a rapid increase in cytoplasmic concentration of a number of compatible solutes (Patchett *et al.,* 1992). The most studied osmolytes are glycine betaine, carnitine, proline, and proline betaine with other compatible solutes including sugars, amino acids, amino acid derivatives, sulphate esters, and small peptides (Kempf and Bremer, 1998). The uptake of glycine betaine and carnitine is mediated by three transporters BetL, Gbu, and OpuC (Fraser *et al.,* 2000; Sleator *et al.,* 2003) that are all regulated at least in part by σ^B.

Another mechanism used by *L. monocytogenes* to combat osmotic stress is the alternation of gene expression leading to increased or decreased synthesis of various proteins (Liu, 2008). Some of the identified osmotic stress proteins are RelA, Ctc, HtrA,

KdpE, LisRK, ProBA, BtlA, DnaK, OpuC, GbuA, and AppA (Duché *et al.,* 2002a, 2002b; Abram *et al.,* 2008). As mentioned above, RelA is a (p)ppGpp synthetase whose product is accumulated under nutrient-limited conditions and induces the so-called stringent stress response (Gentry and Cashel, 1996). Along with its role in osmotolerance, (p)ppGpp has also been implicated in biofilm formation (Okada *et al.,* 2002; Taylor *et al.,* 2002), and is recognized as a regulatory component of σ^B in *B. subtilis* (Zhang and Haldenwang, 2003). Ctc belongs to the L25 family of ribosomal proteins and has been found to be involved in osmotic stress tolerance in the absence of any osmoprotectants in the medium (Duché *et al.,* 2002a; Duché *et al.,* 2002b; Gardan *et al.,* 2003). LisRK, like Ctc, appears to function independently of compatible solutes (Sleator and Hill, 2005) and also regulates HtrA and probably many additional systems (Stack *et al.,* 2005). DnaK functions as a heat shock protein, stabilizing cellular proteins (Gandhi and Chikindas, 2007). The remaining proteins are involved in K^+ and subsequent osmolyte uptake as previously mentioned.

Lastly, *L. monocytogenes* responds to osmotic stress by making a switch from zwitterionic lipids such as phosphatodylethanolamine (PE), to anionic lipids such as diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) (McGarrity and Armstrong, 1975; Kogut and Russell, 1984; Hosono, 1992; Russell, 1993; Russel *et al.,* 1995). These changes are necessary to prevent a membrane phase transition that would disrupt membrane function.

2.4. Desiccation Survival of *L. monocytogenes* and Other Bacteria

Recently, *L. monocytogenes* has been shown to survive desiccation for three months in a simulated food processing environment (Vogel *et al.,* 2010). Furthermore, the same strains have been repeatedly isolated from the same processing plants for up to eight

years, despite proper sanitation efforts (Møretrø and Langsrud, 2004; Gudmundsdóttir *et al.,* 2006; Keto-Timonen *et al.,* 2007). Given that bacterial persistence in an environment is described as the relationship between introduction and the subsequent ability to survive, grow, and resist removal (Carpentier and Cerf, 2011), it is hypothesized that *L. monocytogenes*' persistence in food plants and subsequent contamination of foods, is partly due to the bacterium's desiccation tolerance. This realization has initiated a series of studies aimed at characterizing the environmental and physiological factors contributing to *L. monocytogenes*' desiccation survival.

2.4.1. Desiccation Induced Stress and Mechanisms of Tolerance

Very little is known about the survival adaptation mechanisms used by *L. monocytogenes* to tolerate desiccation stress. Studies that report on the expression or identification of genes involved in the desiccation survival of similar Gram-positive bacteria (although information is limited), Gram-negative bacteria, and prokaryotes as a whole; provide some insight into the general mechanisms used to adapt to low water environments. In addition, osmotic stress responses can provide information on how bacteria survive intracellular water loss. Planktonic cells suspended in a liquid will desiccate in the presence of an osmotic pressure gradient between the intra- and extracellular water potential (i.e., osmotic stress). Surface adhering sessile cells located in the interphase between the aqueous and gaseous environment will desiccate due to differences in water potential between the surrounding gas phase and the cells (i.e., matric stress). In reality, most surface located cells will experience a mixture of osmotic and matric stress. There is likely an overlap in some of the tolerance mechanisms used to survive both solute and matric stress and to an equal or lesser extent to some of the other

adaptive responses including heat, cold, acid, alkaline, and oxidation stress, etc. A larger number of genes were reported to be up regulated in *P. putida* during matric stress than in the thermodynamically equivalent solute stress, suggesting that adaptation to matric stress is much more complex (van de Mortel and Halverson, 2004). Cellular dehydration can lead to protein denaturation, DNA damage, and membrane phase transitions (Crowe and Crowe, 1986; Potts, 1994). In addition, during the drying process, the efficiency of enzymes such as RNA and DNA polymerases are restricted due to the limited amount of water available for mobility, which would also affect the diffusion of nutrients to the cell (Potts, 1994).

2.4.1.1. Membrane Damage and Fatty Acid Compositional Changes

The bacterial cell envelope consisting of the cell membrane and cell wall, is in direct contact with any hostile environments it encounters and when water availability is low, the membrane endures the most drying-induced damage. The surface tension of water holds membranes together. When water is removed it causes the polar head groups of phospholipids to pack more closely together resulting in increased van der Waals interactions among the hydrocarbon chains (Potts, 1994). This causes the transition temperature (also known as the melting temperature, T_m) of the membrane to increase considerably under desiccation conditions (i.e., from -10 to 60°C) (Oliver *et al.,* 1998; Hoekstra, 1993). The T_m also depends on the length and saturation of the carbon chains and the electrostatic properties of the head groups of membrane lipids (Potts, 1994). Above T_m, the membrane is fluid-like, whereas below T_m the membrane is in a gel phase where the lipids are packed more orderly and are relatively immobile (Tokumasu *et al.,* 2002). This increase in lipid stacking produces a thicker membrane by as much as 1 nm

(Tokumasu *et al.,* 2002). Around the transition temperature the two phases can co-exist resulting in a mixture of membrane properties. This occurs during the drying process as the T_m rises towards and ultimately above ambient temperature. When both phases co-exist, membrane permeability is very high and this can be catastrophic for cells if free water becomes available for solute transport (Crow and Crowe 1986, 1992). In *Rhizobium* spp. death that resulted from desiccation was shown to be due to changes in membrane permeability (Bushby and Marshall, 1977).

It is thought that desiccation tolerant bacteria must have mechanisms to resist the dehydration induced rise in T_m in order to postpone the membrane fluid to gel phase transition. The synthesis of phospholipids with elevated degrees of unsaturation is a strategy used by pollen to retain a fluid membrane in dry environments (Hoekstra *et al.,* 1991). In *Salmonella*, the highest expressed gene during desiccation was that encoding FadA (3-ketoacycl-coA thiolase) which introduces double bonds into growing fatty acid chains of membrane phospholipids (Li *et al.,* 2012) thus lowering the membrane T_m. The only problem with this strategy is that increases in unsaturated phospholipids will lead to higher levels of oxidation during desiccation (Mckersie *et al.,* 1990) and as a result, cells utilizing this strategy have a very short lifetime in the dry state (Hoekstra and Roekel, 1988).

Other changes in membrane lipid composition have also been noted. Halverson and Firestone (2000) reported fatty acid composition changes in *P. putida* that led to increased cis to trans isomerase activity in 16:1 and 18:1 FAs during matric stress conditions. They also found that under <-1.0 MPa of matric stress *P. putida* developed a thicker outer membrane. Similarly, a *Pseudomonas aureofaciens* strain in sand cultures exhibited a twofold increase in the content of trans FAs as well as increased levels of

saturated FAs in response to an environment with water content of 5% (Kieft *et al.,* 1994). Hermann *et al.* (1996) also reported an increase in the trans/cis FA ratio when *P. putida* and *Vibrio spp.* cells were exposed to high temperatures and lethal salt levels.

Changes in the expression of genes involved in FA biosynthesis and compositional changes have been observed in transcriptome studies of cells under desiccation stress. When *Salmonella* was air-dried for 2 h on filter disks, FA metabolism was the most significantly induced physiological activity (Li *et al.,* 2012). The authors hypothesized that the drastic increase in FA metabolism may be due to the starvation response eliciting a need for energy (oxidation of FA produces more ATPs per C atom than glucose) or the matric stress inducing a need for a more rigid membrane with low fluidity. Along with the upregulation of FadA which was mentioned earlier, genes associated with the metabolism of short-chain FAs propionic and butyric acid were also among the top with upshifted expression. Another study with *Salmonella* saw the induction of *ddg*, encoding lipid A biosynthesis palmitoleoyl acyltransferase during desiccation at 40% RH, 25°C for 22 h (Gruzdev *et al.,* 2012). Ddg modifies lipid A composition by replacing laurate with palmitoleate at low temperatures to maintain membrane fluidity (Carty *et al.,* 1999). Van de Mortel and Halverson (2004) found that *P. putida* cells under matric stress upregulated genes involved in the degradation of damaged phospholipids. The exact reasons why these membrane FA composition changes take place during desiccation remain speculative but it is evident that changes in membrane fluidity are necessary for survival.

2.4.1.2. Protein Damage, Modifications, and Synthesis

Cellular dehydration subjects proteins to transitions in proton distributions leading to the reordering of disulfides, and the prevention of side-chain and backbone

conformational shifts (Potts, 1994). These changes leave proteins tense as opposed to loose and in most cases, causes loss of function (Potts, 1994). There are several theories on how desiccation tolerant cells retain protein functions, 1) proteins of tolerant cells are more stable than those of sensitive cells 2) the proteins of tolerant cells are no different from those of sensitive cells but are able to remain in a stable state due to one or more extrinsic factors exclusive to desiccation tolerant cells, and 3) the proteins of tolerant cells are no different from those of desiccation sensitive cells and survival under low water activity is due to alternative stress mechanisms (Potts, 1994). The enzyme phosphofructokinase is one example of the second theory. Under water limiting conditions phosphofructokinase irreversibly dissociates into inactive dimers (Carpenter and Crowe, 1988). When proline was added to cells prior to desiccation, the enzyme remained stable but only during partial dehydration. However, when trehalose was added, the enzyme remained completely stable even after extreme drying (Carpenter and Crowe, 1988). The role of trehalose in desiccation survival will later be discussed in more detail.

A very desiccation tolerant strain of *Salmonella* showed significant decreases in protein biosynthesis after 2 h of air drying at 11% RH in comparison to a control strain (Li *et al.,* 2012). The authors hypothesized that redirecting the energy into other metabolic needs may be more beneficial for survival under desiccation/starvation stress. Decreased expression levels of universal stress proteins were observed in *P. putida* after 5 days of exposure to matric stress (-0.4 MPa Ψ_m) (Gülez *et al.,* 2012). On the contrary, another desiccation (22 h, 40% RH) study with *Salmonella* revealed the upregulation of numerous transcription- and translation-associated genes encoding DNA-directed RNA polymerase subunits (RpoA, RpoB, RpoC) and ribosomal proteins (Gruzdev *et al.,* 2012). Other proteins upregulated were proteases (HflB, HslU) likely to be involved in the degradation

of misfolded proteins. The differences in the expression of protein biosynthesis genes across desiccation studies is likely due to the time point in which the expression levels were measured as well as the rate of desiccation and the means by which dehydration was induced. Whether the nutrients for protein biosynthesis are obtained exogenously or endogenously remains unknown but during times of carbon starvation, peptidase-dependent autophagy has been reported (Navarro Llorens *et al.,* 2010). The same study with *Salmonella* also found that the histidine and arginine biosynthetic pathways were induced during dehydration. The increased expression of histidine (*hisABCDGH*) as well as leucine and cysteine biosynthesis genes was also observed in *Salmonella* desiccated on food-grade stainless steel (Finn *et al.,* 2013). These observations suggest that proteins with increased histidine content may provide bacteria with some form of protection or stabilization in low-water environments.

2.4.1.3. Oxidation Induced Cellular Damage and Repair Mechanisms

Cellular dehydration imposes oxidative stress when increased cytoplasm ion concentrations cause the formation of reactive oxygen species. Furthermore, biomolecules in low water activity environments are more susceptible to oxidation (Potts, 1994). Damage to both DNA and proteins as well as lipid peroxidation, is mediated through reactive oxygen species (Brawn and Fridovich, 1981; Hansen *et al.,* 2006). Nucleic acids are easily damaged and DNA breaks accumulate under oxidative stress conditions imposed by desiccation. *E. coli* experienced 10 breaks per single stranded genome in cells caused by drying for only 12 min (Bieger-Dose *et al.,* 1992). The transcriptome analysis of desiccated *Brayrhizobium japonicum* revealed the induction of nucleic acid modification and repair proteins including ATP-dependent RNA helicase, MutL DNA mismatch repair

protein, and a RecF DNA recombination and repair protein (Cytryn *et al.,* 2007). Similar results were also seen in *P. putida* under water stress (Gülez *et al.,* 2012). Therefore, survival under dry conditions may be dependent on the protection of RNA-binding proteins including ribosomal proteins, transcription factors, and DNA-dependent RNA polymerase (Potts, 1994).

In desiccation sensitive cells of cyanobacteria, free radicals caused fatty acid de-esterification from phospholipids resulting in the intracellular accumulation of free FAs and reduced membrane integrity (Potts, 1994). This may explain the up-regulation of genes involved in the metabolism of damaged phospholipids in *P. putida* under matric stress (van der Mortel and Halverson, 2004). Desiccation associated oxidative damage has been shown to cause protein damage and lipid peroxidation that results in a loss of a diffusion barrier to membrane-impermeable markers and ultimately cell lysis in cyanobacteria (Dadheech, 2010). Furthermore, exposure of freeze-dried *E. coli* to oxygen resulted in a damaged cytoplasmic membrane that became leaky to potassium (Israeli *et al.,* 1974).

The transcriptome analysis of desiccated *Salmonella* (Li *et al.,* 2012; Gruzdev *et al.,* 2012), *B. japonicum* (Cytryn *et al.,* 2007), and *P. putida* (van de Mortel and Halverson, 2004; Gülez *et al.,* 2012) all revealed the upregulation of genes involved in scavenging reactive oxygen species such as peroxidases and superoxide dismutases. Furthermore, genes of the oxidative pentose phosphate pathway (OPPP) were also upregulated in *Salmonella* (Li *et al.,* 2012). One of these genes, glucose-6-phosphate dehydrogenase, was found to be important for oxidative stress survival in *Salmonella* and yeast cells (Lundberg *et al.,* 1999; Slekar *et al.,* 1996). These studies clearly demonstrate that bacteria under desiccation stress employ mechanisms to cope with the subsequently induced cellular oxidative damage.

2.4.1.4. Uptake and or Biosynthesis of Compatible Solutes

During desiccation the main focus of cells is survival since the low water activity does not permit growth. However, during the early stages of desiccation when the water activity still permits enzyme activity, cells may achieve water balance through *de novo* synthesis or extracellular uptake of compatible solutes (Potts, 1994). Many desiccation tolerant cells accumulate large amounts of either one or both of the disaccharides trehalose or sucrose (Womersley and Smith, 1981; Hottiger *et al.,* 1987; Guo *et al.,* 2000). Adding a disaccharide such as trehalose before drying, lowers the transition temperature (T_m) of the dry membranes by replacing the water between the lipid headgroups and therefore preventing the phase transition and its accompanying leakage upon rehydration (Crowe *et al.,* 1983). In addition, trehalose and sucrose have been shown to preserve both structure and function in isolated proteins during drying (Carpenter *et al.,* 1987a; Carpenter *et al.,* 1987b; Crowe *et al.,* 1987). This is a result of the disaccharides forming hydrogen bonds with the proteins when water is removed, thus preventing protein denaturation (Carpenter and Crowe, 1988; Carpenter and Crowe, 1989). Leslie *et al.* (1995) showed that the survival of *E. coli* and *Bacillus thuringiensis* was improved by 62 and 48% and 43 and 30% in the presence of trehalose and sucrose respectively, during freeze-drying. Similar results have been reported for several strains of freeze-dried lactic acid bacteria (Zayed and Roos, 2004; Conrad *et al.,* 2000).The survival of *B. japonicum* increased by 294% when cells were loaded with trehalose prior to desiccation on soya beans for 24 h (Streeter, 2003). In *E. coli*, osmotic stress induced intracellular trehalose synthesis provided cross protection against subsequent desiccation stress (Welsh and Herbert, 1999). Trehalose production via trehalose synthase was found to be upregulated in both *Salmonella* and *P. putida* exposed to desiccation stress (Li *et al.,* 2012; Gülez *et al.,* 2012). Unlike these

bacteria, *Listeria* cannot naturally synthesize or accumulate trehalose, however, a *L. monocytogenes* 568 *ΔTreA* mutant capable of accumulating the disaccharide, showed increased resistance to harsh conditions including desiccation and hyper-osmolarity (Ells and Truelstrup Hansen, 2011).

Other compatible solutes which have been recognized to provide protection in osmotically stressed cells include glycine betaine, glutamine, proline, and carnitine (Kempf and Bremer, 1997). Huang (2011) showed that the desiccation survival of *L. monocytogenes* was most improved by the addition of glycine betaine to the pre-culture and desiccation mediums, followed by carnitine and to a lesser extent proline. Similarly, Dreux *et al.* (2007) reported that exogenous glycine betaine improved the desiccation (50-60% RH) survival of *L. monocytogenes* on parsley leaves independent of intracellular accumulation as demonstrated by a deletion mutant of three glycine betaine uptake systems. In *Pantoea agglomerans*, osmoadaptation leading to the intracellular accumulation of trehalose and glycine betaine, significantly increased survival under low RH conditions (Bonaterra *et al.,* 2005). Li *et al.,* (2012) observed the induction of genes related to glycine, choline, and proline betaine transport when *Salmonella* strains were air dried. Similarly, genes involved in osmoprotectant transport systems (*proP, proU, osmU*) were overexpressed in *Salmonella* cells desiccated on stainless steel (Finn *et al.,* 2013). All deletion mutants of these genes as well as *rpoE* (encoding σ^E), showed significant decreases in survival upon desiccation in comparison to the wildtype.

2.4.1.5. Production of Extracellular Polymeric Substances

Several desiccation tolerant bacteria are known to secrete copious amounts of exopolymeric substances (EPS). EPS layers are formed by the accumulation of various

polymeric substances of high viscosity (proteins, polysaccharides, extracellular DNA) around bacterial cell walls. These polymers also tend to be hygroscopic (Roberson and Firestone, 1992) and may decrease the rate of water loss from cells. Cyanobacteria produce large amounts of excreted polysaccharides that is thought to contribute to the extreme desiccation tolerance of *Nostoc commune*, which can survive for centuries under 0% RH conditions (Wright *et al.,* 2005; Tamaru *et al.,* 2005). The extrapolysaccharide-associated genes (*exoM, exoN, exoP*) as well as *rpoE* were significantly induced in desiccated *B. japonicum* (Cytryn *et al.,* 2007). The *rpoE* gene (encoding σ^E also known as sigma factor AlgU) was shown in *Pseudomonas fluorescens* and *P. putida* to regulate extrapolysaccharide biosynthesis in response to desiccation and other environmental stresses (Schnider-Keel *et al.,* 2001; van de Mortel and Halverson, 2004). Some Gram-negative bacteria produce colanic acid, a highly viscous capsular polysaccharide (Stevenson *et al.,* 1996). EPS is considered capsular when it is covalently attached to lipids, if there are no visible means of attachment then it is known as secreted EPS (Roberts, 1996). A study done by Ophir and Gutnick (1993) showed that mucoid strains of *E. coli*, *Acinobacter calcoaceticus*, and *Erwinia stewartii* were significantly more resistant to desiccation than their corresponding non-mucoid mutants.

In *Salmonella* the presence of thin aggregative fimbriae (known as curli) and cellulose in the extracellular matrix, contributed to desiccation resistance (White *et al.,* 2006). The authors hypothesized that cellulose polymers may directly remove or neutralize reactive species as suggested by Scher *et al.,* (2005), or trap additional polysaccharides on the cell surface to enhance protection (White *et al.,* 2003). Curli have been implicated in the colonization of inert surfaces, cell-cell interaction during biofilm development, and virulence (host protein binding and internalization) (Prigent-Combaret *et al.,* 2000; Vidal

et al., 1998; Sjöbring *et al.,* 1994; Olsén *et al.,* 1998; Herwald *et al.,* 1998). *E. coli* and *Salmonella* have both been reported to produce lipopolysaccharide O-polysaccharides EPS known as O-antigen (O-Ag) capsules (Whitfield and Roberts, 1999; Gibson *et al.,* 2006). Garmiri *et al.* (2008) found that *Salmonella* Typhimurium mutants lacking O-Ag expression were more susceptible to drying. In *Salmonella* the O-Ag, fimbria- and cellulose-associated EPS are coregulated by the protein AgfD from the Tafi (thin aggregative fimbriae) operon (Gibson *et al.,* 2006). Activation of the promoter for *agfD* is regulated by RpoS which has been shown to also regulate the expression of genes involved in virulence in *Salmonella* (Fang *et al.,* 1992), acid, heat, and salt tolerance in *E. coli* (Cheville *et al.,* 1996) and genes expressed in stationary phase (Tanaka *et al.,* 1993).

Although EPS production has been shown to increase desiccation survival, this is not always the case. A recent study, Daneshvar Alavi (2012) showed that despite extensive biofilm formation and EPS production by *P. fluorescens*, its desiccation survival was poor in comparison to *L. monocytogenes* and *Serratia proteamaculans*.

2.4.1.6. Other Genes Which May Contribute to Desiccation Survival

Thanks to a number of recent transcriptome studies of desiccation bacteria, several other genes and proteins that are either up or down-regulated during survival in low water environments have been identified. Flagellum biosynthesis and motility genes have been commonly reported as being down-regulated during desiccation (van de Mortel and Halverson, 2004; Gruzdev *et al.,* 2012; Li *et al.,* 2012; Finn *et al.,* 2013). Van de Mortel and Halverson (2004) hypothesized that under matric but not solute stress, the water film surrounding cells is not thick enough for flagellar rotation and as a result the flagella breaks during surface contact and negatively regulates flagellum biosynthesis. Other authors

suggest that by down-regulating flagellum synthesis the cell can redirect energy into more important survival strategies. On the contrary, the induction of flagella and pili related genes have also been reported as being induced during desiccation stress (Cytryn *et al.,* 2007; Gülez *et al.,* 2012).

Another group of proteins found to be commonly up-regulated under cellular dehydration are those involved in carbohydrate metabolism and transport. Such genes include those encoding glycogen synthase, a galactose transport protein, and phosphoenolpyruvate synthase (Gruzdev *et al.,* 2012; Li *et al.,* 2012). Similar genes have also been reported as down-regulated in desiccated *Salmonella* on stainless steel (Finn *et al.,* 2013). It is likely that the differences observed in gene expressions across studies are due to the variations in the experimental protocols, bacterial species and the time points (early, middle, late stages of desiccation) at which the transcripts are analyzed. Some studies also applied the matric stress in the form of non-permeating substrates such as PEG 8000 which present bacteria with a similar but not identical form of desiccation stress.

In addition to carbohydrate transport mechanisms, the upregulation of genes involved in phosphate (e.g., *pstABCS*) (Finn *et al.,* 2013), and potassium transport (e.g., *kdpFABC*) (Finn *et al.,* 2013; Gruzdev *et al.,* 2012; Cytryn *et al.,* 2007) have also been identified in desiccation stress studies. It appears that when cells are exposed to both osmotic and desiccation stress, K^+ transport is required for the uptake of osmoprotectants (Lee and Gralla, 2004; Booth and Higgins, 1990; Epstein, 1986; Balaji *et al.,* 2005).

2.4.2. Environmental Factors Influencing Bacterial Desiccation Survival

In addition to the tolerance mechanisms exploited by *L. monocytogenes* and other bacteria to survive dehydration/starvation stress, several environmental factors also have

a significant impact on cellular survival under dry conditions. Such factors include temperature, humidity levels, organic residues, salt, and biofilm formation. McEldowney and Fletcher (1988) conducted a multifactorial desiccation study which determined the survival of *Pseudomonas spp.*, *Acinetobacter calcoaceticus*, a coryneform, and *Staphlyococcus* spp., on glass coverslips in response to various temperatures (4, 15, 25°C) and levels of RH (0, 34, 75%). They found that temperature considerably influenced survival with the most rapid decline in viability being observed at 25°C and the longest survival at 4°C, whereas varying RH had no overall effect on cell survival. The survival of *Enterobacter sakazakii* exposed to 43% RH also decreased with increasing temperature (4, 25, 37°C) (Kim *et al.,* 2008). A similar drying study with *L. monocytogenes* concluded that survival was longer at 5°C than at 25°C whereas again the impact of different RHs (1-75%) on survival was less pronounced (Palumbo and Williams, 1990). However, the desiccation survival of *S. aureus* at 25°C on glass, ceramic tile, and steel, was highest under 11% RH out of a range from 11-53% RH (McDade and Hall, 1963).

Of interest to the food industry is how food residues on surfaces may impact desiccation survival. Food components contain nutrients that bacteria may utilize to extend their survival on otherwise dry surfaces. *L. monocytogenes* dried on glass coverslips showed enhanced survival when suspended in beef extract, glycerol, corn syrup, skim milk, and canned milk compared to distilled water (Palumbo and Williams, 1990). *Enterobacter sakazakii* survived statistically better when desiccated (43% RH) in a medium of infant formula as opposed to distilled water (Kim *et al.,* 2008). In a similar study, Helke *et al.* (1994) found that the desiccation survival of *L. monocytogenes* and *Salmonella* Typhimurium at 25°C and 32.5 or 75.5% RH was increased in dilute pasteurized whole milk compared to PBS. *Acinetobacteria* spp. also showed improved

survival when desiccated in bovine serum albumin compared to distilled water (Jawad *et al.,* 1996). In a more recent study by Takahashi and others (2011), the pathogens *L. monocytogenes*, *S. aureus*, and *S.* Typhimurium were all air dried at 25°C on stainless steel coupons previously coated in minced tuna, ground pork, and cabbage. Minced tuna and ground pork increased the survival of all bacteria types during the first 14 days of the 30 day experimental period with *L. monocytogenes* showing the greatest survival throughout. Smoked and non-smoked salmon juice has also been shown to increase the desiccation (43% RH) survival of *L. monocytogenes* strains on stainless steel coupons (Vogel *et al.,* 2010). These results all suggest that survival is enhanced when cells are desiccated in the presence of increased nutrients. The increased survival of both *L. monocytogenes* (Vogel *et al.,* 2010) and *Salmonella enteritis* (De Cesare *et al.,* 2003) desiccated in TSB as compared to physiological peptone saline (PPS) or PBS, also supports these observations.

Given the similar impacts of both matric and solute stress on intracellular water levels, several studies have focused on the role of osmoadaptation and the presence of salt during desiccation, on cell survival. Dreux *et al.* (2008) reported increased survival of *L. monocytogenes* when desiccated at 48% RH on parsley leaves when cells were pre-cultured in 3% NaCl. Vogel *et al.* (2010) also reported enhanced desiccation survival when *L. monocytogenes* cells were pre-cultured in 5% NaCl and even higher survival counts when cells were also subsequently desiccated in 5% NaCl media. It is possible that pre-culture in high salt may induce a physiological state or the production of stress proteins which allow cells to better survive desiccation stress.

Lastly, desiccation survival is greatly improved when cells are surrounded in a biofilm matrix of EPS (Truelstrup Hansen and Vogel, 2011; van de Mortel *et al.,* 2004; Espinal *et al.,* 2012). Such circumstances occur when surface attached biofilm forming

cells are initially exposed to a high moisture environment where upon biofilm development takes place and is then followed by an extended period under low RH conditions. Biofilm polymers are very hydrophilic and may aid in reducing the rate of biofilm and cellular drying. In addition, the fact that stationary phase cells show better desiccation survival than exponential phase cells (Welsh and Herbert, 1999; Billi *et al.,* 2000) suggests that the physiological changes in stationary phase cells may also contribute to the increased resistance of biofilm cells. Studies have shown the bacterial transfer from biofilms to foods (cheese, bologna, salami) was enhanced when biofilms were first dried as opposed to wet (Rodriguez *et al.,* 2007a; Rodriguez *et al.,* 2007b). This demonstrates that not only do biofilms enhance bacterial survival during desiccation; but dry biofilms are more successful at cross contaminating foods. Furthermore, biofilms formed by *E. sakazakii* in infant formula were significantly more desiccation resistant than those formed in minimal nutrient media (Kim *et al.,* 2008). This emphasizes the importance of removing food soils from surfaces.

2.5. Determination of Genes Contributing to Desiccation Survival

When aiming to determine genes which may contribute to a specific phenotype of interest, one can either study 1) the up/down-regulation of genes in response to a particular stimulus via localized or global transcription of genes using DNA microarrays or RNA sequencing or 2) perform random mutagenesis which allows for the identification of processes disrupted when a gene is mutated (Johnson *et al.,* 2002). In the latter approach, which was employed in this study, the known DNA sequence of the randomly inserted transposon element is then used in the subsequent identification of the disrupted gene.

2.5.1. Insertional Mutagenesis

One of the most widely used genetic tools for studying microbial systems is the transposon (Hayes, 2003). These mobile DNA sequences are used for random insertion mutagenesis, creating large libraries of mutants that can be screened for factors related to specific bacterial functions. An older widely used transposon delivery system for Gram – positive bacteria is Tn917 which is 5.2 kb and delivered on an 11 kb temperature sensitive plasmid (Gudmundsdóttir *et al.,* 2006). However, this system has a low efficiency of transposition and a high rate of vector retention (Camilli *et al.,* 1990). In recent years, a *Himar1* mariner transposon has been used for more effective transposition in low-GC-content Gram-positive bacteria (Akerley *et al.,* 1998; Bae *et al.,* 2004; Le Breton *et al.,* 2006). Originally isolated from the horn fly, *Himar1* is a member of the most widespread transposon superfamily in nature, Tc1/mariner (Plasterk *et al.,* 1999). Insertion of the mariner into a genome only requires the dinucleotide TA, allowing for high efficiency and improved overall genome coverage in low-GC-content bacteria such as *L. monocytogenes* (39%) (Cao *et al.,* 2007). In this study the 1,395 bp mariner transposon was designed to be contained on the pMC39 delivery vector (Cao *et al.,* 2007). The vector consists of the *E. coli* p15A low-copy-number replication origin (Chang and Cohen, 1978), the RP4 ori for conjugative transfer (Pansegrau *et al.,* 1988), the pE194ts Gram-positive temperature sensitive replication origin (Gryczan *et al.,* 1982), a Gram-negative chloramphenicol resistance gene (*cat*) (Chang and Cohen, 1978), a Gram-positive noninducible erythromycin resistance gene (*ermC*) (Villafane *et al.,* 1987) flanked by the 29 bp inverted terminal repeats (ITR) of the *Himar1* mariner (Lampe *et al.,* 1999), a Gram-positive kanamycin resistance gene (*kan*) (Guérout-Fleury *et al.,* 1995) as a screening marker for loss of the plasmid, and the *Himar1* mariner transposase gene (*tpase*) (Lampe *et al.,* 1996)

for insertion of the transposon into the chromosome (Figure 2-1). By using this system, Cao *et al.* (2007) reported a 10-fold increase in the number of random mutants compared to the Tn917-based vector and less than 2.5% plasmid retention in comparison to 50% with the Tn917-based vector.

To successfully achieve transposon insertion in the bacterial chromosome, the plasmid is first extracted from *E. coli* cells, purified and then transferred to the donor cells via common methods such as electroporation. Once the temperature-sensitive plasmid has been introduced into the cell, the culture temperature is raised above the permissive temperature such that the plasmid is unable to replicate, which will force the integration of the transposon into the chromosome resulting in the delivery plasmid being lost (Fernandez and Hoeffler, 1998). Successful integrants are isolated on erythromycin antibiotic agar.

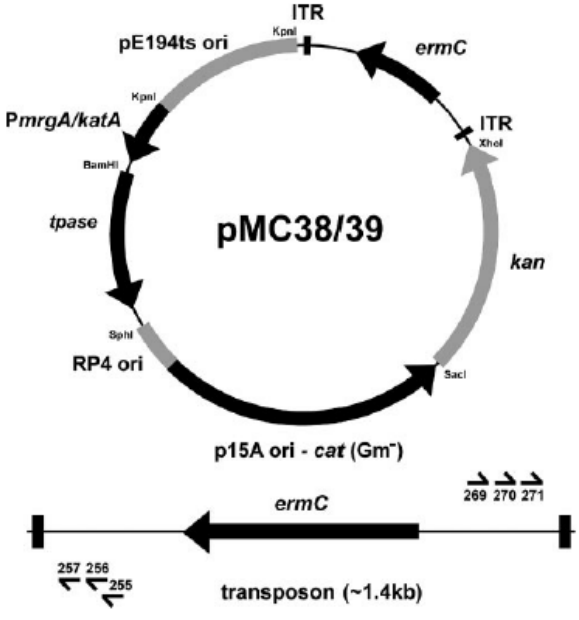

Figure 2-1. Physical map of the mariner-based transposon delivery vectors pMC38/39. Below the plasmid, the binding sites for the transposon specific primers are indicated. These are used together with unspecific primers in the arbitrary PCR protocol to identify the transposon insertion site in selected mutants. (Cao *et al.,* 2007 – permission attached in Appendix B).

2.5.2. Arbitrary PCR Method

To determine the location of the transposon insertion in *L. monocytogenes* mutants, the DNA flanking the insertion sites is amplified from either end of the transposon involving two rounds of PCR with Taq polymerase in the first round and High Fidelity polymerase in the second round (Cao *et al.,* 2007). This arbitrary PCR method has been used widely for the identification of transposon insertions (O'Toole *et al.,* 1999; Bahrani-Mougeot *et al.,* 2002; Knobloch *et al.,* 2003; Burall *et al.,* 2004). Arbitrary primers are used that can anneal to multiple sites in the whole genome and generate multiple PCR amplicons (Liu, 2008). In the first round of PCR two separate reactions are prepared for each mutant containing a primer specific to one end (left or right) of the transposon and an arbitrary primer with a constant region at the 5' end (Garsin *et al.,* 2004). The same is done in the second round using transposon specific primers further nested, and a primer specific to the constant region of the arbitrary primer (Garsin *et al.,* 2004). These products can then be separated by gel electrophoresis and the banding patterns observed, however, it is expected that banding patterns will differ between isolates due to the different locations of the transposon inserts. The resulting product is then purified and sequenced using a third nested primer specific to the transposon (Figure 2-2) (Garsin *et al.,* 2004).

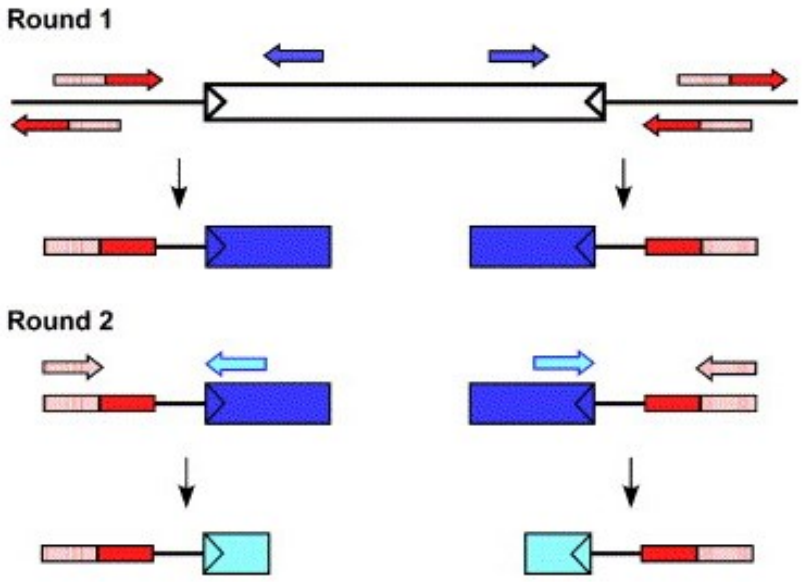

Figure 2-2. Diagram depicting the principle behind arbitrary PCR for the identification of transposon insertions in bacterial chromosomes. Round I primers specific to the transposon (open box with triangles at each end) are shown as blue arrows. Each of these primers is paired in a separate reaction with an arbitrary primer (solid and striped red arrow). Products from the first-round reactions are used as templates for second-round reactions involving further nested, sequence-specific primers (turquoise arrows) paired with an arbitrary primer (red-striped arrows) identical to the 5′ sequence of the first-round arbitrary primer. Amplicons from second-round reactions are subjected to DNA sequence analysis (Das *et al.,* 2005 – permission attached in Appendix B).

Until fairly recently, inverse PCR has been the method of choice for identifying transposon insertion sites in bacterial chromosomes (Ochman *et al.,* 1988). This method involves the digestion of the bacterial chromosome using a restriction enzyme (not recognizing transposon sequences) and subsequent circulation via self-ligation at low DNA concentrations (to avoid intermolecular ligation) (Smith and Sockett, 1999). The resulting ligation products are then used as substrates for enzymatic amplification by PCR using oligonucleotide primers homologous to the ends of the core sequence but facing in opposite orientations (Figure 2-3) (Ochman *et al.,* 1988). In comparison to this method, arbitrary PCR provides the advantages of avoiding 1) the artificial construction of a region for which the primer sequence is known and brackets the unknown sequence, 2) the

creation of gene fragments which are either too large to be amplified (>10 kbp) or too small to become circular, and 3) the use of multiple steps (ligation and circulization) which increase the chances of contamination and are laborious (Parker *et al.,* 1991).

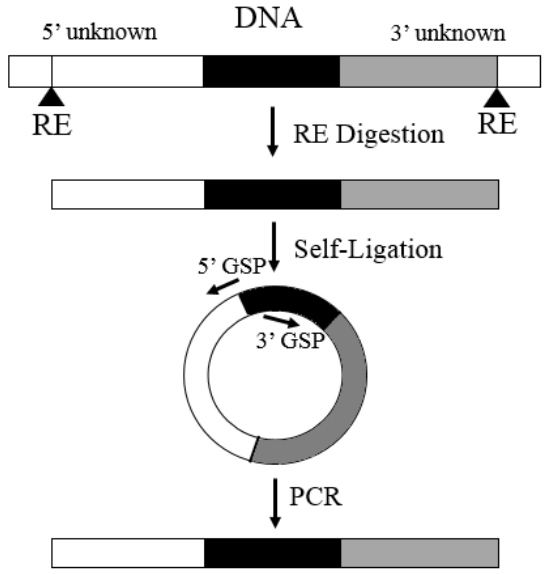

Figure 2-3. Inverse PCR method diagram showing the restriction enzyme (RE) sites and resulting gene products, self- ligation, and subsequent PCR (adapted from Jong *et al.,* 2002).

CHAPTER 3

Role of Initial Contamination Levels, Biofilm Maturity and Presence of Salt and Fat on Desiccation Survival of *Listeria monocytogenes* on Stainless Steel Surfaces

Hingston, P.A., E.C. Stea, S. Knøchel, L. Truelstrup Hansen. 2013. Role of initial contamination levels, biofilm maturity and presence of salt and fat on desiccation survival of *Listeria monocytogenes* on stainless steel surfaces. *Food Microbiology, 36*(1), 46-56.

3.1. Introduction

Listeria monocytogenes is a foodborne bacterial pathogen routinely isolated from food products, including lightly preserved ready-to-eat (RTE) foods, often in combination with a long refrigerated shelf life (Ryser and Marth, 2007). RTE food products that support growth of the bacterium are often implicated in listeriosis outbreaks (Gray *et al.,* 2006). In 2008, Canada experienced an outbreak of listeriosis resulting in 56 confirmed cases and 22 deaths due to consumption of contaminated sliced chilled luncheon meats (Weatherill, 2009). The cause of the outbreak was traced to contaminated slicers in one food processing plant, indicating failure of the sanitation protocols in the removal of the pathogen. Consequently, new Canadian regulations place emphasis on environmental sampling (Farber *et al.,* 2011). In spite of increased efforts to obtain control with *L. monocytogenes* in the food supply, the number of infections reported in the European Union has been going up (Allerberger and Wagner, 2010). Recently, consumption of contaminated Colorado grown cantaloupes caused 146 cases and 30 deaths in the US (CDC, 2011). Clearly, proper intervention strategies (e.g., cleaning/sanitation chemistry, application and management, construction of equipment, consumer education) for control of *L. monocytogenes* are currently missing. However, to devise improved intervention strategies

a better understanding of the survival mechanisms of this pathogen during food processing is urgently needed.

L. monocytogenes can colonize food processing plants resulting in the same genotype being re-isolated for years despite intensified cleaning efforts and periods with inactivity (Wulff *et al.,* 2006). Since survival of bacterial pathogens on food contact surfaces increases subsequent cross-contamination of foods (Midelet *et al.,* 2006; Rodriguez *et al.* 2007; Keskinen *et al.,* 2008), research has begun to look into mechanisms for survival and persistence in that environment. Bacterial persistence in an environment has been described as the relationship between introduction and subsequently the ability to survive, grow, and resist removal (Carpentier and Cerf, 2011). It was recently shown that *L. monocytogenes* survives desiccation for three months in a simulated food processing environment (Vogel *et al.,* 2010). We, therefore, hypothesize that the bacterium's persistence in food plants is partly related to its desiccation tolerance.

Desiccation tolerance is a survival strategy common to many prokaryotes (Potts, 1994). Planktonic cells suspended in a liquid will desiccate in the presence of an osmotic pressure gradient between the intra- and extracellular water potential (i.e., osmotic stress). Surface adhering sessile cells located in the interphase between the aqueous and gaseous environment will desiccate due to differences in water potential between the surrounding gas phase and the cells (i.e., matric stress). In reality most surface located cells will experience a mixture of osmotic and matric stresses, and as the intracellular water activity decreases it may ultimately fall below the threshold required for growth and/or survival of the cell. At this point it is not known whether *L. monocytogenes'* ability to tolerate desiccation is due to cellular mechanisms designed to minimize the impact of osmotic and matric stresses. Similarly, it remains unclear whether cells being desiccated would benefit

from the lysis of adjacent cells and the associated local increases in the amount of extracellular water and organic material.

L. monocytogenes forms a biofilm when left on surfaces in high RH environments (Rieu *et al.,* 2008; Pan *et al.,* 2010) and cells become surrounded by exopolymeric substances (EPS) including proteins, extracellular DNA (eDNA) and carbohydrates (Jahn and Nielsen, 1995; Chae *et al.,* 2006; Harmsen *et al.,* 2010). Aside from physiological changes in sessile cells, the extra layer of hydroscopic polymers will increase the amount of water surrounding the cell, thereby potentially altering the desiccation kinetics. Our preliminary study showed that biofilm formation increased residual survivor levels of *L. monocytogenes* N53-1 (serotype 1/2 a) and this in turn resulted in the transfer of more viable cells upon contact with salmon products in cross-contamination model experiments (Truelstrup Hansen and Vogel, 2011). It is, however, not known how desiccation affects *L. monocytogenes* biofilms of varying developmental stages.

Different types of food soils have been shown to have a protective effect on *L. monocytogenes* cells during desiccation including fresh and cold-smoked salmon (Vogel *et al.,* 2010) and tuna, cabbage and ground pork (Takahashi *et al.,* 2011). However, relevant to many segments of the food industry is how fat soils on the surface affects the desiccation tolerance of the bacterium.

In this study, the impact of initial surface contamination levels, biofilm development and maturity, and presence of common food soils (salt and two types of fat in varying initial concentrations) on the desiccation survival of *L. monocytogenes* was examined. To simulate conditions in the food processing industry, the desiccation survival studies were carried out on food grade stainless steel surfaces at 15°C and a constant RH of 43%.

3.2. Materials and Methods

3.2.1. Bacterial Strain and Culture Conditions

The *Listeria monocytogenes* strain 568 (serotype 1/2a, Kalmokoff *et al.,* 2001), originally isolated from a food processing plant, was used in the desiccation survival experiments. Routine culturing and pre-culturing was carried out on Brain Heart Infusion (BHI, Difco, Fisher Scientific, Whitby, ON, CA) agar (Technical agar, Difco, Fisher Scientific) or in Tryptic Soy Broth (TSB, Difco, Fisher Scientific) supplemented with 1% (w/v) glucose (Fisher Scientific) to create TSB-glu. Stock cultures were stored at -80°C in BHI broth containing 20% glycerol.

3.2.2. Preparation of Stainless Steel Coupons

Stainless steel (SS, Food grade AISI 316, type 4 finish, thickness 1 mm) was cut into coupons (0.5 × 0.5 cm). Prior to use, coupons were boiled in 1% (w/v) Sodium-Dodecyl-Sulphate (SDS) solution for 10 min, sonicated (60 min, 60°C, 50/60 kHz) in a 1000 W sonication bath (Elmo S120H Heat/Drain, Fisher Scientific) and immersed in 100% isopropanol to remove any remaining residues. They were then rinsed in distilled water, autoclaved and stored in 95% ethanol until further use. Before surface inoculation, coupons were flame sterilized, left to cool, and placed in sterile plastic Petri dishes.

3.2.3. Inoculation of Stainless Steel Coupons

Five ml of TSB-glu (0.5% NaCl, already present in the media) or TSB-glu supplemented with NaCl to achieve a final concentration of 5% NaCl (to osmoadapt cells) were inoculated with *L. monocytogenes* 568 (from colonies) and pre-cultured for 48 h at 15°C to obtain non-osmoadapted and osmoadapted cells, respectively, in the stationary phase. Cells were harvested by centrifugation (2,824 × *g*, 15 min) and re-suspended in low

(0.5%) or high (5% NaCl) salt TSB-glu to an $A_{450\ nm}$ of 1.0 to reach final cell concentrations of approximately 1×10^9 CFU/ml as determined by spread plating on BHI agar. For the desiccation survival experiments, each pre-culture salt treatment was diluted in both the low and high salt TSB-glu media (see below section 2.4). Ten µl from the appropriate dilutions were then placed on the surface of one side of the SS coupons to yield initial contamination levels of approximately 3.5, 5.5 and 7.5 log CFU/cm^2.

3.2.4. Desiccation of Non-Biofilm and Biofilm L. monocytogenes on Stainless Steel Surfaces

In non-biofilm experiments desiccation followed immediately after contamination of the SS coupons. These were placed in Petri dishes without lids, and transferred to a desiccator (Mini 1 Desiccator, Bohlender GmbH, Grünsfeld, Germany). The desiccator had been conditioned to the desired environmental conditions (15°C and 43% RH) by placing two Petri dishes with saturated solutions of K_2CO_3 (Fisher Scientific) in the bottom of the chamber at least 3 h at 15°C prior to the experiment.

To allow a biofilm to form, contaminated coupons were placed in desiccators containing two Petri dishes with demineralised water to obtain 100% RH and incubated for 48 h at 15°C. After 48 h, new Petri dishes filled with saturated K_2CO_3 were used to shift the RH from 100% to 43% (± 2%) to begin the desiccation period (43% RH attained after < 3 h).

During all experiments, both temperature and RH were continuously monitored using a data logger (Gemini Tinytag View 2, Interworld Electronics and Computer Industries Inc., Markham, ON, CA).

For each contamination level (3.5, 5.5, 7.5 log CFU/cm^2), the following four salt treatments were used to prepare cells for the non-biofilm (cells desiccated immediately

after placement onto the SS coupons) and biofilm desiccation survival experiments which were undertaken at 43% RH and 15°C for 20 days: 1. Non-osmoadapted cells re-suspended and desiccated in low (0.5%) salt media, 2. Non-osmoadapted cells re-suspended and desiccated in high (5%) salt media, 3. Osmoadapted cells re-suspended and desiccated in low salt media and 4. Osmoadapted cells re-suspended and desiccated in high salt media. As described above, for the biofilm desiccation experiments, cells were allowed to form biofilm in the re-suspension medium before being desiccated.

3.2.5. Desiccation of L. monocytogenes in the Presence of Food Lipids

L. monocytogenes was pre-cultured in TSB-glu for 48 h at 15°C, adjusted to $A_{450\ nm}$=1.0, centrifuged and re-suspended in fresh TSB-glu containing canola oil (0, 5, 10%) (Rapsolie, budget, DK) or animal lard (0, 20, 60%) (Raffinol, DK). Tween 80 (1% w/v, Sigma-Aldrich, Oakville, ON, CA) was added to assist in emulsifying both types of fat in the TSB-glu. The suspensions were inoculated onto SS coupons at a density of 7.5 log CFU/cm^2 and desiccated as previously described.

3.2.6. Enumeration of Surviving Bacteria on the Stainless Steel Coupons

Six coupons were pulled for each treatment on the sampling days (n = 6 or 7), which were distributed unevenly over the 20-day desiccation period to capture the changes in desiccation survival. Once the coupons were removed from the desiccators, they were individually placed in microcentrifuge tubes containing 1 ml of Peptone Saline (PS, 0.1% Peptone (Oxoid), 0.85% NaCl). Adhering cells were released from the coupons by sonication for 4 min in a sonication bath (Elmo, 50/60 kHz, 1000 W) followed by vortexing for 20 s before continuing the serial dilutions in PS. This release method follows the procedure developed by Leriche and Carpentier (1995). Appropriate dilutions were

spread plated on BHI agar and colonies were enumerated after incubation for 48 h at 35°C and expressed as log CFU/cm^2. For sampling days where coupons were estimated to contain <100 CFU, 500 µl of the original dilution was deposited on BHI agar as five 100 µl spots and enumerated following incubation as stated above.

3.2.7. Fluorescence and Scanning Electron Microscopy

Coupons from the biofilm and non-biofilm experiments were examined after 2 days of desiccation using fluorescence and scanning electron microscopy (SEM). For fluorescence microscopy, coupons were stained by depositing 20 µl of LIVE/DEAD® stain (*Bac*Light™ Bacterial Viability Kit L7007, Molecular Probes, Burlington, ON, CA) and leaving them in the dark for 15 min at room temperature. Excess stain was removed and the coupons were rinsed with 20 µl of distilled water before being examined under a Nikon Eclipse 80i light microscope with a D-FL epi-fluorescence attachment (Nikon Canada, Mississauga, ON) using a Nikon triple band DAPI-FITC-Texas Red Filter (Excitation: 395-410 nm DAPI, 490-505 nm FITC, 560-580 nm Texas Red; Emission: 450-470 nm DAPI, 515-545 nm FITC, 600-650 nm Texas Red). Images were taken at 1000× magnification with a Nikon DS-Fil camera connected to NIS-Elements BR 2.30 image analysis software program (Nikon Canada).

To prepare the coupons for SEM examination a protocol previously described by Ells and Truelstrup Hansen (2006) was used. Coupons were first submerged in 0.1 M cacodylate buffer (Fisher Scientific) containing 2% glutaraldehyde (Fisher Scientific) for 2 h, rinsed three times in 0.1 M cacodylate buffer + 3% glucose, and then post-fixed in 1% OsO_4 (Osmium Tetroxide, Electron Microscopy Sciences, Hatfield, PA, US) in 0.1 M cacodylate buffer for 4 h. To remove residual osmium, coupons were rinsed in 0.1 M

cacodylate buffer three times, then dehydrated in increasing concentrations of ethanol (35, 50, 70, 90 and 100%) for 15 min each except the final 100% ethanol treatment which was repeated three times. Next the coupons were dried using ascending gradients of hexamethyldisilazane (HMDS):ethanol (25:75, 50:50, 75:25 and 100:0) for 15 minutes each, with the final 100% HMDS (Acros Organics, Fisher Scientific) step repeated twice. After the final dehydration step the coupons were mounted on stubs, sputter coated with gold-palladium (27 nm) and stored under vacuum until the time of viewing. Specimens were viewed using a Hitachi S-4700 FEG Scanning Electron Microscope (Hitachi High Technologies Canada, Toronto, ON, Canada) operated at 3.0 kV. The magnification of the images taken ranged from 500 to 25,000×.

3.2.8. Modelling of L. monocytogenes Desiccation Survival Curves

The survival of *L. monocytogenes* during the desiccation treatments was modelled using the Weibull model (Mafart *et al.,* 2002), which is parameterized as follows:

$$\log(N_t) = \log(N_0) - (t/\text{delta})^p \qquad (1)$$

where delta is the time to the first decimal (log) reduction, N_0 is the initial population density, t is the time and p is the shape parameter. For downward concave curves, p will be greater than 1, while upward concave curves will have p-values that are below 1. If traditional first order kinetics is observed, then p=1 (delta=D-value).

The Weibull model was fitted to the data using the Microsoft Excel® Add-in software, GInaFIT (version 1.5), available at the KULeuven/BioTec homepage (http://cit.kuleuven.be/biotec) and developed by Geeraerd *et al.* (2005) to generate estimates for delta, p and $\log(N_0)$ for each of the six replicates. The fitting performance of

the model was statistically evaluated based on three indices (r^2, MSE_{model} and the F test) as previously described (Truelstrup Hansen and Vogel, 2011). These indices were calculated by fitting the Weibull model to all data from the replicates at once.

In addition to the two model parameters of interest (delta, p), the overall loss in viability (Δ log CFU/cm^2) after 20 days of desiccation was calculated for all treatments as:

$$\Delta \log CFU/cm^2 = \log CFU/cm^2_{day20} - \log CFU/cm^2_{day0} \qquad (2)$$

3.2.9. Statistical Analysis

The Multivariate Analyses of Variance (MANOVA) were performed to test the effect of the independent main factors (three initial bacterial contamination levels, presence or absence of bacterial osmoadaptation prior to desiccation, initial salt levels (0.5 or 5%) in desiccation menstruum (TSB-glu), presence or absence of biofilm formation) on the dependent vector variable composed of the Weibull model's delta- and p-parameters and the Δ log CFU/cm^2 values. Univariate Analysis of Variance (ANOVA) was also performed to test the effect of the independent main factors (same as above for MANOVA) and their interactions on bacterial survival as expressed by the Δ log CFU/cm^2 values (dependent). Finally, separate ANOVAs were computed to test the effect of the initial unsaturated (5 and 10%) and saturated (20 and 60%) fat levels in desiccation menstruum and the other main factors on the bacterial desiccation survival. The statistical analyses were carried out using SYSTAT® 11.0 software package (Systat Software Inc., San Jose, CA, USA) and R (http://www.r-project.org/). The Tukey post hoc test was used to compare the means at the 95% confidence level.

3.3. Results

3.3.1. Impact of Initial Contamination Levels on Survival of Non-Biofilm L. monocytogenes During Desiccation on Stainless Steel Coupons

Our results reveal that the density of cells on the SS surface generally has no impact on *L. monocytogenes'* survival kinetics. As shown in Figure 3-1a (no salt stress), all initial contamination levels (3.5, 5.5 and 7.5 log CFU/cm^2) exhibited similar survival curves with most loss in viability (1.5-2.0 log CFU/cm^2) occurring in the first 2 days, after which survivor plateaus (no further reduction in survival) proportional to the original cell densities were observed with final losses of 2.9-3.0 log CFU/cm^2. This result was observed for all four salt treatments (± osmoadaptation prior to desiccation and ± desiccation in high salt media) indicating that the desiccation inactivation kinetics were independent of the initial level of surface contamination (Figure 3-1 a-d).

The non-linear inactivation kinetics of these curves was suitably modelled using the Weibull model (Mafart *et al.,* 2002) as revealed by the statistical analysis of the model fits (Table 3-1). The time to the first log reduction (90% loss in viability) was mostly equal to or less than two days (Table 3-1). Although the delta parameter is a useful tool in comparing survival curves, the size of the remaining population is also highly relevant for food safety reasons. Therefore, the overall reduction in cell survival after 20 days was used to compare the effect of contamination levels and salt treatments. After 20 days of desiccation, average final cell reduction values of 2.0 and 2.9 log CFU/cm^2 were significantly different ($p<0.000$, Supplemental material Table A3) for cells desiccated in high and low salt media, respectively, on the SS coupons (Figure 3-1 a-d and Table 3-1). In general, Δ log CFU/cm^2 values were similar among the three contamination levels within each of the salt treatments (Table 3-1). This trend was, however, not observed for

the high and low contamination levels of osmoadapted cells desiccated in high and low salt media, respectively, where Δ log CFU/cm^2 values significantly differed ($p<0.05$) from the other two contamination levels (Table 3-1).

The experimental survivor detection limit of 0.90 log CFU/cm^2 should be taken into consideration when statistically comparing the desiccation survival curves of the lowest contamination level. With the exception of non-osmoadapted cells desiccated in high salt media (Δ log CFU/cm^2 = −1.42), the largest possible cell reduction of 2.60±0.50 log CFU/cm^2 was reached by the low contamination levels of the remaining three salt treatments (Table 3-1).

The 20-day desiccation period was best survived by non-osmoadapted cells desiccated in high salt media, regardless of the contamination level (Figure 3-1b). Consequently, the delta-values of 9.28 and 10.29 days obtained for this treatment's low and medium contamination levels were the highest observed under the conditions used in this study (Table 3-1, Figure 3-1b).

Microscopic imagery of the SS coupons after 2 days of desiccation showed increasing bacterial coverage of the stainless steel surface as the initial contamination level rose from 3.5 to 7.5 log CFU/cm^2 (Figure 3-2). Fluorescence microscopy (Figure 3-2 a-c) with the LIVE/DEAD stain showed an approximately equal distribution of dead or damaged cells (red) and live cells (green) on the coupons. The morphology of cells ranged from normal to elongated rod shapes. In the high contamination level samples (Figure 3-2c), clusters of cells and chains were also observed. The SEM (Figure 3-2d-f) images provided further evidence of the rising concentrations of monolayer cells on the SS coupons as the initial contamination levels increased.

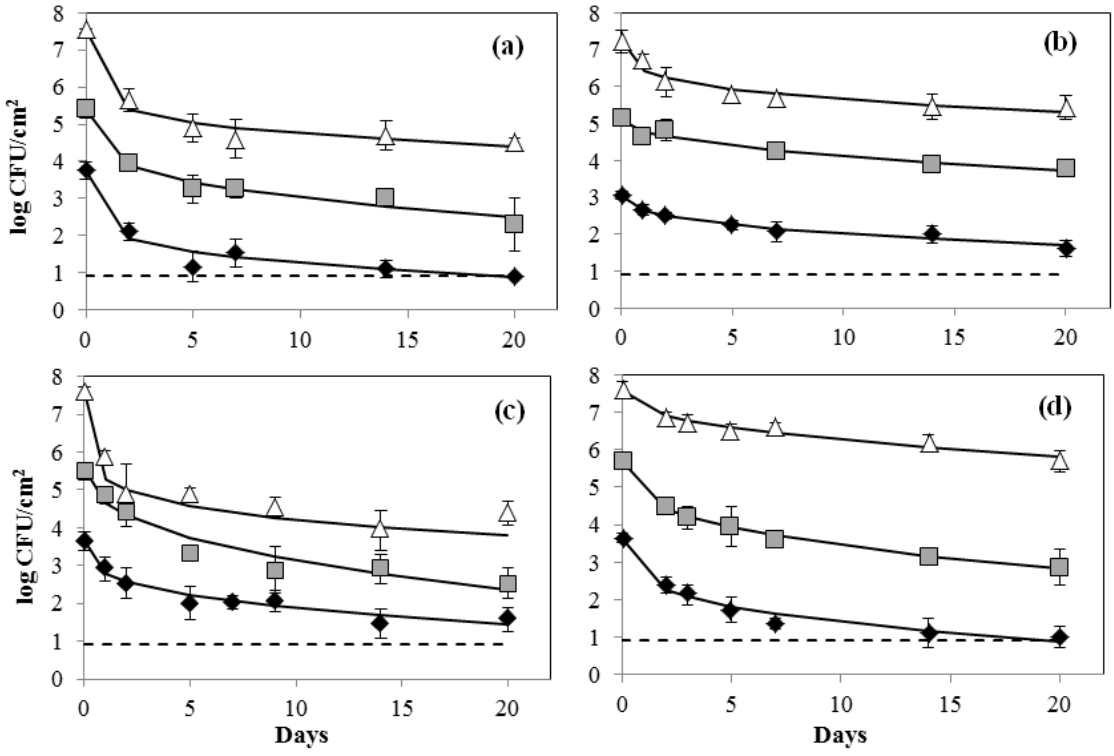

Figure 3-1. Desiccation survival of different concentrations of non-biofilm *L. monocytogenes* cells on stainless steel coupons. Cells were pre-cultured in TSB-glu and re-suspended in (a) TSB-glu or (b) TSB-glu+5% NaCl, or pre-cultured in TSB-glu+5% NaCl and re-suspended in (c) TSB-glu or (d) TSB-glu+5% NaCl, adjusted to three different initial contamination levels representing high (Δ) medium (■), and low (♦) surface densities and desiccated (43% RH, 15°C) for 20 days. The horizontal dashed line corresponds to the detection limit of the experiment. Symbols are averages of replicates (n=6) and error bars indicate standard deviation.

Table 3-1. Desiccation survival kinetics of non-biofilm *L. monocytogenes* cells on stainless steel coupons over 20 days. Osmoadapted and non-osmoadapted cells were re-suspended in TSB-glu or TSB-glu+5% NaCl and adjusted to three initial contamination levels (7.5, 5.5, 3.5 log CFU/cm^2) before being spotted on stainless steel coupons and desiccated (43% RH, 15°C). Survivors were enumerated on BHI agar and survivor curves modelled using the Weibull model (n=6).

Salt treatment	Osmo-adaptation	Desicca-tion salt level	Initial contamination level	Δ log CFU/cm^2 (day20-day0)	Model parameter estimates		Statistical indices of model fit		
					Delta (days to first log red.)	*p* (shape parameter)	MSE_{model}	r^2	*f-value*
1	No	Low	High	−3.03±0.07 $^{AB\ a\ 2}$	0.02±0.03 $^{A\ b\ 1}$	0.17±0.04 $^{ACD\ a}$	0.14	0.89	1.23*
			Medium	−3.04±0.74 $^{BE\ a\ 1}$	0.45±0.29 $^{A\ a\ 1}$	0.28±0.05 $^{ABCD\ a}$	0.14	0.87	1.03*
			Low	−2.85±0.22 $^{ABE\ a\ 1}$	0.08±0.09 $^{A\ a1}$	0.19±0.04 $^{ACD\ a}$	0.11	0.90	1.33*
2	No	High	High	−1.78±0.43 $^{CDF\ a\ 12}$	1.81±0.93 $^{AB\ b\ 12}$	0.28±0.05 $^{ABCD\ a}$	0.09	0.81	1.24*
			Medium	−1.39±0.24 $^{D\ a\ 2}$	10.29±2.07 $^{D\ a\ 2}$	0.47±0.08 $^{BCE\ a}$	0.04	0.86	1.32*
			Low	−1.42±0.20 $^{CD\ a\ 2}$	9.28±2.04 $^{CD\ a\ 2}$	0.39±0.06 $^{CE\ a}$	0.03	0.85	1.02*
3	Yes	High	High	−1.86±0.28 $^{CD\ b\ 1}$	4.97±1.36 $^{BC\ b\ 2}$	0.41±0.05 $^{BE\ a}$	0.04	0.87	1.13*
			Medium	−2.80±0.42 $^{AB\ a\ 1}$	0.96±0.38 $^{A\ a\ 1}$	0.35±0.04 $^{ABCD\ a}$	0.10	0.89	0.94*
			Low	−2.63±0.34 $^{ABF\ a\ 1}$	0.61±0.28 $^{A\ a\ 1}$	0.29±0.03 $^{ABCD\ a}$	0.09	0.89	1.26*
4	Yes	Low	High	−3.69±0.56 $^{E\ a\ 3}$	0.01±0.01 $^{A\ b\ 1}$	0.16±0.05 $^{D\ b}$	0.18	0.87	1.09*
			Medium	−2.94±0.48 $^{ABE\ a\ 1}$	1.20±0.55 $^{A\ a\ 1}$	0.41±0.06 $^{ABC\ a}$	0.15	0.86	1.14*
			Low	−2.10±0.57 $^{ACD\ b\ 3}$	1.53±0.78 $^{A\ a\ 1}$	0.31±0.14 $^{ABCD\ a}$	0.11	0.80	1.03*

Values in the same column followed by a different capital letter are significantly ($p<0.05$) different
Values in the same column and salt treatment followed by a different small letter are significantly ($p<0.05$) different
Values in the same column corresponding to the same contamination level and followed by a different number are significantly ($p<0.05$) different
*Indicates that the fit of the model was suitable

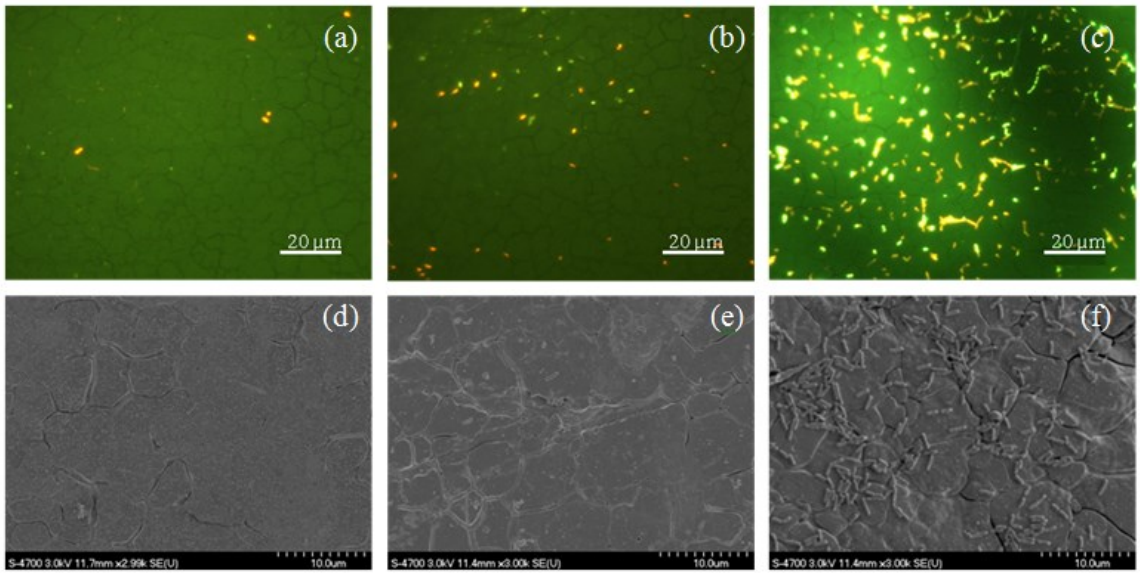

Figure 3-2. Scanning electron and fluorescence microscopy of non-biofilm *L. monocytogenes* after 48 h of desiccation on stainless steel coupons. Fluorescence microscopy (×1000; a, b, c) and SEM (×3000; d, e, f) examination of coupons with initial contamination levels of 3.5 (**a** and **d)**, 5.5 (**b** and **e**), and 7.5 log CFU/cm^2 (**c** and **f**), respectively. For fluorescence microscopy cells were stained with *Bac*Light™ LIVE/DEAD® stain where live cells with intact membranes fluoresce green and dead or damaged cells fluoresce red.

3.3.2. Desiccation Survival of L. monocytogenes in Biofilms of Different Developmental Stages

To further study the survival of *L. monocytogenes* on food processing surfaces, the impact of different initial contamination levels and salt treatments on *Listeria's* ability to form a biofilm (48 h, 100% RH) and survive subsequent desiccation was investigated. When non-osmoadapted cells were re-grown and desiccated in low salt media, all three initial contamination levels (3.5, 5.5, 7.5 log CFU/cm^2) reached the maximum surface cell density of 8.50 log CFU/cm^2 after 48 h (Figure 3-3a). In spite of the 48 h of re-growth eliminating the difference in cell densities among the three individual contamination levels, the desiccation survival kinetics differed between the contamination levels. Coupons inoculated with the lowest initial contamination level exhibited a statistically

($p<0.05$) larger reduction in survival (−3.24 log CFU/cm^2) after 20 days when compared to the medium and high initial contamination levels (−1.49 and −1.71 log CFU/cm^2, respectively) where the latter were not found to be significantly ($p>0.05$) different from each other (Table 3-2). Based on these results it was hypothesized that the lowest initial contamination level may just have reached the maximum surface cell density after 48 h and not had sufficient time to either form a biofilm at all or form a biofilm to the same level of maturity as the two higher initial contamination levels. This hypothesis is supported by the lack of difference ($p>0.05$, Supplemental material Table A5) between delta-values obtained for biofilm and non-biofilm treatments with low initial contamination levels (Table 3-1, Table 3-2) and indicates the inability of immature biofilms to protect cells during desiccation. For the medium and high initial contamination levels, however, significantly ($p<0.05$) different delta values were found for *L. monocytogenes* desiccated on coupons following biofilm formation (2.52-4.98 days) as compared to desiccation without biofilm formation (0.02-0.45 days) (Table 3-1, Table 3-2, salt treatment 1).

Osmoadapted cells re-grown and desiccated in low and high salt media, showed similar desiccation survival patterns (Figure 3-3 c,d) to non-osmoadapted cells desiccated in low salt (Figure 3-3a). For osmoadapted cells desiccated in low salt, all initial contamination levels attained the maximum surface cell density during the 48 h re-growth/biofilm formation period (Figure 3-3c). Delta-values of 6.47, 11.47 and 1.15 days for the high, medium, and low initial contamination levels, respectively, show that cells of the medium and high initial contamination levels were significantly ($p<0.05$) more resistant to desiccation than those of the low initial contamination level (Table 3-2). This is also supported by log reduction values of −1.25 and −1.38 log CFU/cm^2 for the medium

and high contamination levels and −2.29 log CFU/cm^2 for the low contamination level after 20 days of desiccation (Table 3-2). When osmoadapted cells were re-grown and desiccated in high salt media, the pre-exposure to salt enabled the cells to grow at a similar rate to those which were re-grown and desiccated in low salt media. The medium and high initial contamination levels grew to the maximum cell surface density of 8.50 log CFU/cm^2 while the low contamination level reached 7.50 log CFU/cm^2 (Figure 3-3d). Again the log reduction value for the low initial contamination level (−2.02) was significantly ($p<0.05$) larger than that of the medium (−0.59) and high (−0.97) initial contamination levels (Table 3-2). Accordingly, delta-values for the medium and high initial contamination level desiccation curves tended to be larger compared to the low initial contamination level although the difference was not significant ($p>0.05$). For the medium and high contamination levels less than a one log reduction was achieved during the 20-day desiccation period resulting in large standard errors being associated with the estimated delta-values for these treatments (Table 3-2).

When non-osmoadapted cells were re-grown and desiccated in high salt media, different survivor trends resulted. Three separate cell densities remained on the coupons throughout the 48 h of biofilm formation with only the high initial contamination level reaching the maximum surface cell density (Figure 3-3b). When desiccated, no significant differences ($p>0.05$) were found between any of the initial contamination levels with respect to delta-values and final log reductions after 20 days (Table 3-2).

Both the SEM and fluorescence micrographs showed the different stages of biofilm formed by the three initial contamination levels (Figure 3-4). With the exception of non-osmoadapted cells desiccated in high salt media, biofilms formed by the high initial contamination levels (Figures 3-4c,f) were observed to form a honeycomb structure with

single layer coverage surrounded by multilayers of aggregated cells (Figure 3-5a). A closer look (Figures 3-5b-c) shows the presence of extracellular fibrils extended both between cells as well as between cells and the stainless steel surface. These fibrils may be remnants of extracellular polymeric substances (EPS) as indicated in Figure 3-5d which shows cells surrounded by a more preserved EPS structure. Coupons with the medium initial contamination level biofilms were mostly covered by a monolayer of cells (Figure 3-4b,e) and showed the beginning stages of fibril formation (image not shown) but not to the extent of the high contamination level. No fibrils were observed on coupons of the low initial contamination levels, suggesting lack of biofilm formation during the 48 h period (Figure 3-4a,d). These features were observed regardless of the salt treatment used. Looking at the individual cells, SEM images revealed elongated biofilm cells (Figure 3-5e) and cells in chains (Figure 3-5f) when re-growth and desiccation took place under high initial salt conditions.

Further statistical analyses revealed that the desiccation survival of *L. monocytogenes* was not significantly ($p>0.05$) impacted by the initial contamination level irrespectively of the biofilm or salt treatments, whereas significant ($p<0.05$) effects of biofilm formation/maturity, osmoadaption and desiccation salt levels were observed (Supplemental material, Tables A1 and A2). However, multiple significant interactions were found in the ANOVA on the Δ log CFU/cm^2 response variable (Supplemental material, Table A1) which suggested the existence of complex relationships between the environmental variables. For example as also noted above, biofilm was found to significantly ($p<0.05$) interact with initial contamination level, suggesting that the protective effect of biofilm depended on the initial level of contamination and vice versa. In addition, the three way interaction between biofilm, desiccation salt, and inoculation

level was found to be significant (Supplemental material, Table A1), suggesting a complicated effect where the protection by the biofilm depended on the initial desiccation salt and contamination levels.

When contrasting survival across all the salt treatments, biofilm cells from the medium and high contamination levels survived desiccation significantly ($p<0.05$, for Δ log CFU/cm^2 values, Supplemental material, Table A6) better compared to non-biofilm cells at the same initial contamination levels. Regardless of the salt treatment, there were no significant differences in survival ($p>0.05$, for both Δ log CFU/cm^2 and delta-values, Supplemental material, Table A5) of non-biofilm and biofilm cells on coupons with low initial contamination levels.

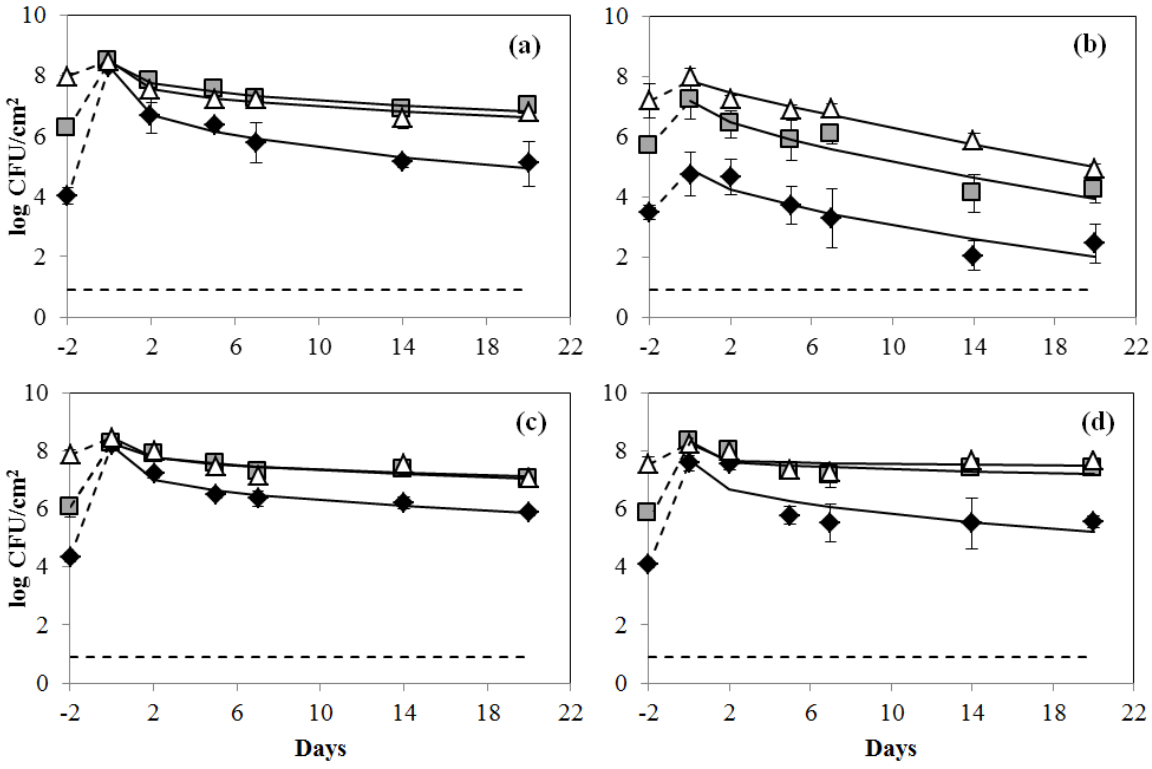

Figure 3-3. Desiccation survival of *L. monocytogenes* cells in different stages of biofilm development on stainless steel coupons. Cells were pre-cultured in TSB-glu and re-suspended in (a) TSB-glu or (b) TSB-glu+5% NaCl, or pre-cultured in TSB-glu+5% NaCl and re-suspended in (c) TSB-glu or (d) TSB-glu+5% NaCl, adjusted to three different initial contamination levels (representing high (Δ), medium (■), and low (♦), initial surface contamination, spotted on stainless steel coupons and kept at 100% RH for 48 h to allow biofilm formation prior to desiccation. The horizontal dashed line corresponds to the detection limit of the experiment. Symbols are averages of replicates (n=6) and error bars indicate standard deviation.

Table 3-2. Desiccation survival kinetics of biofilm *L. monocytogenes* cells on stainless steel coupons over 20 days. Osmoadapted and non-osmoadapted cells were re-suspended in TSB-glu or TSB-glu+5% NaCl, adjusted to three initial contamination levels (7.5, 5.5, 3.5 log CFU/cm^2), spotted on stainless steel coupons and allowed to form a biofilm for 48 h prior to being desiccated (43% RH, 15°C, 20 days). Survivors were enumerated on BHI agar and survivor curves modelled using the Weibull model (n=6)

Salt treatment	Osmo-adaptation	Desicca-tion salt level	Initial contamination level	Δ log CFU/cm^2 (day20-day0)	Model parameter estimates		Statistical indices of model fit		
					Delta (days to first log red.)	*p* (shape parameter)	MSE_{model}	r^2	*f-value*
1	No	Low	High	−1.71±0.03$^{A\ a\ 1}$	2.52±1.10$^{A\ ab\ 1}$	0.31±0.07$^{A\ a}$	0.05	0.92	1.10*
			Medium	−1.49±0.11$^{AC\ a\ 1}$	4.98±1.35$^{A\ a\ 2}$	0.36±0.06$^{A\ a}$	0.03	0.93	1.25*
			Low	−3.24±0.62$^{D\ b\ 2}$	0.45±0.33$^{A\ b\ 2}$	0.32±0.06$^{A\ a}$	0.18	0.91	0.91*
2	No	High	High	−3.07±0.22$^{DE\ a\ 2}$	5.92±0.87$^{A\ a\ 1}$	0.86±0.09$^{B\ a}$	0.07	0.94	1.74*
			Medium	−2.84±0.72$^{DE\ a\ 2}$	3.44±1.47$^{A\ a\ 2}$	0.66±0.14$^{B\ a}$	0.34	0.79	1.28*
			Low	−2.31±1.24$^{BDE\ a\ 12}$	3.86±2.09$^{A\ a\ 1}$	0.64±0.18$^{B\ a}$	0.46	0.70	1.30*
3	Yes	High	High	−0.59±0.14$^{C\ a\ 3}$	325±1272$^{A\ a\ 1}$	0.10±0.10$^{C\ a}$	0.09	0.63	2.99
			Medium	−0.97±0.21$^{C\ b\ 1}$	11.88±8.04$^{A\ a\ 1}$	0.20±0.08$^{AC\ b}$	0.09	0.63	2.30*
			Low	−2.02±0.34$^{ABE\ c\ 1}$	2.06±1.62$^{A\ a\ 12}$	0.39±0.12$^{A\ c}$	0.44	0.62	2.12*
4	Yes	Low	High	−1.38±0.13$^{AC\ a\ 4}$	6.47±2.99$^{A\ a\ 1}$	0.28±0.06$^{A\ a}$	0.06	0.76	5.51
			Medium	−1.25±0.16$^{AC\ a\ 1}$	11.57±2.51$^{A\ b\ 1}$	0.39±0.06$^{A\ b}$	0.03	0.86	1.59*
			Low	−2.29±0.13$^{BE\ b\ 12}$	1.15±0.40$^{A\ c\ 12}$	0.29±0.03$^{A\ a}$	0.04	0.94	1.54*

Values in the same column followed by a different capital letter are significantly ($p<0.05$) different
Values in the same column and salt treatment followed by a different small letter are significantly ($p<0.05$) different
Values in the same column corresponding to the same contamination level and followed by a different number are significantly ($p<0.05$) different
*Indicates that the fit of the model was suitable

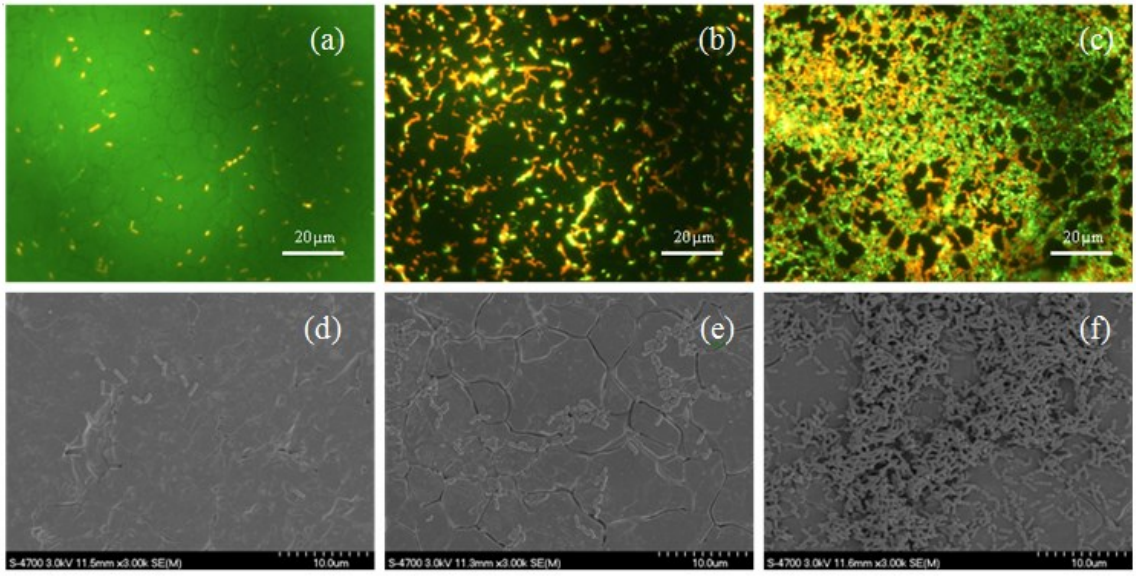

Figure 3-4. Scanning electron and fluorescence microscopy of *L. monocytogenes* cells in three different stages of biofilm development, obtained after 48 h of desiccation (15°C, 43% RH) on stainless steel coupons. Fluorescence microscopy (×1000; a, b, c) and SEM (×3000; d, e, f) examination of coupons with initial inoculation levels of 3.5 (**a** and **d**), 5.5 (**b** and **e**), and 7.5 log CFU/cm^2 (**c** and **f**), respectively. For fluorescence microscopy cells were stained with *Bac*Light™ LIVE/DEAD® stain where live cells with intact membranes fluoresce green and dead or damaged cells fluoresce red.

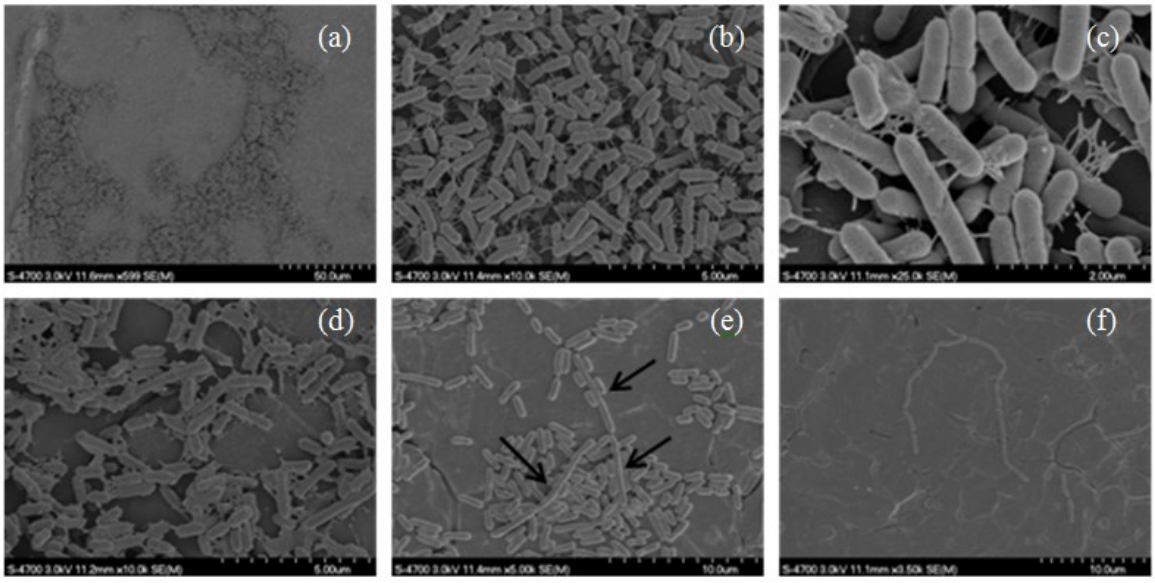

Figure 3-5. Scanning electron microscopy images showing details of *L. monocytogenes* mature biofilms (high initial contamination level, 15°C, 100% RH, 48 h) after desiccation (48 h, 15°C, 43% RH) on stainless steel coupons. **(**a) Overview of the biofilm showing heterogeneous coverage. (b) Extracellular fibrils anchoring cells to each other as well as to the stainless steel surface. (c) Details of the high initial contamination level biofilm structure. (d) Preserved EPS structure in biofilms on coupons with the high initial contamination level. (e) Long cells and (f) chains of cells observed when biofilm developed in the presence of high salt.

3.3.3. Protective Effect of Food Lipids During Desiccation of L. monocytogenes on Stainless Steel Coupons

Lastly, the desiccation survival of *L. monocytogenes'* in the presence of various levels of saturated and unsaturated fats simulated to be left on food processing surfaces by incomplete cleaning procedures, was investigated. When cells were desiccated in a matrix consisting of 0, 5 and 10% canola oil, no trending differences were observed between the model parameters estimated from the survival curves (Figure 3-6a, Table 3-3). A one log reduction in survival was reached in less than one day for all treatments. After 20 days of desiccation, final log reductions ranged from −4.91 to −5.81 log CFU/cm^2.

With regard to the survival of *L. monocytogenes* in a matrix of saturated animal lard on stainless steel, cells desiccated in the presence of 20 and 60% lard showed initial

resistance to desiccation in comparison to the control (0% lard) (Figure 3-6b). This was supported by delta values of 1.37 and 4.24 days for 20 and 60% lard, respectively, in comparison to 0.02 days for the control (Table 3-3). In fact, a linear relationship was found between the level of saturated fat on the stainless steel surface and the delta value in the tested range of lard concentrations (delta-value = 0.0771×(%lard)−0.0007, R^2 = 0.993). After approximately 5 days of desiccation the difference between the three levels began to diminish as the survivors in the control (0% lard) reached a plateau and the 20 and 60% lard survivor counts continued to descend. At the end of the 20-day desiccation period, final cell reductions were −3.76, −3.13 and −2.93 log CFU/cm^2 for cells desiccated in the presence of initial levels of 0, 20 and 60% lard, respectively, with no significant difference ($p>0.05$) between 20 and 60% lard.

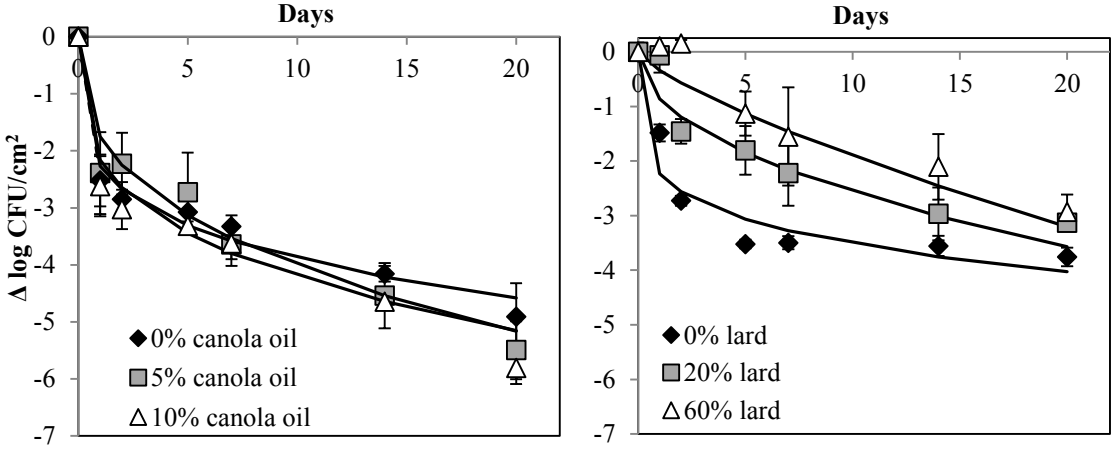

Figure 3-6. Desiccation survival of *L. monocytogenes* cells in fat matrices on stainless steel coupons. Cells were pre-cultured in TSB-glu, re-suspended in TSB-glu containing either 20 or 60% animal lard, spotted on stainless steel coupons at a cell density of 7.5 log CFU/cm^2 and desiccated (15°C, 43% RH) for 20 days. Coupons were sampled on days 0, 1, 2, 5, 7, 14, and 20. The y-axis displays the reduction in cells from the initial inoculation amount. Symbols are averages of replicates (n=6) and error bars indicate standard deviation.

Table 3-3. Survival kinetics of *L. monocytogenes* cells desiccated in fat matrices on stainless steel coupons. Cells (7.5 log CFU/cm^2) were desiccated (43% RH, 15°C) in TSB-glu containing either canola oil (0, 5, 10%) or animal lard (0, 20, 60%) for 20 days. Survivors were enumerated on BHI agar and survivor curves modelled using the Weibull model (n=6).

Fat type	Fat level (%)	Δ log CFU/cm^2 (day 20-day0)	Model parameter estimates		Statistical indices of model fit		
			Delta (days to first log red.)	*p* (shape parameter)	MSE_{model}	r^2	*f-value*
Canola Oil	0	−4.91±0.59 [a]	0.03±0.02 [a]	0.23±0.02 [a]	0.15	0.93	1.32*
	5	−5.50±0.51 [ab]	0.21±0.13 [b]	0.36±0.04 [b]	0.36	0.89	1.24*
	10	−5.81±0.28 [b]	0.07±0.04 [ab]	0.29±0.03 [ab]	0.15	0.95	1.78*
Animal Lard	0	−3.76±0.17 [b]	0.02±0.02 [a]	0.20±0.02 [a]	0.15	0.92	9.01
	20	−3.13±0.12 [a]	1.37±0.62 [a]	0.47±0.07 [b]	0.24	0.85	1.74*
	60	−2.93±0.32[a]	4.24±1.33 [b]	0.75±0.13 [c]	0.27	0.82	1.24*

Values in the same column and fat type followed by a different letter are significantly ($p<0.05$) different
*indicates that the fit of the model was suitable

3.4. Discussion and Conclusions

3.4.1. Impact of Initial Contamination Levels on Survival of L. monocytogenes During Desiccation on Stainless Steel Coupons

The desiccation inactivation kinetics of *L. monocytogenes* on stainless steel was independent of the initial cell surface densities. This meant that after 20 days of desiccation residual survivor levels were proportional to the initial levels of contamination (Figure 3-1). Originally it was hypothesized that cells in close proximity of each other might induce quorum sensing to initiate stress adaptive mechanisms or benefit from the expulsion of extracellular water and organic matter from the lysing of surrounding cells. However, our findings indicate that desiccation tolerance in *L. monocytogenes* may be a function of the cell itself and independent of its proximity to neighbouring cells. Similar desiccation survival experiments involving three concentrations of *L. monocytogenes* (Dreux *et al.,* 2007) and *Staphylococcus aureus* (Kusumaningrum *et al.,* 2003) on parsley leaves and stainless steel surfaces, respectively, produced similar non-linear inactivation kinetics showing no overall protective effect of a higher concentration of cells. In both studies, the presence of a subpopulation which appeared to have enhanced survival during desiccation stress was also noted. However, the inactivation kinetics was not modelled in these studies. Further work is needed to understand whether this extended survival is due to environmental, physiological, or genetic factors.

In agreement with previous work (Vogel *et al.,* 2010), *L. monocytogenes* cells desiccated in high salt (5% NaCl) TSB-glu showed ≥1 log CFU/cm^2 improvement in survival after 5 days of desiccation in comparison to the survival of cells desiccated in low salt (0.5% NaCl) TSB-glu (Figure 3-1). This mediated survival may be due to the accumulation and/or synthesis of salt stress proteins and/or osmolytes (Ghandi and

Chikindas, 2007; Schmid *et al.,* 2009; Sleator *et al.,* 2003). In fact, osmoadaptation prior to desiccation had a significant ($p<0.05$, for delta and Δ log CFU/cm^2, Supplemental material Table A4) impact on the desiccation survival of non-biofilm cells, leading to cells surviving equally well in the presence of low and high initial desiccation salt levels in four out of six cases (Table 3-1).

3.4.2. Desiccation Survival of L. monocytogenes in Biofilms of Different Developmental Stages

The protective effect of a biofilm during desiccation appeared to rely on the bacterium reaching and/or spending sufficient time at the maximum cell surface density on the SS coupons and the formation of extracellular fibrils or EPS (Figure 3-5). With the exception of non-osmoadapted cells re-grown in high salt media (all contamination levels) and the low contamination level of osmoadapted cells re-grown in high salt media, all other initial contamination levels reached the maximum cell surface density observed for *L. monocytogenes* in the SS model system, of 8.50 log CFU/cm^2 after 48 h under 100% RH at 15°C. Although this cell surface density was reached, different desiccation kinetics were observed. Except for non-osmoadapted cells re-grown in high salt media, cells in biofilms formed by the medium and high initial contamination levels were statistically ($p<0.05$) more resistant to desiccation than those in low initial contamination level biofilms (Table 3-2). In turn, the desiccation kinetics of the low initial contamination level biofilm cells resembled that of their non-biofilm counterpart. The same was true for all initial contamination levels of non-osmoadapted cells re-grown and desiccated in high salt where biofilms never reached the maximum cell density. An earlier study showed that 48 h old mature biofilms enhanced the desiccation survival of *L. monocytogenes* (Truelstrup Hansen and Vogel, 2011). The present study is, however, to the best of our knowledge the

first to report the impact of varying developmental stages of biofilm on *Listeria's* desiccation survival on food-grade SS surfaces. This information is highly relevant for food processors as it shows how inadequate cleaning procedures may enable surviving *L. monocytogenes* cells to form a mature biofilm if left for a 2-day inactive period at high RH. Mature biofilm formation will in turn lead to increased environmental resistance including enhanced desiccation survival.

Fluorescence and SEM images of the coupons revealed three distinct levels of biofilm formation (Figure 3-4). Biofilms formed by the high initial contamination levels took on a honeycomb structure (also called a net) described by other researchers as a single layer coverage of cells surrounded by multilayers of aggregated cells (Marsh *et al.,* 2003; Thar and Kuhl, 2002**;** Schaudinn *et al.,* 2007). An intense system of extracellular fibrils interconnected cells to each other as well as to the SS surface. The presence of similar structures has been noted in other isolates of *L. monocytogenes* bound to solid surfaces (Herald and Zottola, 1988; Kalmokoff *et al.,* 2001; Marsh *et al.,* 2003). It is hypothesized that the fibrils may represent extracellular polymeric substances which have condensed as a result of specimen preparation (Herald and Zottola, 1988). The medium initial contamination level biofilm showed only the beginning stages of fibril formation and the majority of the coupon surface was covered by a monolayer of cells. Cells on the low initial contamination level coupons were randomly located on the surface and showed no sign of fibril formation.

Discrepancies existed between the plate count data and microscopy images of the biofilm coupons. When enumerating using the plate count method, the medium and high contamination level coupons appeared to contain the same number of cells, however, microscopy revealed very different cell densities. It may be that the mature fibril formation

seen on the high initial contamination level coupons allowed cells to adhere more strongly to the surface than cells on the two lower initial contamination level coupons resulting in more cells being washed away from these coupons during specimen preparation for microscopy.

The combination of desiccation kinetics and microscopy images presented here verify that *L. monocytogenes'* desiccation survival is enhanced by the presence of a biofilm which is created when cells have spent sufficient time at the maximum cell density to begin the production of EPS. Interestingly, no significant differences ($p>0.05$) were observed between the desiccation kinetics of the medium and high initial contamination level biofilms (not including non-osmoadapted cells re-grown in high salt) in spite of their attaining distinctively different stages of biofilm formation. This may indicate that after a certain stage of biofilm development, further maturity and increased structural complexity does not subsequently increase desiccation survival of *L. monocytogenes*. Increased desiccation tolerance of biofilm cells may be due to a shift in cell metabolism, cell envelope composition and/or protective effect of the biofilm matrix. Surface adhering *L. monocytogenes* cells produce a significant amount of EPS and the amount varies among serotypes and depends on environmental factors such as temperature, nutrients, salt, etc. (Djordjevic *et al.,* 2002; Di Bonaventura *et al.,* 2008; Pan *et al.,* 2010; Zameer *et al.,* 2010). It has been reported that *Listeria* EPS and biofilm formation involve extra-cellular DNA (eDNA) and peptidoglycans including N-acetylglucosamine (Harmsen *et al.,* 2010; Böckelmann *et al.,* 2006). These polymers are highly hydrophilic and may aid in reducing the rate of biofilm and cellular drying.

While non-osmoadapted cells re-grown and desiccated in high salt did not form a biofilm, osmoadapted cells re-grown in high salt showed no delay in growth or biofilm

formation and, in turn displayed increased desiccation survival (Figure 3-3b,d and Table 3-2). *Listeria* is often exposed to high salt environments during both food processing and preservation and this study shows the significance such osmoadaptation has on biofilm development in the continual presence of salt.

The re-growth/biofilm formation of *L. monocytogenes* under high salt conditions produced long and chain linked cells (Figure 3-5). These elongated cells have been reported in *L. monocytogenes* cultures grown in >5% salt and are composed of several normal size cells which fail to divide during low a_w stress (Jørgensen *et al.,* 1995; Zarei *et al.,* 2012; Isom *et al.,* 1995). It is believed that elongated cells are actually close to dividing and when transferred to more favourable conditions, will split up into single cells and start growing (Hazeleger *et al.,* 2006). This is a large concern for the food industry since cross contamination from a high to low salt food product and/or environment could result in the rapid division of elongated pathogens into many cells resulting in highly contaminated food products.

3.4.3. Protective Effect of Food Lipids During Desiccation of L. monocytogenes on Stainless Steel Coupons

The current study is to the best of our knowledge the first to report the effects of fat on *Listeria's* desiccation survival on SS surfaces used in the production of food products. The fat percentages were chosen to resemble those found in food products associated with listeriosis including smoked salmon which commonly contains between 5-10% unsaturated fat and luncheon meats which range from 20-60% saturated fat. The results from this study showed that levels of canola oil up to 10% had no overall impact on *L. monocytogenes* desiccation survival (Figure 3-6a, Table 3-3). However, when *L. monocytogenes* was desiccated in animal lard, rising levels (20 to 60%) of saturated fat

were positively correlated with increased desiccation survival during the first 14 days of desiccation (Figure 3-6b). After this time, no significant difference ($p>0.05$) was found between residual survivor levels in 20 and 60% lard (Table 3-3). This trend was also noted in the work done by Takahashi *et al.* (2011) where initial differences in survival of *L. monocytogenes* suspended in slurries of ground tuna, pork, and cabbage began to disappear after 14 days of desiccation.

Hiramatsu *et al.* (2005) reported that the desiccation survival of pathogenic *E. coli* and *Salmonella* inoculated into a cocoa drink without sucrose and then placed on filter paper discs, was enhanced by 51 and 370 times, respectively, compared to saline solution, leading the authors to suggest that the cocoa butter fat likely was a contributing factor. In support of this, two other research groups (Tamminga *et al.,* 1976; Ostovar, 1973) concluded that *Salmonella* and *Staphylococcus* strains died off more rapidly in chocolate containing <6% fat than in chocolate containing 20-55% fat which had more than twice the amount of solid fat (cocoa butter). In a cross-contamination experiment using slicers, the prolonged transfer of *L. monocytogenes* by contaminated salami in comparison to turkey and bologna, was thought to be a result of the former's higher fat content which formed a visible layer on the blade (Vorst *et al.,* 2006). Together with our findings this suggests that high fat levels on processing surfaces may lead to better bacterial protection, survival, and extended transfer to food products.

Recently, a *Salmonella enteritidis* strain was found to express less than 5% of its genes when subjected to desiccation and starvation stress in 100% peanut oil while it expressed 78% of its genes when growing in Luria-Bertani Broth (Deng *et al.,* 2012). The ability to enter such a "dormancy" state might explain the survival observed for several

bacteria including *L. monocytogenes* in low water activity environments, and should be further investigated.

In conclusion, the current work shows that the non-linear desiccation inactivation kinetics observed for sessile *L. monocytogenes* cells on SS surfaces is independent of initial contamination levels. Furthermore, this study highlights the importance of preventing the formation of mature biofilms and ensuring complete removal of salt and fat soils from food contact surfaces in order to limit the subsequent survival of *L. monocytogenes* in low RH environments.

CHAPTER 4

Insertional Mutagenesis Reveals Genes that may Contribute to *Listeria monocytogenes*' Desiccation Tolerance

4.1. Introduction

The food-borne pathogen *L. monocytogenes* is capable of surviving desiccation under a number of conditions (Truelstrup Hansen and Vogel, 2011) with survival extending up to three months in a simulated food processing environment (Vogel *et al.,* 2010). Despite the importance of preventing the cross-contamination of foods by pathogens such as *L. monocytogenes*, the mechanisms used by these bacteria to adapt and survive in low water content environments is largely understudied.

Cellular dehydration can lead to protein denaturation, DNA breaks, and membrane phase transitions from a fluid to a gel phase (Potts, 1994). The damage to proteins and nucleotides as well as lipid peroxidation are largely due to the oxidative stress imposed by the increased concentration of cytoplasm ions which leads to the formation of reactive oxygen species (Potts, 1994). In addition, bacteria living on dry surfaces are also faced with starvation stress.

Responses to osmotic stress provide some insight into the general mechanisms used by bacteria to survive intracellular water loss. Furthermore, cells exposed to matric stress also experience osmotic stress as dehydration causes the concentration of solutes to increase both outside and inside the cell. However, van de Mortel and Halverson (2004) reported that in *P. putida* a larger number of genes were upregulated during matric stress than in the thermodynamically equivalent solute stress, suggesting that adaptation to desiccation stress is much more complex. There is likely a considerable overlap in bacterial

responses to a wide variety of environmental stresses due to redundancies in regulatory networks (Martinez-Bueno *et al.,* 2002).

A number of recent transcriptome studies of Gram-negative bacteria under either desiccation or matric (presence of non-permeating high-molecular-weight molecules such as PEG 8000) stress conditions, have identified several genes upregulated in response to desiccation stress. Some of these genes include those involved in heat shock, extracellular polysaccharide synthesis and transport, DNA repair, transcription and ribosome structure, oxidative stress, amino acid and lipid biosynthesis, trehalose synthesis, osmolyte and potassium transport, oxidative stress, envelope modification, stress response, energy production, and virulence (Cytryn *et al.,* 2007; Li *et al.,* 2012; Gruzdev *et al.,* 2012; van de Mortel and Halverson, 2004; Gülez *et al.,* 2012).

The objective of this study was to investigate some of the possible molecular mechanisms which may contribute to *L. monocytogenes*' desiccation tolerance. A library of over 11,000 *Himar1 L. monocytogenes* 568 transposon insertion mutants was screened for strains displaying increased or decreased desiccation survival. This represents the first study to use insertional mutagenesis to identify important genes/mechanism which contribute to *L. monocytogenes*' desiccation tolerance. Furthermore, the cross protection of these genes against osmotic stress was analyzed.

4.2. Materials and Methods

4.2.1. Insertional Mutagenesis of L. monocytogenes 568

Insertional mutagenesis was carried out using the shuttle vector pMC39 (kindly obtained from the Marquis laboratory, Cornell University, Ithaca, NY, US), which contains a temperature sensitive origin of replication and the mariner transposon (*Himar1*).

The pMC39 was electroporated into competent *L. monocytogenes* strain 568 cells according to the method described by Alexander *et al.* (1990). The procedure was carried out in 0.1 cm cuvettes in a Micropulser^{TM} (BioRad Laboratories, Inc., Mississauga, ON, Canada) set to a field strength of 1.0 kV. Following electroporation, the cells were re-suspended in 1.0 ml of SOC medium (2% Tryptone (Oxoid, Fisher Scientific), 0.5% Yeast Extract (Oxoid, Fisher Scientific), 10 mM NaCl (Fisher Scientific), 2.5 mM KCl (Fisher Scientific), 10 mM $MgCl_2$ (Fisher Scientific), 10 mM $MgSO_4$ (Mallinckrodt, Fisher Scientific), 20 mM glucose (Fisher Scientific)) and transferred to a 15 ml centrifuge tube. The cells were allowed to recover for 3 h at 28°C without shaking.

Transposon mutagenesis was performed following the protocol described by Cao *et al.* (2007). Lm568 transformants were selected at 30°C on BHI (Difco, Fisher Scientific, Whitby, ON, CA) agar (15%, Technical Agar, Difco, Fisher Scientific) plates supplemented with erythromycin (erm, Sigma-Aldrich, Oakville, ON, CA) at 5 µg/ml. Individual colonies were picked and grown overnight in BHI with erm and kanamycin (kan) (10 µg/ml) (Sigma-Aldrich) at 30°C with shaking. The cultures were diluted 1/200 in broth with erm (10 µg/ml), grown for 1 h at 30°C with shaking, and then shifted to 40°C for about 6 h until the absorbance at 600 nm was between 0.3 and 0.5 (NanoPhotometer® P330, Implen, Westlake Village, CA, US). Aliquots of the culture were spread plated on BHI agar supplemented with erm (10 µg/ml) and incubated at 40°C for two days. Approximately 11,700 mutants were picked from these plates and inoculated into microwell plates (Costar* Clear Polystyrene 96-Well Plates, Fisher Scientific) containing 100 µl of TSB (Difco, Fisher Scientific) and 100 µl of 30% glycerol (Fisher Scientific) (for a final concentration of 15% glycerol) and grown for two days at room temperature (20-22°C) before being stored at -80°C until further use.

Plasmid retention was restimated to be less than 2.5 % and 25 mutants were sequenced to confirm random insertion of the transposon in the Lm568 chromosome.

4.2.2. Selection of Desiccation Mutants – First Screening and Growth Curves

Lm568 transposon insertion mutants were subjected to a two-stage screening process to identify genes contributing to increased or decreased tolerance to desiccation stress. Frozen library stocks were thawed and 10 µl from each well was used to inoculate 190 µl of fresh TSB (Difco, Fisher Scientific) distributed in microwell (96 wells) plates. These cultures were then grown at room temperature overnight (24 h) to allow all transposon mutants to reach the same approximate cell density and final absorbance readings were recorded at 490 nm using a BioTek* ELx808* Absorbance Microplate Reader (Fisher Scientific) connected to a computer operating Gen5™ 2.0 Reader Control software (BioTek, Fisher Scientific). To determine the growth kinetics of the mutants, 10 µl from each well of the overnight cultures was inoculated into microwell plates containing 190 µl of fresh TSB and then were incubated at 15°C. Plates were read (490 nm) every 3 h until stationary phase was reached by most mutants (approx. 20 h). Subsequently, 10 µl from the overnight cultures were spotted on the bottom of empty microwell plates and placed in desiccators pre-conditioned to 43% RH and 15°C (See section 3.2.4). After 5 days the plates were removed from the desiccators. Cells in each well, representing one unique transposon mutant each, were rehydrated with 190 µl of TSB followed by plates being incubated at 15°C. Every 3 h the plates were briefly shaken and A_{490} was recorded until stationary phase was achieved by most isolates (approx. 28 h). The resulting regrowth curves were used in selecting desiccation mutants for the second round of screening. This selection was based on the premise that mutants with increased

and impaired desiccation survival would show higher and lower regrowth-after-desiccation rates, respectively, in comparison to the wildtype strain Lm568, which underwent the same desiccation followed by regrowth treatment.

To be chosen for the second round of screening, transposon mutants had to display regrowth-after-desiccation curves which strongly deviated from both the wild type (Lm568) and the majority of isolates. Resistant mutants were chosen based on displaying short lag phases and reaching A_{490}=0.40 at least 2 h before the majority of mutants. Similarly, sensitive mutants displayed extended lag phases, reached A_{490}=0.10 at least 2 h later than the majority of isolates, and were unable to enter stationary phase within 25 h at 15°C. Mutants also had to display growth kinetics similar to that of the wildtype to avoid misjudging slow and fast growing isolates as having decreased and increased desiccation tolerance, respectively.

4.2.3. Desiccation Survival of Selected Transposon Mutants on Stainless Steel Coupons

In total, 190 isolates were selected from the frozen stocks and streaked onto both erm (10 µg/ml) and kan (10 µg/ml) BHI agar plates to confirm the presence of the transposon and absence of the pMC39 plasmid, respectively. From the BHI+erm plates, colonies were inoculated into 5 ml of TSB-glu (1% w/v glucose, Fisher Scientific) and incubated at 15°C for two days. Following incubation, cultures were centrifuged, re-suspended in fresh TSB-glu to A_{450}=1 and diluted to obtain a cell density of 7.5 log CFU/cm^2 when inoculated onto the cleaned and sterilized SS coupons (section 3.2.2). Coupons were desiccated (43% RH, 15°C) for 7 days and sampled as previously described (section 3.2.6). Three coupons were prepared for each mutant per sampling day (days 0, 1, 2, 4, 7) and survivors were enumerated via the plate count method on BHI agar. Mutants,

which continued to exhibit increased or decreased (>±0.50 Δ log CFU/cm^2 after 4 days) desiccation survival on the SS coupons relative to the wildtype Lm568, were selected for arbitrary PCR and DNA sequencing.

4.2.4. Modelling and Statistical Analysis of Mutant Desiccation Survival Curves

The desiccation survival of Lm568 insertion mutants was modelled using the Weibull model as described in section 3.2.8. As previously done, the fitting performance of the model was evaluated using three indices (r^2, MSE_{model} and the F test). In addition to model parameters (delta, p), and final cell reductions (Δ log CFU/cm^2 day 7), Δ log CFU/cm^2 day 4 values were also included to help distinguish between low, high, and intermediate low and high survivors. The statistical analyses were performed in Microsoft Excel using the two-tailed Student t tests to determine whether desiccation survival differed significantly ($p<0.05$) between each mutant and the wildtype.

4.2.5. Arbitrary PCR

To elucidate the possible functions of the genes interrupted in the isolated mutants, the arbitrary primer PCR method (Garsin *et al.,* 2004; Cao *et al.,* 2007) was used to determine nucleotide sequences flanking the transposon insertions. This method used two sequential PCRs to amplify the transposon-chromosome junctions. The first PCR uses primers complimentary to the left (L) and right (R) ends of the transposon (255-L, 269-R) and an arbitrary primer containing 3' random nucleotides (207). At low temperatures the arbitrary oligonucleotides will prime at random sites in the chromosome. Due to the arbitrary nature of the priming, this first PCR yields fragments of mixed sizes, many of which will be the irrelevant products of two arbitrary primers landing near one another. In

the second PCR the transposon specific PCR products are re-amplified at a higher temperature and with greater specificity using two transposon-specific primers (256-L, 270-R) that are proximal to the junctions and a primer (208) complimentary to the non-random 5' end of the original arbitrary primer. The final PCR products are sequenced using the transposon specific primers (257-L, 271-R). See Table 4-1 for a complete list of primers (Sigma-Aldrich) and their corresponding nucleotide sequences.

The first round of arbitrary PCR was performed using an IDTaq DNA Polymerase Kit (ID labs Manufacturing Inc., London, ON, CA) and mutant colonies on BHI-erm plates. Each 24.5 µl reaction contained: 2.5 µl 10× $MgCl_2$ free buffer, 0.5 µl dNTP mixture (2.5 mmol each), 2.0 µl $MgCl_2$, 0.25 µl ID Taq polymerase, 18.25 µl RNA free dH_2O, 0.5 µl of each primer stock (10 mmol each), and one colony. The following PCR cycle was conducted using a Tgradient thermocycler (Whatman Biometra, Goettingen, Germany): a 5 min initial denaturation step at 95°C followed by 30 cycles of 95°C denaturation for 30 s, 34°C annealing for 45 s and 72°C elongation for 1 min. This was followed by a final extension step of 10 min at 72°C. The resulting PCR products were diluted 0.5 µl in 24.5 µl of RNA free dH_2O and 0.5 µl of this was used in the second PCR reaction. Each reaction consisted of: 5 µl 5 × buffer, 0.5 µl high fidelity taq (HotStar Taq DNA Polymerase, Qiagen, Toronto, ON, CA), 2.5 µl of each primer stock, and 14 µl RNA free dH_2O for a total of 25 µl including the diluted products from round I. The PCR cycle was as follows: 5 min initial denaturation step at 95°C followed by 40 cycles of 95°C denaturation for 30 s, 34°C annealing for 30 s and 72°C elongation for 1 min. The final extension step was 72°C for 10 min. Gel electrophoresis on an agarose gel (1.5% agarose (Fisher Scientific), 1% TAE (Tris-acetate EDTA, Fisher Scientific) was used to confirm the presence of amplified regions prior to sequencing (See Appendix A for a photo example).

Table 4-1. Primers used in arbitrary PCR reaction as described by Cao *et al.* (2007).

Primers		*Sequence (5' to 3')*	*Annealing temperature (°C)*
Round I			
Arbitrary	207	GGCCACGCGTCGACTAGTACNNNNNNNNNN GTAAT	74.3
Left	255	CAGTACAATCTGCTCTGATGCCGCATAGTT	73.2
Right	269	GCTCTGATAAATATGAACATGATGAGTGAT	65.7
Round II			
Arbitrary	208	GGCCACGCGTCGACTAGTAC	66.9
Left	256	TAGTTAAGCCAGCCCCGACACCCGCCAACA	82.6
Right	270	TGTGAAATACCGCACAGATGCGAAGGGCGA	82.0
Sequencing			
Left	257	CTTACAGACAAGCTGTGACCGTCT	65.6
Right	271	GGGAATCATTTGAAGGTTGGTACT	64.8

4.2.6. DNA Purification, Sequencing, and Identification of Interrupted Genes

Purification of sequencing products obtained by the arbitrary PCR method was performed using a QIAquick® PCR Purification kit (Qiagen) as directed. The purified DNA samples were then diluted in pH adjusted dH_2O (pH 7.0-8.5) to a final concentration of 1-10 ng/µl using the NanoPhotometer® P330. Sequencing of the insertion sites was performed using the Sanger sequencing reaction (McGill University and Génome Québec, Montréal, CA). The sequences were aligned using BLASTn online software (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) and comparisons were made using the GeneBank database for the complete sequence of *Listeria monocytogenes* strain EGD-e (Glaser *et al.,* 2001; GeneBank ID NC 003210), or other suitable sequenced *L. monocytogenes* 1/2a serotype genomes if there was no match in the EGD-e genome.

4.2.7. Phenotyping of Desiccation Mutants

4.2.7.1. Cross Protection of Desiccation Genes Against Osmotic Stress

Sequenced desiccation mutants were tested for their ability to survive high osmotic stress conditions. Colonies from BHI-erm plates were pre-cultured in 5 ml TSB-glu for two days at 15°C, centrifuged (2,824 g for 5 min), and re-suspended in 5 ml TSB-glu containing 20% (w/v) NaCl (Fisher Scientific). A 0.315 µl aliquot of this culture was then diluted to 10 ml with TSB-glu + 20% NaCl in a 15 ml centrifuge tube to obtain a final concentration of 7.5 log CFU/ml. The tubes were stored at 15°C for 49 h and survivors were enumerated periodically using plate count method on BHI agar. Survivors after 49 h were statistically compared between the mutant strains and the wildtype using a two-tailed Student t-test as described above. A Bivariate Pearson Correlation analysis was also performed using IBS SPSS Statistics 21.0.0 (available for download at http://www14.software.ibm.com/download/data/web/en_US/trialprograms/W110742E06714B29.html) to determine if there was a relationship between the desiccation survival of Lm568 transposon mutants after 4 days (Δ log CFU/cm^2) and osmotic stress survival after 49 h (Δ log CFU/ml).

4.2.7.2. Motility Assay and Flagella Staining

Lm568 and select mutants containing transposon insertions in flagella related genes were tested for motility using a soft agar assay. Colonies for the wildtype and mutant strains were picked from BHI or BHI+erm agar plates, respectively, and stabbed into a petri dish containing BHI agar (0.3% agar). The plates were incubated at room temperature (20-22°C) for two days. A large area of bacterial growth surrounding the initial point of the stab inoculation indicated bacterial motility.

Flagella staining was performed on the wildtype and flagella-related insertional mutants to confirm the presence or absence of flagella on non-motile mutants as determined by the soft agar assay. The stain was prepared using the protocol described by Heimbrook *et al.* (1989). There are two components; Solution 1, the mordant, contains 10 ml of 5% aqueous solution of phenol (Fisher Scientific), 2 g of tannic acid (Mallinckrodt inc., Fisher Scientific), and 10 ml of saturated aqueous solution of aluminum potassium sulfate-12 hydrate (0.15 g/ml, Acros Organic, Fisher Scientific). Solution 2, the stain, is a saturated solution of crystal violet (Gurr, BDH, Toronto, ON, CA) in 95% ethanol (0.12 g/ml). The final stain was prepared by mixing 10 parts mordant with 1 part stain and then filtering this solution through a filter paper to remove coarse precipitate. The stain was kept in a syringe attached to a 0.22-µm-pore-size membrane (Corning, Fisher Scientific) until further use. Cells grown for two days on fresh BHI plates at room temperature were used for flagella staining. A single colony was gently mixed with 10 µl of sterile dH_2O on an ethanol cleaned microscope slide and then a coverslip was immediately applied on top. The slides were left for 10 min to allow cells to attach to the glass and then 2 drops of stain was applied to the edge of the coverslip and allowed to flow under and mix with the cell suspension. After 15 min the cells were examined under 1000× magnification with a Nikon Eclipse 80i light microscope (Nikon Canada, Mississauga, ON) for the presence or absence of flagella using the wildtype Lm568 as a the flagella expressing standard for comparison.

4.2.7.3. Heat Tolerance Assay

One of the most desiccation resistant mutants, 71D9, was found to contain a transposon insertion in the same gene as a previously reported Lm568 heat resistant mutant (Ells *et al.,* 2009). To determine if this phenotype also exists in mutant 71D9, the mutant

and the wildtype were exposed to a mild heat treatment. Cells were first cultured in TSB-glu for two days at 15ºC and then centrifuged and re-suspended in PS to a concentration of 7.5 log CFU/ml. From here, 110 µl aliquots were distributed in PCR tubes in triplicate and heated to 55ºC in a Tgradient thermocycler for 30 min. After this time, aliquots were serially diluted in PS, plated on BHI agar, and incubated for two days at 37ºC before being enumerated.

4.3. Results

4.3.1. Screening Insertion Library for Mutants Displaying Increased or Decreased Desiccation Survival

A total of 11,700 mutants were screened for increased or decreased desiccation survival leading to the selection of 190 mutant candidates, which were further tested on SS coupons. The regrowth-after-desiccation microwell plate assay proved to be successful in identifying mutants of interest. There was a high correlation between slow growers in both the growth curve and regrowth-after-desiccation assay, demonstrating that the protocol was effective in distinguishing slow growing mutants from those with impaired desiccation survival although some overlap may still have occurred.

Figure 4-1 illustrates the detection of both types of desiccation mutants. In general, the microwell assay detected a higher number of low desiccation survival mutants (129) than high survivors (61). However, in the more detailed desiccation survival screening on SS surfaces, a larger portion of the high desiccation survivors (25%) from the initial screening maintained their desiccation resistant phenotype in comparison to the low survivors, where only 13% of strains met the elected criteria of having a decreased survival of at least 0.50 CFU/cm^2 after 4 days compared to the wildtype. Ultimately, 15 high and 16 low desiccation survival strains were selected for further analysis and DNA sequencing.

Four of these strains (61B9, 9D11, 31G10, 45C5) expressed resistance to kan indicating either plasmid retention or incorporation into the chromosome. Given that two strains were high survivors and the other two low survivors, plasmid retention appeared to not have a uniform impact on *L. monocytogenes'* desiccation survival. Subsequent transfers onto BHI+erm agar was successful in eliminating kan resistance in three of the mutants but was ineffective for 31G10 which most likely had incorporated the plasmid into its chromosome. For the other three mutants, no differences in desiccation survival were found between cells of the same mutant with and without kan resistance (data not shown).

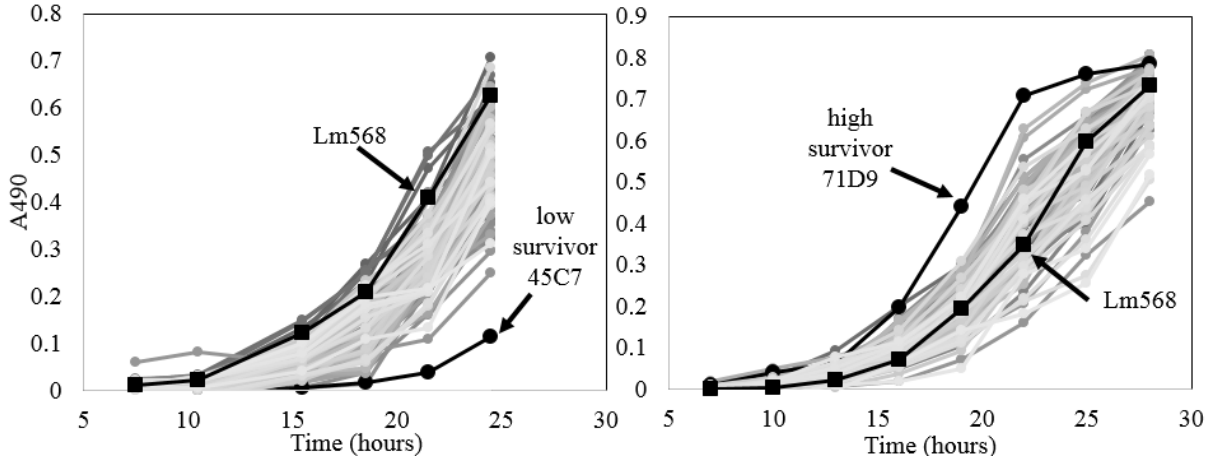

Figure 4-1. Examples of regrowth-after-desiccation curves displaying mutants with low and high desiccation survival. Ten µl from overnight cultures was spotted on the bottom of empty 96-well plates and placed in desiccators pre-conditioned to 43% RH and 15°C. After 5 days the plates were removed and each well was rehydrated with 190 µl of TSB. Plates were then incubated at 15°C and absorbances (490 nm) were recorded every 3 h until stationary phase was achieved by most mutants.

Desiccation tolerant mutants were divided into two categories: high survivors and intermediate high survivors. High survivors were classified as displaying less than a 1 log reduction in survival by day 4 of the desiccation period whereas intermediate high survivors displayed >1 log reduction in survival by day 4 and a significantly ($p<0.05$) improved survival (Δ log CFU/cm^2 day 4) in comparison to the wildtype (Figure 4-2). At

the end of the 7 day desiccation period high survivors and intermediate high survivors decreased by < -1.15 and < -1.51 log CFU/cm^2, respectively, while the wildtype was reduced by -1.75 log CFU/cm^2 (Table 4-2). Similarly, delta values (with some exceptions) ranged from 5.04-9.93 days for high survivors, 1.76-2.82 days for intermediate high survivors compared to 0.01 days for the wildtype. The majority of strains (9/15) showed significantly larger ($p<0.05$) delta values than Lm568. It is important to note that the accuracy of the predicted delta values is dramatically reduced if a one log reduction is not reached within the experimental period. This was the case for four highly desiccation resistant mutants (71D9, 51F6, 32D4, 9D11) and as a result the Weibull model predicted a horizontal line for the survival kinetics, producing infinite delta values denoted as ∞. Therefore, although these delta values were not significantly different ($p>0.05$) from the wildtype, these strains were among some of the most resistant mutants identified in this study.

When final survival levels (Δ log CFU/cm^2 day 7) were statistically compared between resistant strains and the wildtype, 10 mutants showed significantly ($p<0.05$) increased desiccation survival after 7 days (Table 4-2). Five intermediate strains which demonstrated initial desiccation tolerance expressed as elevated delta-values, did not differ significantly ($p<0.05$) from the wildtype by the end of the desiccation period (Table 4-2). This suggests that some gene interruptions responsible for initial increases in desiccation tolerance may have less of an impact on survival after desiccation for 4 days and longer.

The two most tolerant strains (51F6, 71D9) exhibited only a -0.20 log CFU/cm^2 decrease in survival throughout the entire desiccation experiment. Notably, both strains were also detected as slow growers however, their desiccation survival was so great that

their regrowth-after-desiccation curves showed no signs of slow growth and recovered more quickly than the wildtype and all other mutants in their respective microwell plates. Although slow growing mutants would most likely be outcompeted in the environment, the mechanisms used by these bacteria to strongly resist desiccation stress can still provide important knowledge on desiccation survival and for this reason they were kept for DNA sequence analysis. Mutant 51F6 was also found to develop a donut shaped colony morphology (Figure 4-10) following 4 or more days of desiccation on SS or when streak plates on BHI were left for extended periods of time at refrigeration temperatures.

Similar to the resistant mutants, strains displaying decreased desiccation tolerance were categorized as being low or intermediate low desiccation survivors displaying > or < 3 log reduction after 4 days, respectively. A few exceptions include the intermediate low desiccation survivor 38B6 located right on the classification border and low desiccation survivors 22G3 and 31G10 which displayed < 3 log reduction after 4 days but exhibited amongst the largest losses in viability by day 7 (-4.09 and -4.58 Δ log CFU/cm^2, respectively) (Table 4-3). All strains displaying impaired desiccation survival showed significantly ($p<0.05$) larger total losses in viability compared to the wildtype after 7 days of desiccation. Final decreases in survival ranged from -2.37 to -4.07 log CFU/cm^2 for intermediate low and -3.27 to -4.58 log CFU/cm^2 for low desiccation surviving strains compared to -1.75 for the wildtype. Some strains exhibited a rapid loss in viability within the first 24 h of desiccation with very little further loss whereas other strains showed continual decreases in survival throughout the 7 day period. This made comparing the low survival mutants slightly more complicated however, mutant 72H7 stood out as consistently being the most susceptible to desiccation stress.

Delta values for low survival mutants ranged from 0.00 to 0.24 days demonstrating the rapid loss in survival exhibited by these strains within the first few hours of desiccation. Given that the delta value for Lm568 also fell within this small range (0.01 days), no significant differences ($p>0.05$) could be detected between the delta-values obtained for impaired desiccation survival strains and the wildtype. All strains experienced the most inactivation within the first 24 h of desiccation. For Lm568 this was followed by an almost horizontal survival plateau which, due to lack of sampling between 0 and 24 h, lead the modeling software to overcompensate the sudden loss in survival possibly resulting in an underestimated delta value (Figure 4-4). Therefore the true delta value for the inactivation of Lm568 may more likely lie around 0.50±0.15 days. Aside from the slight limitations in the experimental design, the Weibull model fit to the data generally resulted in suitable descriptions of the survival kinetics of all the desiccation mutants as determined by the F test and r^2 values (Table 4-2, Table 4-3).

The most compelling result from the desiccation of the low survival *L. monocytogenes* mutants was that most strains still survived at concentrations of more than 3.5 log CFU/cm^2 on the SS coupons after 7 days of desiccation. This implies that even the most impaired desiccation survival strains of *L. monocytogenes* still may out compete a large majority of commensal microorganisms found in the food processing environment.

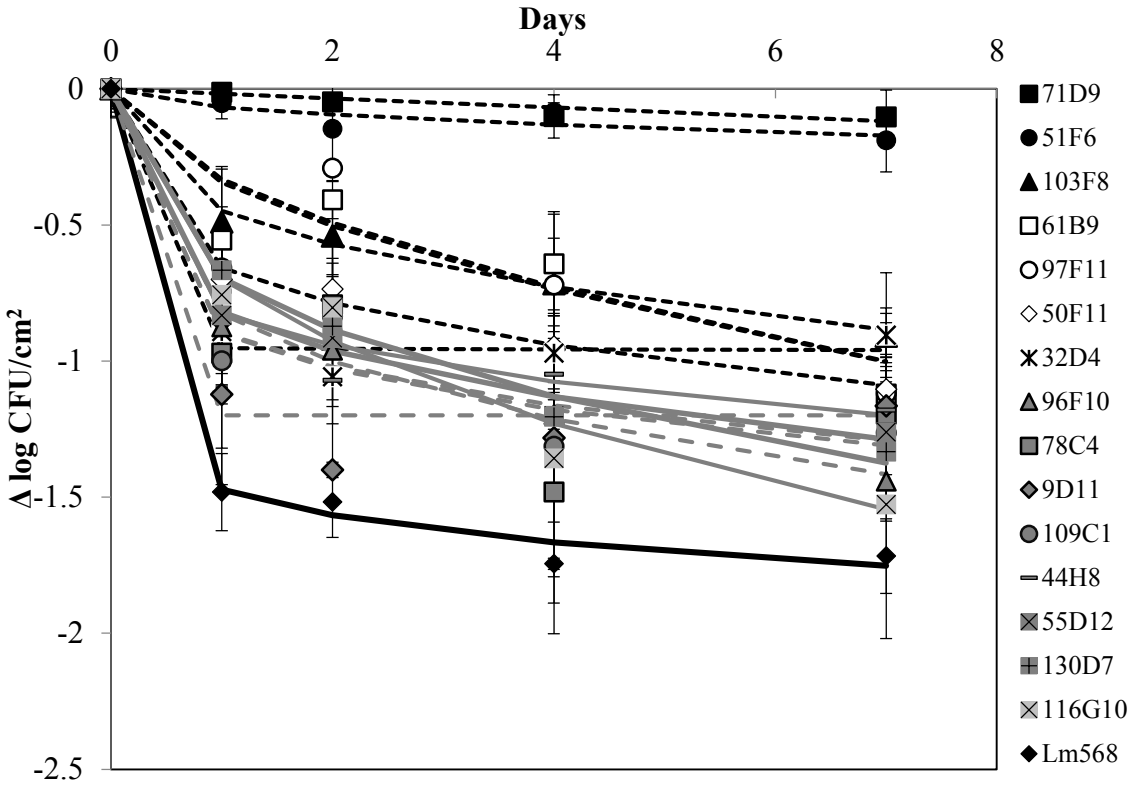

Figure 4-2. *L. monocytogenes* 568 transposon mutants displaying increased desiccation survival in comparison to the wildtype. High and intermediate high survivors were characterized by displaying viability losses of <1 and >1 Δ log CFU/cm^2 after 4 days of desiccation, respectively. Strains were cultured and re-suspended in TSB-glu, spotted on stainless steel coupons at 7.5 log CFU/cm^2 and desiccated for 7 days at 43% RH, 15°C. Grey dashed lines indicate intermediate high survivors and black dashed lines represent high survivors. The wildtype is presented as a solid back line for comparison. Symbols are averages of replicates (n=3) and error bars indicate the standard deviation.

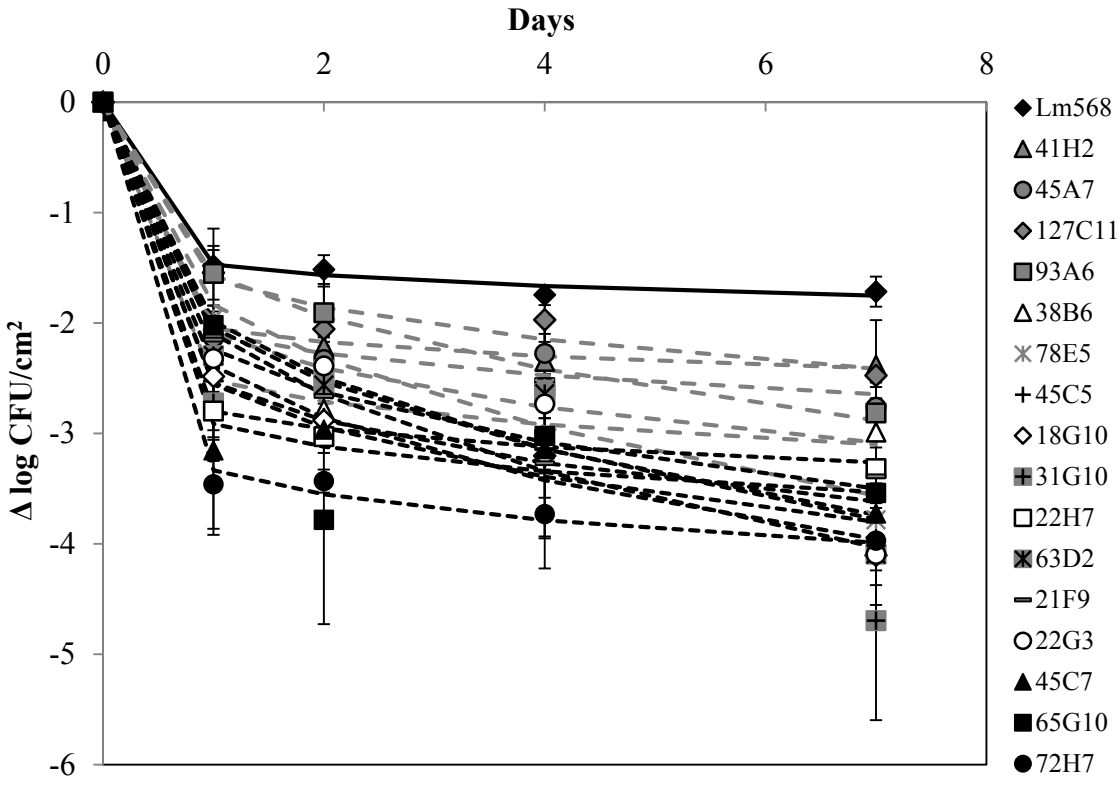

Figure 4-3. *L. monocytogenes* 568 transposon mutants displaying decreased desiccation survival in comparison to the wildtype. Low and intermediate low survivors were characterized by displaying viability losses of > 3 and < 3 Δ log CFU/cm^2 after 4 days of desiccation, respectively. Strains were cultured and re-suspended in TSB-glu, spotted on stainless steel coupons at 7.5 log CFU/cm^2 and desiccated for 7 days at 43% RH, 15°C. Grey dashed lines indicate intermediate low survivors and black dashed lines represent low survivors. The wildtype is presented as a solid back line for comparison. Symbols are averages of replicates (n=3) and error bars indicate the standard deviation.

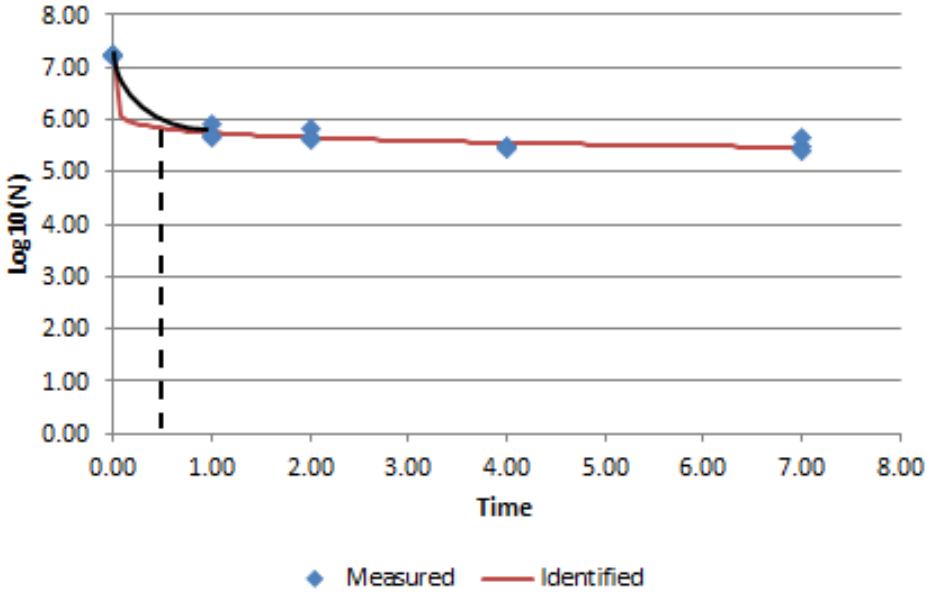

Figure 4-4. *L. monocytogenes* 568 inactivation kinetics during desiccation at 43% RH at 15°C as predicted by the Weibull model using the data set with sampling on days 0, 1, 2, 4 and 7. The black line may represent the more likely desiccation survival trend for Lm568 with the dashed line predicting a delta value of around 0.5 days in comparison to 0.01 days predicted based on the current data set.

Table 4-2. Desiccation survival kinetics and model parameters of *Listeria monocytogenes* 568 transposon mutants displaying increased desiccation survival. Mutants were cultured and re-suspended in TSB-glu, spotted on stainless steel coupons and desiccated at 43% RH, 15°C for 7 days (n=3). F-values lower than 2.75 indicate that the fitting performance of the model is statistically accepted. Values in the same column followed by a * are statistically ($p<0.05$) different from the wildtype.

	Mutant #	*Delta (time to 1 log reduction)*	*P (shape parameter)*	*Δ Log CFU/cm²* *day 4*	*Δ Log CFU/cm²* *day 7*	*Statistical indexes* MSE_{model}	r^2	*f-value*
Wildtype	Lm568	0.01±0.02	0.09±0.03	-1.74±0.02	-1.72±0.15	0.01	0.98	1.05
High survivors	71D9	∞	0.97±0.94*	-0.10±0.08*	-0.10±0.19*	0.00	0.31	0.88
	51F6	∞	0.47±0.47	-0.09±0.03*	-0.19±0.13*	0.01	0.29	0.95
	103F8	9.93±4.60*	0.35±0.13*	-0.72±0.25*	-0.91±0.23*	0.03	0.78	0.86
<1 log reduction after 4 days	61B9	6.99±2.15*	0.57±0.21*	-0.64±0.19*	-1.12±0.33*	0.05	0.73	1.26
	97F11	6.95±2.23*	0.55±0.21*	-0.72±0.17*	-1.12±0.10*	0.05	0.73	2.65
	50F11	5.04±1.48*	0.26±0.06*	-0.95±0.03*	-1.10±0.17*	0.01	0.94	0.92
	32D4	∞	0.00±0.06	-0.97±0.15*	-0.91±0.14*	0.02	0.91	1.08
Intermediate high survivors	96F10	1.98±0.59*	0.28±0.05*	-1.20±0.05*	-1.44±0.19	0.01	0.95	0.90
	78C4	1.68±1.63	0.19±0.10	-1.48±0.41	-1.20±0.04*	0.07	0.78	1.82
	9D11	∞	0.00±0.09	-1.28±0.31	-1.16±0.24*	0.05	0.85	1.51
	44H8	2.73±1.80*	0.19±0.08	-1.05±0.48	-1.18±0.02*	0.04	0.86	0.99
>1 log reduction after 4 days	109C1	1.79±2.29	0.19±0.14	-1.31±0.18*	-1.26±0.31	0.13	0.67	0.96
	55D12	2.35±1.30*	0.23±0.08*	-1.26±0.12*	-1.26±0.26	0.04	0.87	0.88
	130D7	2.82±0.86*	0.35±0.07*	-1.21±0.25*	-1.32±0.26	0.03	0.92	0.91
	116G10	2.41±1.45*	0.41±0.16*	-1.36±0.45	-1.51±0.49	0.13	0.73	0.89

Table 4-3. Desiccation survival kinetics and model parameters of *Listeria monocytogenes* 568 transposon mutants displaying decreased desiccation survival. Mutants were cultured and re-suspended in TSB-glu, spotted on stainless steel coupons and desiccated at 43% RH, 15°C for 7 days (n=3). F-values lower than 2.75 indicate that the fitting performance of the model is statistically accepted. Values in the same column followed by a * are statistically ($p<0.05$) different from the wildtype.

	Mutant #	*Delta (time to 1 log reduction)*	*P (shape parameter)*	*Δ Log CFU/cm² day 4*	*Δ Log CFU/cm² day 7*	*Statistical indexes*		
						MSE_{model}	r^2	*f-value*
Wildtype	568	0.01±0.02	0.09±0.03	-1.74±0.02	-1.72±0.15	0.01	0.98	1.05
Intermediate	127C11	0.10±0.11	0.21±0.05*	-1.97±0.20	-2.61±0.07*	0.07	0.93	1.29
low	93A6	0.24±0.23	0.31±0.09*	-2.59±0.75	-2.81±0.20*	0.17	0.88	0.87
survivors	41H2	0.00±0.00	0.08±0.04	-2.35±0.25*	-2.37±0.37*	0.06	0.94	1.06
	45A7	0.00±0.00	0.12±0.03	-2.28±0.26*	-2.76±0.04*	0.04	0.97	1.33
<Δ3 log	78E5	0.17±0.10	0.34±0.05*	-2.60±0.27*	-3.78±0.24*	0.09	0.96	1.95
CFU/cm^2 by	63D2	0.10±0.09	0.31±0.06*	-2.64±0.06*	-4.07±0.45*	0.15	0.93	2.78
day 4	38B6	0. 00±0.00	0.11±0.02	-3.06±0.07*	-2.99 ±0.16*	0.02	0.99	2.03
	45C5	0.01±0.01	0.20±0.04*	-3.06±0.30*	-4.06±0.33*	0.11	0.95	1.86
Low	18G10	0.03±0.02	0.26±0.03*	-3.20±0.13*	-4.10±0.21*	0.04	0.98	1.54
survivors	31G10	0.11±0.15	0.34±0.11*	-2.66±0.14*	-4.58±0.99*	0.49	0.83	2.08
	22H7	0.00±0.00	0.08±0.05	-3.05±0.34*	-3.27±0.96*	0.14	0.93	0.86
>Δ3 log	21F9	0.03±0.03	0.23±0.05*	-3.27±0.68*	-3.39±0.05*	0.10	0.95	0.94
CFU/cm^2 by	22G3	0.13±0.08	0.33±0.05*	-2.73±0.13*	-4.09 ±0.02*	0.11	0.95	10.13
day 4	45C7	0.00±0.00	0.10±0.06	-3.14±0.79*	-3.71±0.27*	0.27	0.89	0.98
	65G10	0.01±0.02	0.18±0.09	-3.02±0.56*	-3.54±0.19*	0.56	0.78	2.23
	72H7	0.00±0.00	0.09±0.03	-3.97±0.49*	-3.97±0.19*	0.09	0.97	0.94

4.3.2. Identification of Genes Flanking Himar1 Insertions

DNA sequences were successfully obtained from the PCR amplicons that were generated from both sides of the transposon insertion site in 26 of the 31 desiccation mutants identified in this study. In addition, sequences flanking one side of the transposon insertion site were obtained for four low survival mutants (45C5, 45C7, 63D2, 31G10). No sequence was obtained for low survival mutant 41H2 since no PCR product could be obtained through arbitrary PCR. Of the 26 identified interrupted genes, the exact site of insertion was determined in 16 mutants. A one base pair gap separated the left and right flanking sequences of an additional five mutants (116G10, 78C4, 65G10, 45A7, 93A6). The remaining mutants (9D11, 96F10, 55D12, 22G3, 72H7) displayed unidentified gaps of varying lengths (34-94 bp), however, all affected genes were still determined. These gaps may have been a result of the arbitrary PCR method or differences in the sequences of Lm568 and the EGD-e strain to which the inserts were mapped to. Table 4-4 and Table 4-5 list the transposon insertion sites and corresponding gene identities of high and low desiccation survival mutants, respectively. Figure 4-6 shows the transposon insertion sites in the high and low desiccation survival mutants mapped to the *L. monocytogenes* EGD-e chromosome.

Of the 15 sequenced highly desiccation tolerant mutants, 7 contained *Himar1* inserts in motility related genes including flagellar biosynthesis, motor rotation, motor switch, basal body rod modification, and flagellar hook-associated proteins (lmo0676-lmo0706, Figure 4-7). Other genes found to be interrupted in desiccation tolerant mutants included those involved in fatty acid metabolism, membrane transport, protein biosynthesis, transcription regulation, and virulence.

Interestingly, the two most resistant mutants, 71D9 and 51F6, which displayed similar phenotypes of increased desiccation resistance and impaired growth, were in fact found to contain *Himar1* insertions in neighbouring ORF's (lmo1370,1371) of the same transcriptional unit encoding butyrate kinase (*buk*) and dihydrolipoamide dehydrogenase (*lpd*), respectively (Table 4-4). Both enzymes are known to be associated with amino acid metabolism and subsequent fatty acid (FA) biosynthesis. Mutants 50F11 and 97F11 also had insertions in neighbouring ORFs (lmo1742, 1744), however, they are not organized under the same transcriptional unit. Lmo1742 encodes adenine deaminase which is important for adenine utilization as a purine and also as a nitrogen source for microorganisms. Lmo1744 is a hypothetical protein showing high similarities to other unclassified proteins.

The remaining five high desiccation survival mutants harboured transposon insertions unrelated to any other desiccation resistant mutants, and encompassed a wide range of functions. Mutant 130D7 was determined to have a transposon inserted in ORF lmo0289. The encoded protein is similar to a regulatory protein (YycH) for a sensory box histidine kinase two-component system (TCS) (YycFG) first found in *B. subtilis* (Kasahara *et al.,* 1997) and responsible for processes such as cell wall homeostasis, cell membrane integrity, and cell division (Szurmant *et al.,* 2007). The interrupted gene (lmo1582) in mutant 55D12 corresponds to an acetate kinase and the affected gene (lmo1786) in mutant 61B9 contains an insert in *inlC* encoding internalin C, a member of the *Listeria* specific internalin family (Gouin *et al.,* 2010). Lastly, the transposon insertion in mutant 44H8 was mapped to ORF lmo0771 and shows similarity to an ATP-sensitive inward rectifier potassium channel 15 which is member 15 of the subfamily J of inward rectifying potassium channels also known as Kir1.2 or Kir4.2.

Sequencing results for the 15 sensitive mutants revealed three high density areas of transposon inserts in genes impacting desiccation survival as well as some isolated genes. The first cluster includes three mutants with *Himar1* insertions in ORFs lmo1174, 1194 and 1219/20 (Table 4-5). These genes encode an ethanolamine utilization protein (EutA), a cobalt-precorrin-6A synthase (cbiD), and a hypothetical protein in mutants 45A7, 72H7, and 63D2, respectively. In mutant 63D2 only one side flanking the transposon insertion was successfully sequenced making it unknown whether the exact insertion site lies in ORF lmo1219 or in the intergenic space between lmo1219 and 1220.

The second area of concentrated transposon insertions with a detrimental effect on desiccation survival includes mutants 45C7, 45C5, 127C11, and 78E5 with interrupted genes mapped to ORFs lmo2443, 2470, 2490, and 2503, respectively. The first insertion site lmo2443, encodes a hypothetical protein similar to glutamate tRNA ligase which is involved in glutamate metabolism and utilization. ORF lmo2470 corresponds to an unknown protein with a leucine rich domain similar to that of an internalin surface protein. The gene lmo2490 encodes a protein similar to the general stress response protein CsbA found in *B. subtilis*. Directly downstream are the genes encoding excinuclease ABC which is involved in DNA repair. Finally, 78E5, the last mutant in this cluster, contains an insert in lmo2503 which shows similarity to cardiolipin synthase.

Associated with the last cluster of genes, whose interruption diminished desiccation survival, are mutants 93A6, 38B6, and 18G10 with insertions in genes lmo2768 and 2778. Mutants 93A6 and 38B6 housed the transposon insert in two different sites in gene lmo2768 encoding a hypothetical permease protein most likely involved in membrane transport. ORF lmo2778 encodes a hypothetical protein of unknown function located directly downstream of proteins similar to a GTP-dependent binding protein engD

and cellobiose phosphotransferase system (PTS) enzyme IIA. Both ORFs lmo2768/78 are in close vicinity to other membrane proteins including ATP-transporters/binding proteins and additional PTS enzymes involved in the transport of sugars across the cellular membrane.

The remaining five low survival desiccation mutants displayed isolated gene insertions in the *L. monocytogenes* EGD-e chromosome. Only one sequence flanking the transposon insertion was obtained for mutant 31G10 making it unknown whether the insertion is in lmo0371 or the intergenic space between lmo0371/72. ORF lmo0371 encodes a protein similar to a transcription regulator of the GntR family and lies directly downstream of proteins similar to beta-glucosidase (lmo0372) and two PTS beta-glucoside-specific enzymes (lmo0373-4). Mutant 65G10 was determined to have an insertion in ORF lmo0565 corresponding to imidazole glycerol phosphate synthase subunit *hisH*. This gene is in a transcriptional unit with several other proteins (*hisB,D,G,Z*) involved in histidine biosynthesis. The affected gene (lmo0616) in mutant 21F9 encodes glycerophosphoryl diester phosphodiesterase which participates in the metabolism of glycerophospholipids, the main component of biological membranes. Lastly, mutants 22G3 and 22H7 have transposon insertions in genes lmo0983 and lmo1728, both encoding hypothetical proteins with similarities to a glutathione peroxidase and cellobiose-phosphorylase, respectively.

Table 4-4. Identified gene interruptions in *Listeria monocytogenes* 568 transposon mutants displaying increased desiccation survival. The position of the mariner insertions are listed relative to the published genome sequence of *Listeria monocytogenes* EGD-e strain (GeneBank ID NC 003210). The term similar is used to indicate genes that share high similarity (>90%) with strains of *L. monocytogenes* other than EGD-e.

Mutant #	*Similar gene in GeneBank entry for L. monocytogenes strain EGD-e (GeneBank ID NC 003210)*	*Himar1 insertion site*
130D7	lmo 0289 – similar to sensory box histidine kinase two-component system regulatory protein YycH	314652/314653
103F8	lmo 0676 – flagellar biosynthesis protein FliP	711770/711771
116G10	lmo 0679 – flagellar biosynthesis protein FlhB	713348/713350
78C4	lmo 0686 – similar to chemotaxis flagella motor rotation protein MotB	721089/721091
9D11	lmo 0696 – flagellar basal body rod modification protein FlgD	730362/730425
32D4	lmo 0699 – flagellar motor switch protein FliM	732735/732736
96F10	lmo 0700 – flagellar motor switch protein FliY	733682/733776
109C1	lmo 0706 – flagellar hook-associated protein FlgL	740188/740189
44H8	lmo 0771 – similar to ATP-sensitive inward rectifier potassium channel 15	797544/797545
71D9	lmo 1370 – butyrate kinase Buk	1393775/1393776
51F6	lmo 1371 – branched-chain alpha-keto acid dehydrogenase E3 subunit	1395345/1395346
55D12	lmo 1582 – similar to an acetate kinase	1624692/1624745
50F11	lmo 1742 – adenine deaminase AdeC	1811951/1811952
97F11	lmo 1744 – putative uncharacterized protein	1814120/1814121
61B9	lmo 1786 – internalin C InlC	1860262/1860263

Table 4-5. Identified gene interruptions in *Listeria monocytogenes* 568 transposon mutants displaying decreased desiccation survival. The position of the mariner insertions are listed relative to the published genome sequence of *Listeria monocytogenes* EGD-e strain (GeneBank ID NC 003210). The term similar is used to indicate genes that share high similarity (>90%) with strains of *L. monocytogenes* other than EGD-e.* indicates that only one side of the transposon was successfully sequenced.

Mutant #	*Similar gene in GeneBank entry for L. monocytogenes strain EGD-e (GeneBank ID NC 003210)*	*Himar1 insertion site*
31G10	lmo 0371 or intergenic gap between lmo0372	398086/397599 *
65G10	lmo 0565 – imidazole glycerol phosphate synthase subunit HisH	603526/603528
21F9	lmo 0616 – glycerophosphoryl diester phosphodiesterase	656212/656213
22G3	lmo 0983 – hypothetical protein	1014647/1014681
45A7	lmo 1174 – reactivating factor for ethanolamine ammonia lyase EutA	1202687/1202689
72H7	lmo 1194 – cobalt-precorrin-6A synthasecobalt-precorrin-6Y C(5)-methyltransferase CbiD	1220748/1220788
63D2	lmo 1219 or intergenic space between lmo1220	1240012/1240351 *
22H7	lmo 1728 – hypothetical protein similar to cellobiose phosphorylase	1790747/1790748
45C7	lmo 2443 – similar to glutamate tRNA ligase	2509030/2509379 *
45C5	lmo 2470 – similar to leucine-rich repeat domain protein	2545402/2545275 *
127C11	lmo2490 – similar to CsbA putative membrane protein	2567681/2567682
78E5	lmo 2503 – similar to cardiolipin synthase	2579130/2579131
93A6	lmo 2768 – similar to permease membrane protein	2848647/2848649
38B6	lmo 2768 – hypothetical permease protein	2849028/2849029
18G10	lmo 2778 – similar to an uncharacterized protein	2863001/2863002
41H2	NA- unable to obtain sequences	

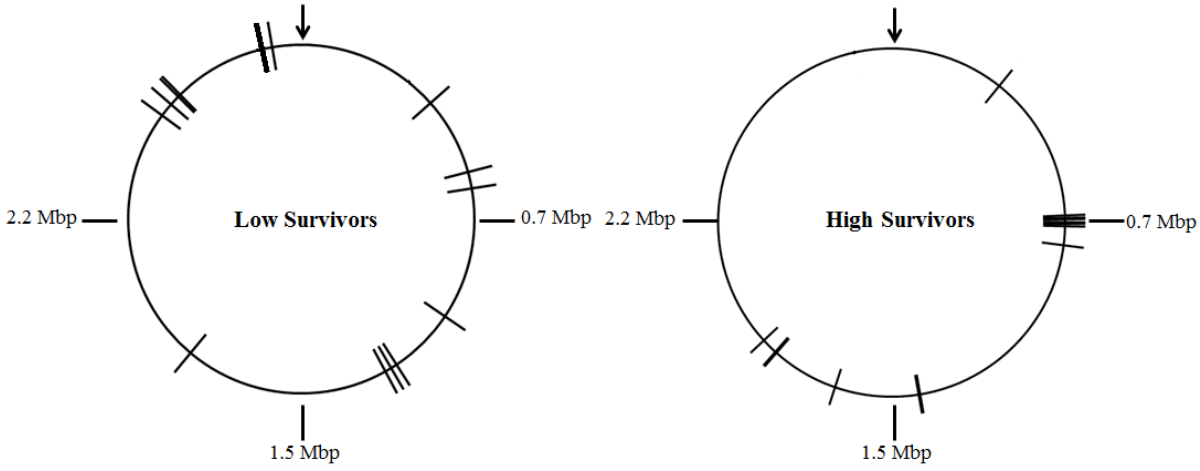

Figure 4-5. Locations of sequenced *Himar1* insertions in the chromosome of *L. monocytogenes* 568 mutants displaying low and high desiccation survival. Each quarter of the chromosome is identified with the approximate base pair, except for bp 1, which is identified by an arrow.

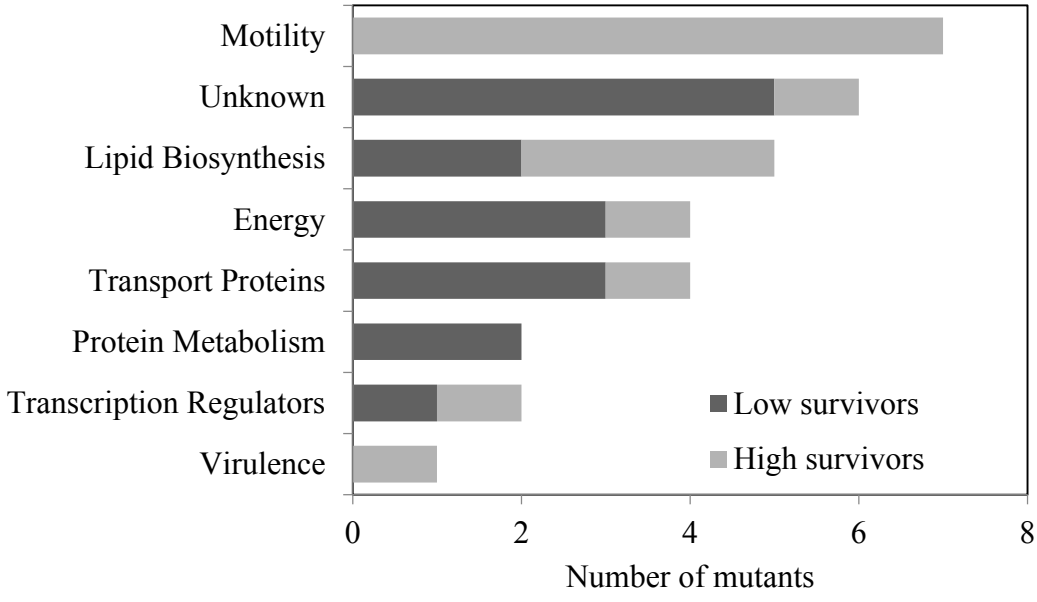

Figure 4-6. Putative classification of the affected gene functional groups in high and low desiccation survival mutants.

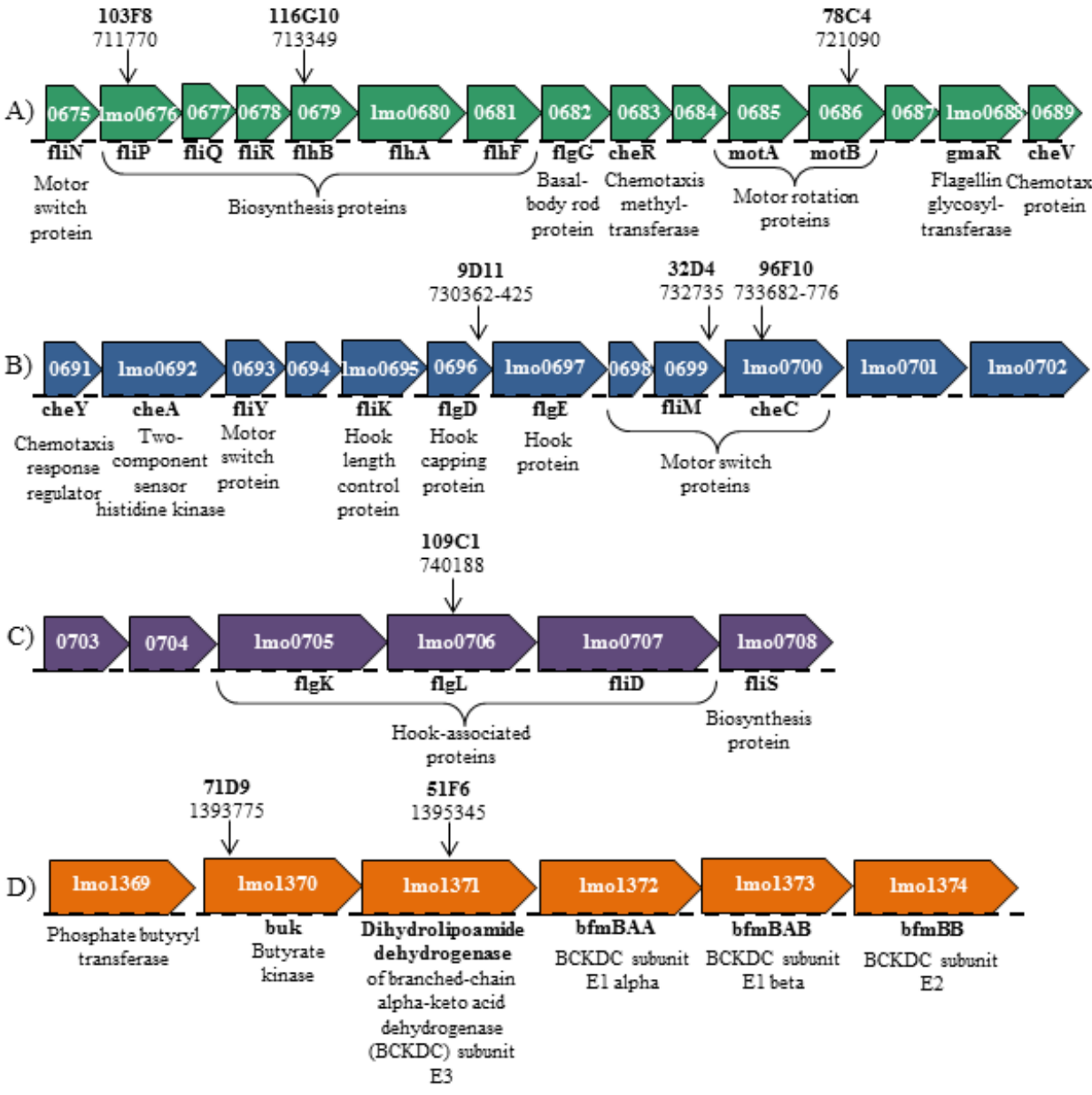

Figure 4-7. Map of genome regions showing the position of *Himar1* insertions for select desiccation resistant mutants. A) Mutants with inserts in *L. monocytogenes* flagella operon 1, B) flagella operon 2 and C) a flagella transcriptional unit. D) Transcriptional unit of mutant 51F6 and 71D9 affected genes. Arrows indicate *Himar1* insertion sites with reference to the *L. monocytogenes* EGD-e strain (GeneBank ID NC 003210).

4.3.3. Phenotyping Desiccation Mutants

4.3.3.1. Cross Protection of Desiccation Genes Against Osmotic Stress

Of interest was whether mutants displaying high and low desiccation survival under low RH environments would maintain the same phenotype when subjected to high osmotic stress (TSB-glu+20% NaCl). Significant ($p<0.05$) increases in the osmotolerance were demonstrated for four high desiccation survival (71D9, 51F6, 103F8, 97F11) and three intermediate high desiccation survival mutants (44H8, 109C1, 116G10). For these transposon mutants, final losses in viability ranged from -0.17 to -0.51 log CFU/ml in comparison to -0.73 log CFU/ml for Lm568 (Table 4-6). Interestingly, it was the intermediate high survival mutants 116G10 and 109C1 which showed the greatest osmotic stress survival throughout the 49 h experimental period (Figure 4-8). Mutants 32D4 and 78C4 showed significantly ($p<0.05$) decreased survival than the wildtype with total losses of -1.24 and -1.31 log CFU/ml, respectively. The colony morphology of mutant 51F6 which previously took on a donut shape when subjected to desiccation, displayed irregular shaped colonies when cultured after being subjected to high osmotic stress (Figure 4-10). The remaining six resistant mutants showed no statistical differences ($p>0.05$) in osmotolerance from the wildtype after 49 h, however, three of these mutants demonstrated consistently higher survival than Lm568 throughout (Figure 4-8). It is important to note that given the relatively small overall loss in viability displayed by all strains, detecting significant differences to osmotic stress were less likely than in the previous desiccation experiments.

Out of the 16 identified low desiccation survival mutants, seven also showed significantly ($p<0.05$) lower osmotolerance than the wildtype (Figure 4-9). Of these mutants four (18G10, 22H7, 22G3, 72H7) were classified previously as being low

desiccation survivors and three (45A7, 78E5, 63D2) were classified as intermediate low desiccation survivors. Final losses in viability for these mutants ranged from -0.92 to -1.25 log CFU/ml (Table 4-6). Mutants 22H7 and 63D2 displayed the lowest overall survival during 49 h of high salt exposure with final losses of -1.25 and -1.16 log CFU/ml, respectively. No significant differences ($p>0.05$) were detected between the remaining ten mutants and the wildtype after 49 h of osmotic stress. Again, although not proven to be statistically different, many of these strains showed a trend toward decreased survival in comparison to Lm568 throughout the experimental period (Figure 4-9).

Overall, survival under high osmotic stress (20% NaCl) was consistently higher for most mutants and the wildtype in comparison to survival under low RH (43%) conditions at 15°C. Exceptions include the extremely desiccation resistant mutants 51F6 and 71D9 which showed higher survival under low RH or matric stress conditions than under osmotic stress conditions. A Pearson correlation showed a significant ($p=0.01$) positive relationship ($y=0.79+1.52$) between the survival of Lm568 transposon mutants under desiccation and osmotic stress (Figure 4-11), demonstrating that similar mechanisms are used to survive both conditions.

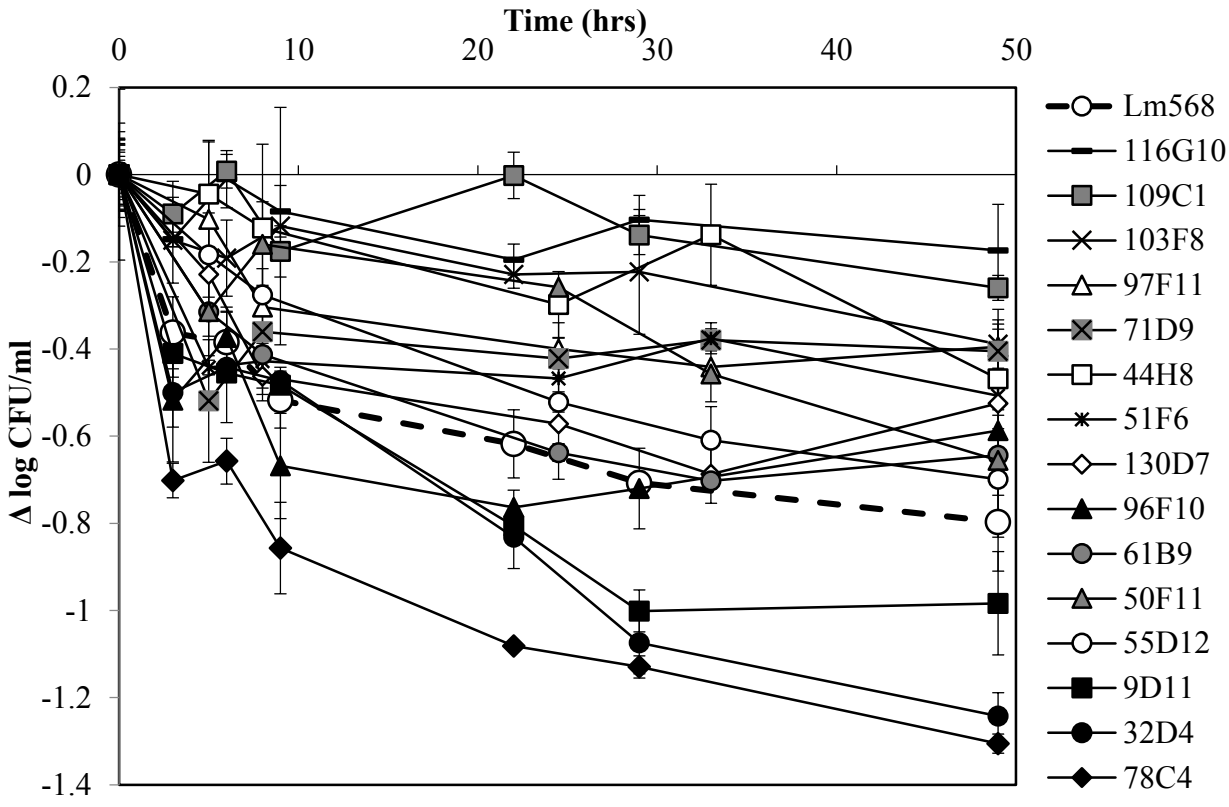

Figure 4-8. Survival of desiccation resistant mutants under high osmotic stress. Cells were pre-cultured in TSB-glu and re-suspended in TSB-glu containing 20% (w/v) NaCl to a level of 7.5 CFU/ml and incubated at 15°C for 49 hours. Symbols are averages of replicates (n=3) and error bars indicate standard deviation.

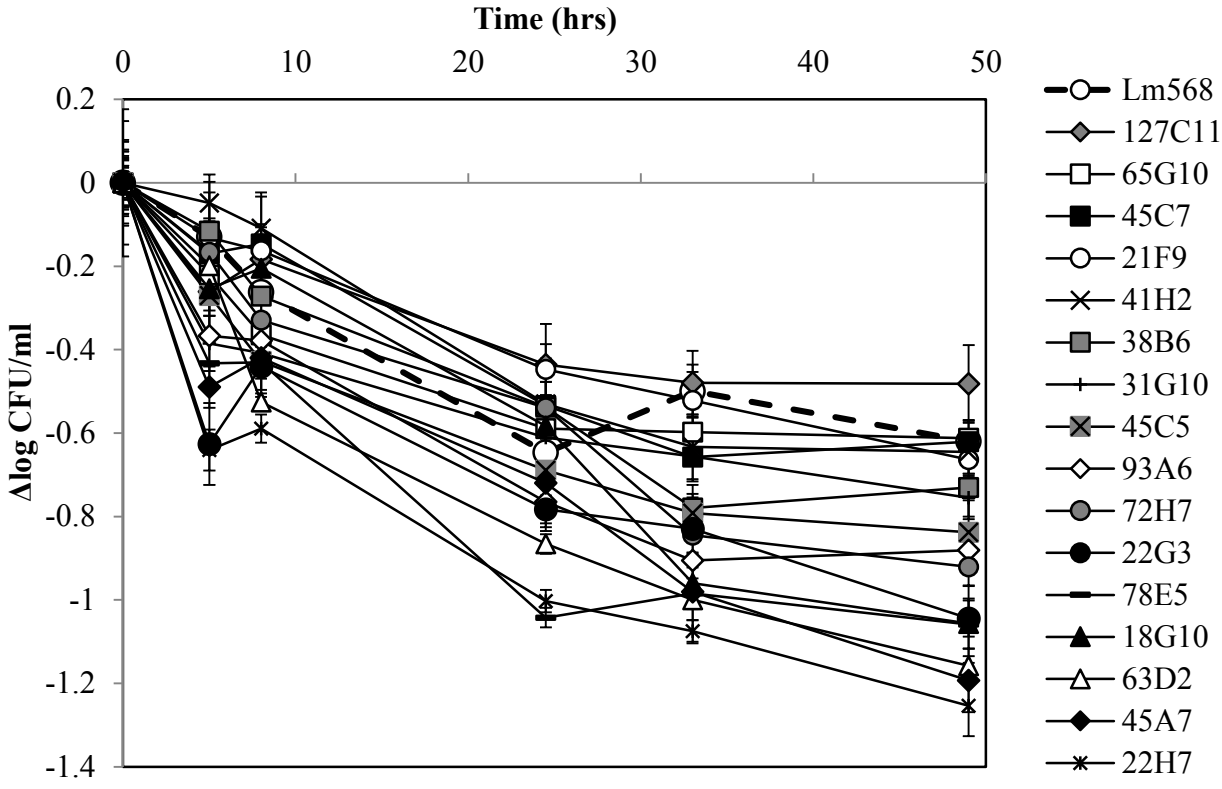

Figure 4-9. Survival of mutants with impaired desiccation tolerance when subjected to high osmotic stress. Cells were pre-cultured in TSB-glu and re-suspended in TSB-glu containing 20% (w/v) NaCl to a level of 7.5 CFU/ml and incubated at 15°C for 49 h. Symbols are averages of replicates (n=3) and error bars indicate standard deviation.

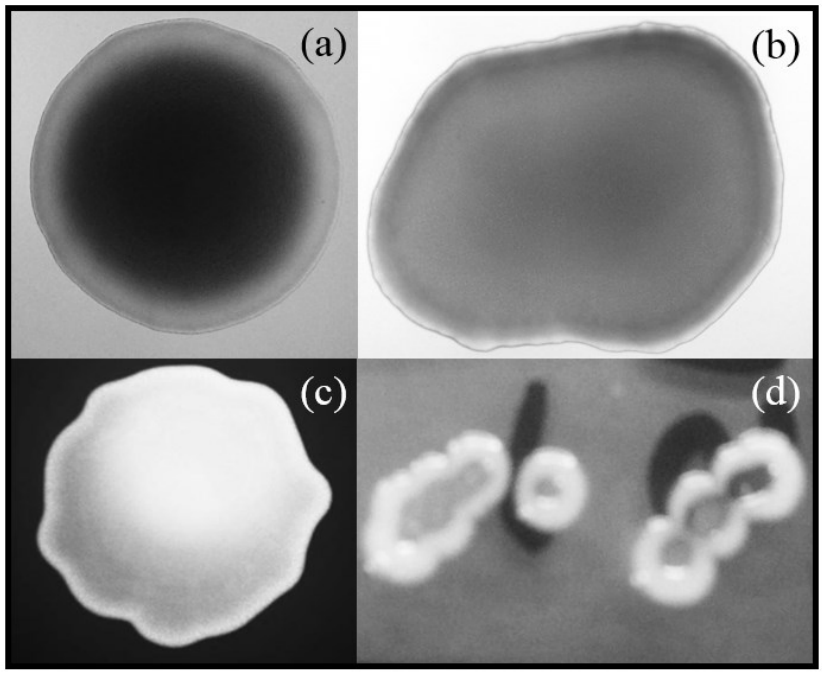

Figure 4-10. Colony morphology on BHI agar after 2 days of incubation at 30°C. a) Lm568, b) high survival mutant 130D7, and high survival mutant 51F6 after c) 4 days of desiccation (43% RH, 15°C), and d) 49 h of osmotic stress (TSB-glu +20% NaCl, 15°C).

Table 4-6. Final losses in viability of desiccation mutants after exposure to high osmotic stress for 49 h at 15°C. Cells were pre-cultured in TSB-glu and re-suspended in TSB-glu containing 20% (w/v) NaCl to a level of 7.5 CFU/ml and incubated at 15°C (n=3).

	Mutant #	*ΔLog CFU/cm^2 (after 49 hr)*		*Mutant #*	*ΔLog CFU/cm^2 (after 49 hr)*
Lm568		-0.73±0.11			-0.73±0.11
High survivors	71D9	-0.41±0.05**	Low survivors	18G10	-1.06±0.10*
	51F6	-0.51±0.02**		31G10	-0.76±0.03
	103F8	-0.39±0.06**		22H7	-1.25±0.07*
	61B9	-0.64±0.09		21F9	-0.66±0.03
	97F11	-0.40±0.09**		22G3	-1.05±0.04*
	50F11	-0.66±0.03		45C7	-0.62±0.05
	32D4	-1.24±0.05*		65G10	-0.61±0.04
				72H7	-0.92±0.05*
Intermediate high survivors	96F10	-0.59±0.24	Intermediate low survivors	127C11	-0.48±0.09
	78C4	-1.31±0.02*		93A6	-0.88±0.02
	9D11	-0.98±0.12		41H2	-0.65±0.05
	44H8	-0.47±0.07**		45A7	-1.19±0.07*
	109C1	-0.26±0.03**		38B6	-0.73±0.07
	55D12	-0.70±0.10		78E5	-1.06±0.10*
	130D7	-0.53±0.06		45C5	-0.84±0.10
	116G10	-0.17±0.11**		63D2	-1.16±0.02*

** indicates that survival was significantly ($p<0.05$) higher than the wildtype
* indicates that survival was significantly ($p<0.05$) lower than the wildtype

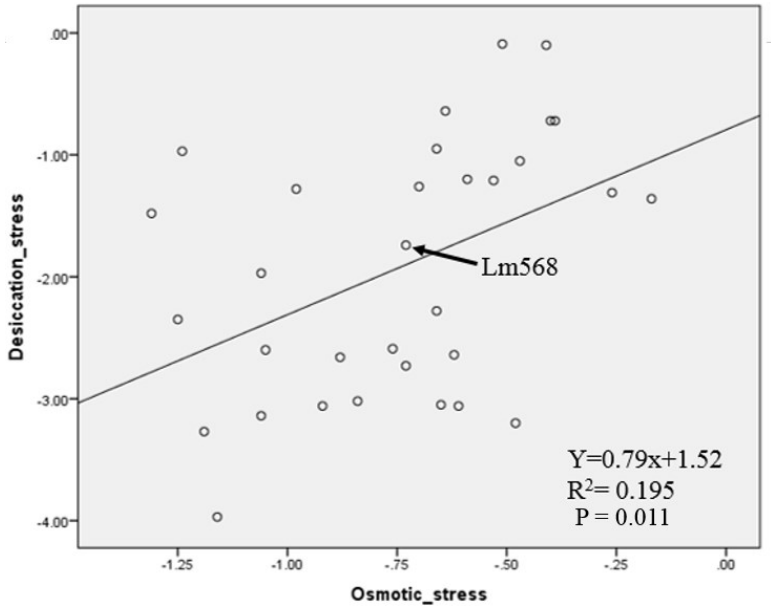

Figure 4-11. Bivariate Pearson Correlation between the survival of Lm568 desiccation mutants under desiccation (Δ log CFU/cm^2 after 4 days) and osmotic stress (Δ log CFU/ml after 49 h).

4.3.3.2. Motility Assay and Flagella Staining

The seven mutants known to contain transposon insertions in flagella related genes were evaluated for motility using a soft agar assay. As seen in Figure 4-12, only the wildtype Lm568 showed motility after 2 days at room temperature (20-22°C). This indicates that the motility mutants either have defective flagella or lack the presence thereof. To further determine if the mutants do in fact possess flagella, cells were stained and viewed using light microscopy. None of the motility mutants were found to produce flagella. This was surprising given that the chemotaxis and motor protein mutants (78C4, 32D4, 96F10) were hypothesized to be flagellated non-motile mutants. Figure 4-13a shows the wildtype with singular and multiple flagella per cell whereas Figure 4-13b demonstrates how the motility mutants lacking flagella appeared after staining. During the microscopic analyses, it was discovered that mutant 9D11 containing a transposon inserted in a flagellar basal body rod modification protein, formed several long chains of cells (Figure 4-13).

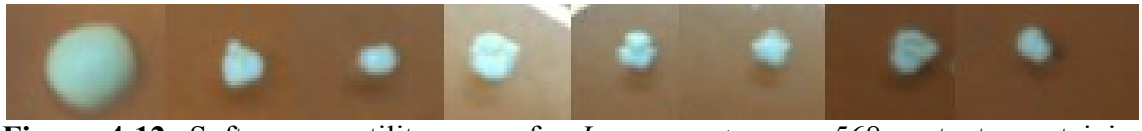

Figure 4-12. Soft agar motility assay for *L. monocytogenes* 568 mutants containing *Himar1* transposon insertions in motility genes. Strains were stabbed into soft BHI agar and incubated at room temperature (20-22°C) for 2 days. A large area of bacterial growth is indicative of bacterial motility. Shown from left to right are the following strains: wildtype Lm568, 116G10, 109C1, 103F8, 9D11, 96F10, 78C4, 32D4.

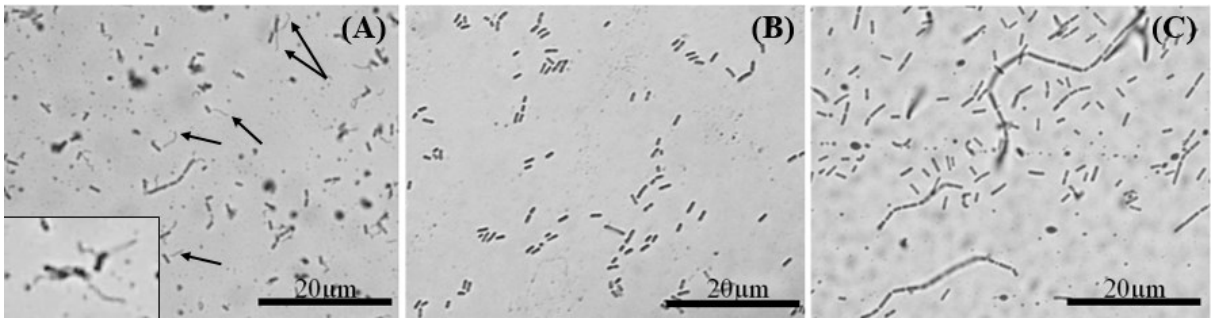

Figure 4-13. Micrographs of flagella stained *L. monocytogenes* 568 cells and its *Himar1* transposon mutants. A) wildtype Lm568 cells displaying flagella at room temperature, B) 109C1 as an example of non-flagellated motility mutants (116G10, 109C1, 103F8, 96F10, 78C4, 32D4) C) long chains of cells formed by non-flagellated motility mutant 9D11. Bacteria were stained as previously described by Heimbrook *et al.* (1989).

4.3.3.3. Heat Tolerance Assay

As suspected, desiccation resistant mutant 71D9 also showed extreme heat tolerance in comparison to the wildtype. After 30 min at 55°C final losses in viability were – 0.84 log CFU/ml for mutant 71D9 and -2.15 log CFU/ml for the wildtype (Table 4-7).

Table 4-7. Thermotolerance of Lm568 and desiccation resistant mutant 71D9. Cells were cultured in TSB-glu for 2 days at 15ºC, re-suspended in PS to 7.5 log CFU/ml and exposed to 55ºC for 30 min (n=3).

	Δ log CFU/ml after 30 min
Lm 568	-2.15 ± 0.05
Mutant 71D9	-0.84 ± 0.06

4.4. Discussion and Conclusions

In this study, insertional mutagenesis with mariner transposon *Himar1*, resulted in the isolation of 31 *L. monocytogenes* 568 mutants displaying increased (15 mutants) and decreased (16 mutants) desiccation tolerance. The majority of the high survival mutants displayed transposon insertions in motility related genes, followed by genes involved in FA biosynthesis and membrane modification. Amongst the low survival mutants, the most identified genes corresponded to unknown proteins, followed by genes involved in energy production and those encoding membrane transport proteins. Specific lipid and protein biosynthesis genes were also determined to be critical for desiccation survival. The remaining desiccation mutants (both high and low survivors) harboured inserts in transcription regulator and virulence related genes.

4.4.1. Impaired Motility Increases Desiccation Survival

Interruptions in flagella-associated genes were responsible for the acquired desiccation resistance of seven mutants. Recently, a highly desiccation tolerant strain of *Salmonella* displayed impaired flagellar gene expression that appeared to be completely shut down once cells entered stationary phase (Li *et al.,* 2012). This phenomenon of flagella inhibition upon entry into stationary phase has also been seen in *E. coli* cells (Serra *et al.,* 2013). In addition, a transcriptional analysis of differentially expressed genes in dehydrated *Salmonella* Typhimurium, did not observe the induction of any motility-related genes (Gruzdev *et al.,* 2012). *Pseudomonas putida* cells showed repressed flagellum biosynthesis during matric, and to a lesser extent solute stress (van de Mortel and Halverson, 2004). The downregulation of motility genes in response to osmotic stress was also reported in *B. subtilis* cells (Hahne *et al.,* 2010). Other studies provided conflicting

results in regards to whether flagella expression is up or down-shifted during the exposure of varying organisms to desiccation stress conditions (Cytryn *et al.,* 2007; Gülez *et al.,* 2011). In the present study, mutants 116G10, 103F8, and 109C1 containing interruptions in two flagella biosynthesis and a hook associated protein, respectively, displayed the greatest survival when exposed to high osmotic conditions (20% NaCl). The remaining four flagella mutants possessing inserts in three motor related and one basal body rod protein, showed decreased osmotolerance with respect to the wildtype thus demonstrating different osmotolerance phenotypes within motility impaired and matric stress resistant mutant strains of *L. monocytogenes*. A reasonable explanation why non-motile mutants showed increased desiccation tolerance is that flagella biosynthesis is very energy consuming and redirecting this energy into other metabolic needs may enable the bacteria to better survive desiccation/starvation stress. This study is the first to report the importance of flagella gene expression and/or regulation in the desiccation survival of *L. monocytogenes*.

Recently, Todhanakasem and Young (2008) showed that *L. monocytogenes* motility defective mutants exhibited decreased initial bacterial surface attachment but ultimately formed hyperbiofilms under dynamic (flow cell) but not static conditions. Similar observations were made for the role of flagella-based motility in *B. cereus* biofilm formation (Houry *et al.,* 2010). Together with the new information obtained in this study, it appears that not only do non-motile strains of *L. monocytogenes* exhibit increased desiccation tolerance but it is hypothesized that they may subsequently form protective hyperbiofilms on surfaces, further increasing the difficulty with which they can be removed from the food supply chain. Mutants in the present study with similarity to those identified in Todhanaksem and Young (2008), contained insertions in *flgL*, the affected

gene in mutant 109C1, and *motA* which lies directly downstream of *motB*, the affected gene in mutant 78C4. In their study, disruption of *motA* produced a non-motile but flagellated mutant, however, in the present study the *motB* mutant was not found to possess flagella, possibly suggesting that the transposon insertion may have caused a polar effect preventing other critical flagella biosynthesis genes from being expressed.

4.4.2. Fatty Acid Metabolism Supporting a Less Fluid Membrane is Important for Desiccation Survival

A large portion of the affected genes identified in both low and high desiccation survivors were associated with FA metabolism. This is not surprising given the role membrane viscosity and integrity plays in the survival of many bacteria exposed to a wide range of environmental stresses including pH extremes, cold and heat tolerance, high osmolarity, high pressure, and transition to stationary phase (Juneja *et al.,* 1998; Annous *et al.,* 1997; Casadei *et al.,* 2002; Russell *et al.,* 1995; Kadner, 1996). The fatty acid composition of the *L. monocytogenes* cellular membrane consists of branched–chain fatty acids (BCFAs, >90% of total fatty acids), unsaturated fatty acids (UFAs), and straight-chain saturated fatty acids (SCFAs). BCFAs and anteiso-C15:0 in particular, are the major determinants of membrane fluidity in *L. monocytogenes* and bacteria alike e.g. *S. aureus*, *Bacillus subtilis*, etc. (Singh *et al.,* 2008). Two enzymes have been deemed critical for BCFA synthesis; branched chain α-keto acid dehydrogenase (BKD) and β-ketoacyl carrier protein synthase III (FabH). BCFA synthesis begins with the transamination of branched chain amino acids followed by decarboxylation by the BKD enzyme complex to produce short branched-chained acyl coenzyme A (acyl-CoA) derivatives. These acyl-CoA primers are then utilized by FabH to initiate BCFA biosynthesis. BKD is a multi-subunit enzyme complex composed of four polypeptides organized in a co-regulated gene cluster that also

includes butyrate kinase (*buk*) and a phosphate acetyl/butyryltransferase (*ptb*). The subunits of BKD in *Listeria* also show considerable homology to those of the pyruvate dehydrogenase (PDH) complex (Glaser *et al.,* 2001) which catalyzes the conversion of pyruvate to acetyl-CoA and later to acetate and ATP via the enzymes phosphotransacetylase and acetate kinase (Ward *et al.,* 2000). PDH has also showed enzymatic activity with branched-chain α-keto acids in *B. subtilis* (Oku and Kaneda, 1988). Together, it is hypothesized that BKD in *Listeria* is involved in the catabolism of branched-chain α-keto acids subsequently generating ATP in a system similar to that of the PDH complex (Ward *et al.,* 2000).

The first gene of the *bkd* locus encodes dihydrolipoamide dehydrogenase (*lpd*) and corresponds to the interrupted gene in one of the two highest desiccation survival mutants, 51F6. Adjacent and downstream of *lpd* is *buk*, which is the interrupted gene in the second highest desiccation survival mutant 71D9. This gene was also reported by Ells *et al.* (2009) as being the transposon insertion site of a Lm568 heat resistant mutant. This phenotype was also found to be true for mutant 71D9 (Table 4-7). Furthermore, a third (55D12) high desiccation survival mutant contains an insert in a gene similar to acetate kinase, one of the active enzymes in the PDH pathway and showing high similarity to *buk* (Walter *et al.,* 1993). It appears that inactivation of the BKD/PDH pathway in *L. monocytogenes* leads to increased desiccation and heat stress survival. Moreover, mutants 51F6, 71D9, and to a lesser extent, 55D12, also showed increased osmotolerance in comparison to the wildtype. Previous studies have shown that BKD-deficient mutants of *B. subtilis*, *L. monocytogenes* and *S. aureus* resulted in membranes with significant reductions in BCFAs (both anteiso and iso), and increased SCFA levels producing a less fluid membrane (Willecke and Pardee, 1971; Zhu *et al.,* 2005; Singh *et al.,* 2008). Also reported were severe growth

defects, longer doubling times, and reduced maximal growth in rich media (Zhu *et al.,* 2005) which is in agreement with the slow growth rates observed for mutants 71D9 and 51F6 and the unusual colony morphologies detected in 51F6 when exposed to both desiccation and salt stress. In this study and that of Zhu *et al.* (2005), *L. monocytogenes* BKD mutations were not lethal, however, a *B. subtilis* strain with mutations in both BKD and PDH was unable to survive suggesting that PDH may compensate for loss of BKD activity to some extent (Willecke and Pardee, 1971).

The effect that membrane FA modification has on the desiccation survival of *L. monocytogenes* has yet to be elucidated. Numerous studies have, however, reported that transcription of FA biosynthesis genes increases in bacteria being subjected to dehydration or matrix stresses (Li *et al.,* 2012; Gruzdev *et al.,* 2012; Cytryn *et al.,* 2007; van de Mortel and Halverson, 2004; Gülez *et al.,* 2012). It is hypothesized that this may be a result of either the starvation response eliciting a need for energy or the osmotic pressure inducing a need for a more rigid cell membrane (low fluidity) (Li *et al.,* 2012). In dehydrated *Salmonella*, transcription of genes responsible for the degradation of long-chain FAs into acetyl-coA, which then are fed directly into the TCA cycle, increased the most (Li *et al.,* 2012). Similarly, transcription analysis of *Pseudomonas putida* cells under matrix stress revealed the up-regulation of enzymes involved in the degradation of damaged phospholipids and modification of membrane FAs (van de Mortel and Halverson, 2004). The oxidation of FAs generates significantly more ATPs per carbon atom than is obtained from an equivalent number of glucose carbon atoms, making FAs very cost-effective energy sources for cells (James *et al.,* 1999). A more in-depth study of FA metabolism in response to desiccation stress would provide more information on the exact mechanisms responsible for *L. monocytogenes'* prolonged survival in low water activity environments.

To help understand the relationship between desiccation tolerance and membrane FA modifications, we can look at the mechanisms used by *Listeria* and other bacteria to survive related stresses. Under a downshift to growth at cold temperatures bacteria would experience reductions in membrane fluidity causing a phase transition from liquid-crystalline to a more rigid gel-like state (Zhu *et al.,* 2005). To restore fluidity at low temperatures, cells respond by incorporating FAs with low melting points (mainly UFAs and BCFAs) into membrane lipids (Zhu *et al.,* 2005). The opposite is observed in bacteria exposed to upshifts to higher temperatures, where a switch from low to high melting point lipids (SCFAs and a switch from anteiso- to iso-BCFAs) is necessary for survival (Annous *et al.,* 1999). *Salmonella* cells pre-exposed to alkaline and acidic conditions displayed increased SCFAs and decreased unsaturated FAs in their membranes, which in turn led to reduced membrane fluidity and subsequent induced thermotolerance (Sampathkumar *et al.,* 2004; Álvarez-Ordóñez *et al.,* 2008). The adaptation and resistance of *B. subtilis* to salt stress was also associated with increased SCFA, unsaturated FA, and cardiolipin production (López *et al.,* 2000). Similar changes have been reported in *E. coli* and *S. aureus* (Lusk and Kennedy, 1972; Kanemasa *et al.,* 1974). These hypertonic cultures developed thicker cell walls, a property that may prevent NaCl entrance and the escape of compatible osmolytes (López *et al.,* 2000). An increased ratio of membrane SFAs may also aid in limiting the effects of lipid oxidation imposed by high temperatures, dehydration and high stress. Therefore, it appears that changes in membrane FA composition leading to decreased fluidity play a large role in alkaline, acid, thermo, osmotic, and desiccation tolerances, whereas the opposite is true for growth at low temperatures. This may explain why interruption of the *buk* gene in the BKD operon of Lm568 produced both a desiccation (71D9) and heat tolerant mutant (Ells *et al.,* 2009) and

why both mutants 71D9 and 51F6 exhibited increased resistance to osmotic stress. Genes associated with the short chain FA butyric acid were among the most upregulated during desiccation of *Salmonella* (Li *et al.,* 2012). Cross-protection of osmotic and heat shock has been reported in several bacteria species (Jørgensen *et al.,* 1995; Trollmo *et al.,* 1988; Skandamis *et al.,* 2008;Völker *et al.,* 1992). Also, in *L. monocytogenes* exposure to starvation stress was shown to lead to increased acid, heat, and oxidative tolerance (Herbert and Foster, 2001). Overlaps amongst stress tolerance mechanisms are logical due to redundancies in regulatory networks contributing to general and specific stress responses (Martínez-Bueno *et al.,* 2002).

Cardiolipin production has long been associated with cellular osmoadaptation and levels have been documented to be positively correlated with increased resistance to cold and acid stress in *L. monocytogenes* (Mastronicolis *et al.,* 2008; Ohniwa *et al.,* 2012). This diphosphatidylglycerol lipid contains four distinct alkyl chains making it a very complex molecule with large potential to participate in a wide range of cellular functions including cell division, energy metabolism and membrane transport (Romantsov *et al.,* 2009). The isolation of low desiccation survival mutant 78E5 containing a disrupted cardiolipin synthase, now suggests that cardiolipin may also be necessary for adaptation to low water activity environments. Not surprisingly, this mutant also demonstrated very low survival during exposure to 20% NaCl. Ter Beek *et al.* (2009) found that decreased levels of the anionic phospholipids phosphatidylglycerol and cardiolipin in the plasma membrane of *B. subtilis* lead to increased susceptibility to weak organic acids and osmotic stress. Cardiolipin synthesis during osmotic stress is upregulated by limited intracellular potassium (K^+) levels. Consequently, the most rapid response to counteract osmotic stress is stimulation of K^+ uptake (Epstein, 1986) by protein transporters that scavenge K^+ from

the environment to help maintain cell turgor pressure (Schniederberend *et al.,* 2010). Interestingly, mutant 44H8 with an interrupted ATP-sensitive inward potassium channel gene, showed increased resistance to both osmotic and desiccation stress. A possible explanation could be that a more efficient K^+ transporter such as KdpFABC, makes the inhibition of less efficient ATP-dependent K^+-transporters an energy saving event during desiccation stress. Several genes of the KdpFABC operon were found to be up-regulated in dehydrated *Salmonella* (Gruzdev *et al.,* 2012) and cyanobacteria (Katoh *et al.,* 2004). The importance of this system in *L. monocytogenes* and *E. coli* survival under osmotic and low temperature stress has also been studied (Brøndsted *et al.,* 2006; Schniederberend et al., 2010), making it likely that this operon is also active in *Listeria* during desiccation.

Another low desiccation survival mutant 21F9, contained a transposon insert in the glycerophosphoryl diester phosphodiesterase gene responsible for glycerophospholipid metabolism. Glycerophospholipids make up the main component of biological membranes and are also used to produce cardiolipin (Romantsov *et al.,* 2009). Unlike the mutant putatively defective in cardiolipin synthase, mutant 21F9 showed an osmotolerance similar to that of the wildtype suggesting that the encoded enzyme is not as crucial for osmoadaptation as it is for desiccation survival.

4.4.3. Putative Role of Virulence Related Genes in Desiccation Survival

A number of both high and low desiccation survival mutants contained transposon inserts in virulence related genes. As an intracellular pathogen, *L. monocytogenes* encounters a wide range of stresses within the host including bile, acid, and nutrient starvation (Gahan and Hill, 2005; Dussurget *et al.,* 2002). In order to invade eukaryotic host cells and grow intracellularly, several stress response and virulence genes are

upregulated (Milohanic *et al.,* 2003). Most of the virulence genes in *L. monocytogenes* are regulated by a transcription factor termed PrfA that is partially regulated by σ^B (Kazmierczak *et al.,* 2006). More than 150 general stress proteins/genes are part of the σ^B regulon, which provides non-growing cells with non-specific preventive stress resistance to oxidative, heat, acid, alkaline, and osmotic stress (Hecker and Völker, 2001). The relationship between environmental stress responses and virulence in bacteria suggest a central role for alternative sigma factors in enabling bacterial survival and subsequent infection of host cells. This overlap may also account for the detection of interrupted virulence genes in desiccation survival mutants.

Two low survival mutants (45A7, 72H7) were found to contain transposon inserts in nearby ORFS encoding genes involved in vitamin B12 synthesis (*cbiD*) and ethanolamine utilization (*eutA*). EutA is thought to be a possible chaperonin protecting ethanolamine ammonia-lyase (EutBC) from inhibition (Tsoy *et al.,* 2009). EutBC allows cells to use ethanolamine as both a carbon and nitrogen source to produce energy (Joseph and Goebel, 2007). Both EutA and EutB/EutC; which lie directly upstream of EutA, are part of a large locus found to be highly upregulated in *L. monocytogenes* under anaerobic growth in host cells (Joseph *et al.,* 2006). Among other upregulated genes in this locus were those involved in cobalamine synthesis (vitamin B12) including *cbiD* (Joseph *et al.,* 2006). Very little is known regarding the induction of the *eut* operon in *Listeria*, but in *S. enterica* serovar Typhimurium it is regulated by both vitamin B12 and ethanolamine (Roof and Roth, 1988; Roof and Roth, 1989). In *L. monocytogenes,* deletion of *eutB* resulted in the inability to utilize ethanolamine as a C or N source which appeared to be critical for intracellular survival (Joseph *et al.* 2006). The results from this study now suggest that *L. monocytogenes* may also require ethanolamine as an energy source to survive the

starvation conditions induced under desiccation stress. Ethanolamine availability is usually at the expense of phosphatidylethanolamine derived from membrane phospholipids (Joseph *et al.,* 2006). This may further explain the upregulation of enzymes involved in the degradation of phospholipids in *Pseudomonas putida* under matrix stress (van de Mortel *et al.,* 2004).

Among the virulence genes regulated by PrfA are those of the *Listeria* specific internalin family (Kim *et al.,* 2004; Kim *et al.,* 2005). Twenty-five proteins have been identified in this family and are characterized by the content of a leucine-rich repeat domain (Ooi *et al.,* 2006). Internalin C (*inlC*), the interrupted gene in the desiccation resistant mutant 61B9, is one of four secreted internalin proteins whereas the remaining 21 have been identified as surface attached proteins (Engelbrecht *et al.,* 1996). The expression of *inlC* is highly induced during late stages of intracellular infection and has been shown to dampen the host innate immune response induced by *Listeria* during the infection process (Gouin *et al.,* 2010). The transcriptional analysis of dehydrated *Salmonella* revealed that four of the seven downregulated genes were contained on the virulence plasmid (Gruzdev *et al.,* 2012) and are known to be induced under simulated host cell conditions, e.g. reduced nutrient supply, high temperature, iron limitation, and low pH (Guiney *et al.,* 1995; Lucas and Lee, 2000; Rychlik *et al.,* 2006). The results from Gruzdev and colleagues (2012) and the present study possibly suggest that inhibition of some virulence related genes may be advantageous for desiccation survival. However, inhibition of *inlC* did not lead to cross-protection against osmotic stress in mutant 61B9. The transposon insert in low desiccation survival mutant 45C5 was also mapped to a putative internalin-like protein (lmo2470) containing a leucine-rich domain. Like 61B9, this mutant also showed osmotolerance comparable to that of the wildtype. The exact role

that internalin proteins play in desiccation survival remains to be discovered. However, the fact that internalin-like genes have been found in 113 non-pathogenic and pathogenic isolates across six species of *Listeria* (Doumith *et al.,* 2004), suggests that these proteins have diverse functions in a number of different hosts and environments, possibly including desiccation tolerance.

4.4.4. Amino Acid Metabolism Genes

Two low desiccation survival mutants identified in this study contained inserts in amino acid/protein metabolism genes, specifically *hisH* (mutant 65G10) involved in histidine biosynthesis and glutamate-tRNA ligase (mutant 45C7), one of 20 aminoacyl-tRNA synthetases essential for protein synthesis. Bron *et al.* (2005) detected the upregulation of both glutamate dehydrogenase and glu-tRNA ligase in *Lactobacillus plantarum* in response to bile stress. In several intestinal microbes, including *E. coli* (De Biase *et al.,* 1999) and *L. monocytogenes* (Cotter *et al.,* 2001), glutamate accumulation by cells has been shown to increase survival during osmotic and acid stress. Interestingly, altered glu-tRNA ligase activity in mutant 45C7 appeared to have no significant impact on osmotic stress survival despite the possibility that glutamate might accumulate in these mutants since it cannot be incorporated easily into new proteins. Interruption of *hisH* in mutant 65G10 also did not impact osmotic stress survival. In *Salmonella*, the second largest group of upregulated genes during desiccation were those involved in amino acid transport and metabolism (Gruzdev *et al.,* 2012). Amongst these genes were those important for histidine biosynthesis (*hisA,H,B,C,D*) and glutamate metabolism (e.g. glutamate synthase, acetylglutamate kinase, acetylglutamate synthase), indicating possible roles for these amino acids in desiccation tolerance.

4.4.5. Oxidative Stress Genes

Oxidative damage is commonly associated with cellular dehydration due to increased cytoplasm ion concentrations leading to the formation of reactive oxygen species. Furthermore, biomolecules in low water activity environments are more susceptible to oxidation (Potts, 1994). These reactive species can damage proteins by modifying amino acid side chains, forming cross-links between proteins, and by causing fragmentation of the polypeptide backbone (Berlett and Stadtman, 1997). Additionally, they can also modify bases and sugars in DNA leading to chain breaks (Storz *et al.*, 1987) and cause lipid peroxidation in cell membranes (Wolff *et al.,* 1986). Therefore, it seems logical that a low desiccation survival mutant (22G3) with an interrupted glutathione peroxidase gene (active in preventing oxidative damage), showed decreased tolerance to both desiccation and osmotic stress.

4.4.6. Genes with Other or Unknown Functions in Desiccation Survival

The remaining desiccation-related genes identified in this study encompassed a wide range of functions. An adenine deaminase (*adeC*) was determined to be the affected gene responsible for increased tolerance to both desiccation and osmotic stress in mutant 50F11. When adenine is taken up it is either phosphoribosylated to AMP or deaminated to produce nucleotide precursors (Nygaard *et al.,* 1996). Under starvation conditions, adenine can act as a sole C and N source for bacteria via the AMP pathway. Inactivation of *adeC* in mutant 50F11 may allow cells to solely utilize adenine as an energy source since cell growth and division are temporarily suspended during periods of low nutrient availability. The desiccation tolerance of another high survival mutant was linked to a regulatory protein (YycH) of the YycFG (recently renamed WalKR) two-component system (TCS)

known to be essential for cell viability in several closely related pathogens (*S. aureus, B. subtilis, L. monocytogenes, E. facecalis*, etc.) (Dubrac and Msadek, 2008). YycH plays a role in negatively controlling WalK activity and abolishing the gene in *B. subtilis* led to associated growth and cell wall defects (Szurmant *et al.,* 2005; 2007). While WalKR TCS activity is strongly linked to cell wall metabolism, a study on *S. pneumonia* also suggests a role in regulating fatty acid metabolism. In this organism, expression of fatty acid biosynthesis genes increased with WalKR expression levels in the cell, resulting in increased fatty acid chain lengths and lowered membrane fluidity (Mohedano *et al.,* 2005). Since YycH represses WalK activity, inhibition of YycH may lead to overexpression of WalK and to formation of a more rigid membrane in mutant 130D7 which has been previously discussed as a possible mode for desiccation resistance. At a microscopic level (×1000 magnification) no cell envelope defects were noticeable; however, this mutant did display irregular shaped colony morphology in comparison to the wildtype (Figure 4-10).

Two low desiccation survivor mutants 38B6 and 93A6 contained different insertions in the same gene (lmo2768) encoding a hypothetical protein with high similarity to a membrane transporter. Both mutants also displayed decreased osmotolerance in comparison to the wildtype, although these differences were not statistically significant. Membrane transport proteins are important for adaptation to low-water activity environments as dehydrated cells may require the uptake of different nutrients, increased uptake of a particular nutrient and/or osmolytes, or the use of alternative transporters that function better under desiccation stress (van de Mortel and Halverson, 2004).

Inhibition of a σ^B controlled general stress response protein CsbA led to decreased survival during exposure to desiccation but not osmotic stress in mutant 127C11. Recently it was reported that the σ^B regulon played a role in the desiccation survival of *L.*

monocytogenes (Huang, 2012). Therefore, it seems reasonable that a defect in a σ^B transcribed stress response protein would invoke decreased desiccation tolerance. Also, CsbA is a putative membrane protein and its interruption may have caused alterations to the membrane integrity.

Other genes whose interruption impacted *L. monocytogenes*' desiccation survival included a possible transcription regular (lmo0371) similar to the GntR family and a protein similar to cellobiose-phosphorylase (lmo1728). Members of the GntR regulatory protein family control the transcription of genes involved in various biological processes. An example of a GntR regulatory protein is FadR found in *E. coli* and *B. subtilis* and the homologous FapR present in *Bacillus, Listeria, Staphylococcus* and other related genera (Schujman and de Mendoza, 2005). Both gene products act as global regulators of FA degradation and UFA synthesis involved in cell membrane homeostasis (Fujita *et al.,* 2007). The role of the putative GntR transcription regulator in the desiccation survival of low desiccation survival mutant 31G10 remains unknown but it appears to serve no purpose in osmotic stress survival. Only one sequence flanking the transposon insert in low desiccation survival mutant 22H7 was obtained and as a result the exact insertion site is either in a protein showing similarities to cellobiose phosphorylase or the intergenic space which follows. The enzyme cellobiose phosphorylase is responsible for the intracellular breakdown of cellulose to glucose monomers which can then be used by the glycolysis pathway to produce energy (Gottschalk, 1986). If the transposon insertion is indeed in this enzyme, then the corresponding decreased desiccation survival may be due to a reduction in available energy. This mutant also demonstrated the lowest survival rate under high osmotic conditions. Cellobiose is present in the TSB-glu+20% NaCl media used in both the osmotolerance and desiccation assays. Therefore, a mutant defective in

cellobiose metabolism may be less able to digest the available nutrients and subsequently exhibit decreased survival compared to the wildtype under both salt and desiccation stress conditions.

The survival of the Lm568 insertion mutants during desiccation and osmotic stress was strongly ($p=0.01$) correlated. However, this was not true for all mutants, suggesting that some desiccation and osmotolerant phenotypes remain separate. A similar result was seen when the transcription of *P. putida* was compared under both matric and osmotic stress (van de Mortel and Halverson, 2004). The authors reported a greater number of loci being transcriptionally regulated during matric stress than solute stress suggesting that adaptation to matric stress is more complex, even though there are some overlaps between the transcriptional responses to the two types of water stress.

Of particular interest was that the most impaired desiccation survival mutants still retained cell viabilities of >3.5 log CFU/cm^2 after seven days of desiccation. Therefore, even with the abolishment of known to be vital metabolic pathways in other bacteria, *L. monocytogenes* can still survive in low water environments significantly better than most other bacterial species. A recent study conducted in our laboratory compared the desiccation survival on stainless steel surfaces of three Gram-negative spoilage bacteria with that of *L. monocytogenes*. It was found that survivors of *Shewanella putrefaciens* and *P. fluorescens* dropped below the detection limit after seven days of desiccation at 43% RH and 15°C, while *Serratia liquefaciens* survival was similar to that of *L. monocytogenes* (Daneshvar Alavi, 2012).

This study is the first to report multiple genes influencing *L. monocytogenes'* desiccation survival with the largest contributors being genes involved in motility and FA membrane composition. While the exact mechanisms of these proteins in response to

desiccation stress remains speculative, this study has increased our understanding of desiccation resistance in *L. monocytogenes*. Further characterization of these cellular responses will help in identifying resistance mechanisms in *L. monocytogenes* and ultimately lead to increased control of *Listeria* in food processing environments.

CHAPTER 5

Isolation of Desiccation Resistant Spontaneous Variants of *L. monocytogenes*

5.1. Introduction

When *L. monocytogenes* is desiccated on surfaces, the most loss in viability occurs during the first day, followed by a much more moderate decrease that eventually leads to a survivor plateau after around 5-10 days of desiccation where very little further loss in viability is observed. The presence of a resistant subpopulation of cells with enhanced survival during desiccation stress has also been noted in a number of other desiccation studies (Dreux *et al.,* 2007; Kusumaningrum *et al.,* 2003; Vogel *et al.,* 2010; Truelstrup Hansen and Vogel, 2011). This tailing can indicate heterogeneity in a population with the presence of desiccation sensitive and desiccation resistant fraction with the latter posing a significant problem for food safety. The probability of detecting resistant variants in a population depends on their frequency in the population, phenotypic characteristic (growth and resistance), and revertance.

The ability of part of a population to survive in a certain environment because of heterogeneity is called persistence. However, there is a difference between survivors that are phenotypically switching between normal cells and persister cells and survivors that are mutated and therefore genetically different (Gefen and Balaban, 2009). New variants establish in a population through mutation and selection. For each new generation, the bacterial DNA is replicated with a spontaneous mutation rate of about 0.0033 per genome, although this number largely depends on the circumstances (Drake, 2006). Van Boeijen *et al.* (2008) determined the fraction of *L. monocytogenes* LO28, Scott A, and EGD-e high pressure processing (HPP) resistant cells in the initial population to be between 10^{-5} and

10^{-6} which included strains that exhibited both phenotypic switching and stable piezotolerant variants. Stable LO28 and Scott A variants were found to comprise 25 to 40% of the fraction of resistant cells, respectively, whereas no stable mutants were discovered for EGD-e (Van Boeijen *et al.,* 2008).

A later study by Van Boeijen and colleagues (2011) used kinetic modelling of HHP and heat inactivation curves of two resistant HHP variants of *L. monocytogenes* LO28 and the wildtype to successfully obtain both HHP and heat resistant variants for all three *L. monocytogenes* strains tested (EDGe, Scott A, LO28). This technique illustrated that three factors impact the detection of resistant variants: 1) the growth and resistance of the variants compared to the wildtype, 2) the frequency of revertance of variants to the wildtype, and 3) the chosen test conditions.

Using the results from Van Boeijen *et al.* (2011) as a guide, the following study aimed to isolate spontaneous desiccation resistant variants of *L. monocytogenes* 568 by selecting survivors after 2, 7, and 20 days of desiccation on SS coupons representing variants which survived the initial rapid loss in viability, variants from the beginning of the survivor plateau, and long term surviving variants, respectively. Isolates were then subjected to a second round of desiccation in microwell plates and variants displaying resistant and sensitive desiccation phenotypes were further tested on SS coupons to determine their stability. In addition, two *L. monocytogenes* 568 freezer cultures from 2004 and 2010 were compared in their desiccation survival to determine if any spontaneous mutations over time might have impacted the bacterium's desiccation tolerance.

5.2. Materials and Methods

5.2.1. Re-Desiccation of Strains Isolated After 2, 7, and 20 Days of Desiccation

To determine whether spontaneous strains of *L. monocytogenes* displaying increased desiccation tolerance exist and could be isolated, Lm568 cells were subjected to two rounds of desiccation stress.

Lm568 was grown in TSB (Difco, Fisher Scientific, Whitby, ON, CA) for two days at 15°C, centrifuged and re-suspended in fresh TSB to A_{450}=1. Appropriate dilutions were performed to achieve a cell density of 7.5 log CFU/cm^2 when 10 µl of the cell suspension was inoculated onto SS coupons. The coupons were placed in preconditioned (43% RH, 15°C) desiccators (Mini 1 Desiccator, Bohlender GmbH, Grünsfeld, Germany) and desiccated for 2, 7 and 20 days representing cells from the early, middle, and late stages of desiccation survival as determined previously in Chapter 3. On these days, coupons were removed from the desiccators and survivors were isolated on BHI (Difco, Fisher Scientific) agar (15%, Difco, Fisher Scientific) as previously described (section 3.2.6).

In total, 376 colonies from each of the 3 desiccation periods (2, 7 and 20 days) were inoculated into four 96-well plates (Costar* Clear Polystyrene 96-Well Plates, Fisher Scientific) containing 190 µl of TSB. Ten µl from each well was transferred to both an empty 96-well plate (desiccation assay) and another containing 190 µl of fresh TSB (growth curve assay). The experimental protocol then followed what was described in section 4.2.2 for the first round of screening of the transposon insertion mutants (section 4.2.2). To preserve the isolated variants, 50 µl of 50% (w/v) glycerol (Fisher Scientific) was added to the original inoculated microwell plates (10% final glycerol concentration). These plates were subsequently stored at -80°C until further use. Isolates which displayed

increased or decreased desiccation survival were further tested on SS coupons (see section 4.2.3) to determine the stability of the desiccation resistant and sensitive phenotypes.

5.2.2. Desiccation Survival of Two Lm568 Freezer Cultures from 2004 and 2010

In addition to screening previously desiccated strains for resistance, the desiccation survival of two freezer cultures of Lm568 from 2004 and 2010 were compared. Like previous experiments the strains were both grown at 15°C for two days in TSB-glu (1% glucose) (Fisher Scientific) before being centrifuged, re-suspended in TSB-glu and spotted on stainless steel coupons in triplicate at a cell density of 7.5 log CFU/cm^2. Coupons were placed in a desiccator preconditioned to 43% RH and 15°C and removed for sampling on days 0, 1, 2, 4, and 7. Survivors were enumerated on BHI agar and reported as log CFU/cm^2. Curves were modelled using the Weibull model and model parameters and Δ log CFU/cm^2 were compared using a Student T-test with 95% confidence.

The thermotolerance of both cultures was also examined using the same protocol described in section 4.2.7.3.

5.3. Results and Discussion

The regrowth-after-desiccation curves from the microwell plate assay of the spontaneous variants were unlike those obtained for the transposon insertion mutants (see Chapter 4); very few strains deviated from the wildtype and the differences between the wildtype and the ones which did was less pronounced. In total 12 resistant and 16 sensitive variants out of the 1128 tested were selected for the second round of screening on SS coupons. Of these, 7 variants were isolated after 2 days of desiccation, 13 were isolated after 7 days, and 8 were isolated after 20 days of desiccation. However, when the

desiccation kinetics of these variants were compared to those of the wildtype on SS coupons, all variants showed comparable survival to the parent strain. This observation could mean a few things. First, since some desiccation resistant strains have been shown to exhibit slow growth rates (Chapter 4), the slow growth of any spontaneous resistant *L. monocytogenes* variants may have interfered with their detection in the regrowth-after-desiccation microplate assay. A second possibility is that the selected variants were not stable and their phenotypes reverted back to that of the wildtype during the two day incubation period at 15°C prior to desiccation on the SS coupons. Lastly, the apparent resistance of sensitive variants may have been a result of experimental error rising from either differences in initial inoculation densities (since no growth step was introduced prior to desiccation); or minor yet detectable differences in the desiccation kinetics of the microwell plate. Of the transposon insertion mutants, 13% of the sensitive desiccation mutants and 25% of the resistant mutants were correctly identified using the regrowth-after-desiccation microwell plate assay. Applying these percentages to the identification of spontaneous variants would mean that hypothetically three resistant and two sensitive stable variants would be detected when desiccated on stainless steel.

When the desiccation survival of two freezer cultures of Lm568 from 2004 and 2010 were compared, the 2004 strain showed significantly ($p<0.05$) lower survival than the 2010 strain (Figure 5-1) displaying delta values of 0.09 and 0.33 days, respectively (Table 5-1). At the end of the 7 day desiccation period final survivor levels were -3.38 Δ log CFU/cm^2 for the 2010 strain and -4.08 Δ log CFU/cm^2 for the 2004 strain (Table 5-1). After 2 and 4 days of desiccation the differences between the strains ranged from 0.77-0.90 log CFU/cm^2. It is clear that somewhere over six years of subculturing, a desiccation tolerant variant was selected which presumably contains a mutation that promotes

desiccation tolerance. In addition, the 2010 Lm568 culture also showed improved thermotolerance (-2.15 Δ log CFU/ml) in comparison to the 2004 culture (-2.80 Δ log CFU/ml) after 30 min at 55°C (Table 5-2). PCR amplification of genes found to be mutated in other spontaneous variants resistant to different stresses may identify possible mutations in the 2010 Lm568 culture compared to the 2004 culture. One example is the stress regulator gene *ctsR* which has been associated with *L. monocytogenes* spontaneous immotile and heat, HHP, acid, and H_2O_2 resistant variants (Karatzas and Bennik, 2002; Joerger *et al.,* 2006; Van Boeijen *et al.,* 2010; Van Boeigen *et al.,* 2011),

The results from this study show that desiccation resistant variants of *L. monocytogenes* 568 do exist but that an alternative approach should be developed for successful isolation. Tay *et al.* (2003) observed that the variability in the number of survivors among nine *L. monocytogenes* strains was greater after a higher pressure (500-MPa) treatment than after a lower-pressure (400-MPa) one. This may suggest that a lower RH imposing a greater desiccation rate, or a less nutrient-rich desiccation medium might improve the detection of resistant variants. Furthermore, Van Boeijen *et al.* (2011) found that none of the 102 *L. monocytogenes* survivors they selected after 25 min at 55°C were stably resistant after culturing and re-testing under the same conditions. After 8 min at 55°C however, 5% of the of the isolated variants showed stability. The authors concluded that at this time point part of the wildtype population would be inactivated and only small parts of the variant population, therefore optimizing the detection of resistant variants. Since we did not have a stable resistant variant of Lm568 to start with, a kinetic modelling approach similar to that applied by Van Boeijen *et al.* (2011), could not be used to determine the best conditions for isolating stable resistant variants. Now that we have identified the Lm568 2010 freezer culture to be stably more desiccation resistant than the

Lm568 2004 culture, a kinetic modelling approach may be used in the future to increase the chances of finding these resistant variants.

In conclusion, although the assay for detecting stable spontaneous desiccation resistant *L. monocytogenes* 568 variants was unsuccessful, the significantly increased desiccation survival of a Lm568 2010 freezer culture in comparison to a 2004 one implies that such variants do exist. Further phenotypic characterization and genomic analysis of these two cultures is needed to determine if there is a mutation in the 2010 strain and if it exhibits increased resistance against other environmental stresses.

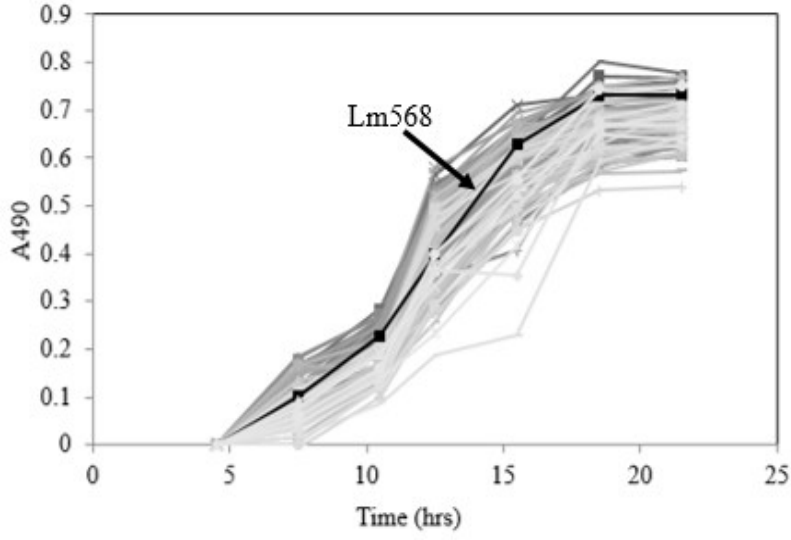

Figure 5-1. Example of a regrowth-after-desiccation curve for survivors isolated after 2 days of desiccation on SS coupons. Survivors were isolated on BHI agar after 2 days of desiccation and inoculated into 96-well plates containing 190 µl of TSB. Ten µl from each well was then spotted on the bottom of an empty 96-well plate and the plate was placed in a desiccator for 5 days at 15°C, 43%RH. After this time, each well in the plate was rehydrated with 190 µl of TSB and then incubated at 15°C and the absorbance of each well at 490 nm recorded every 3 h until stationary phase was reached.

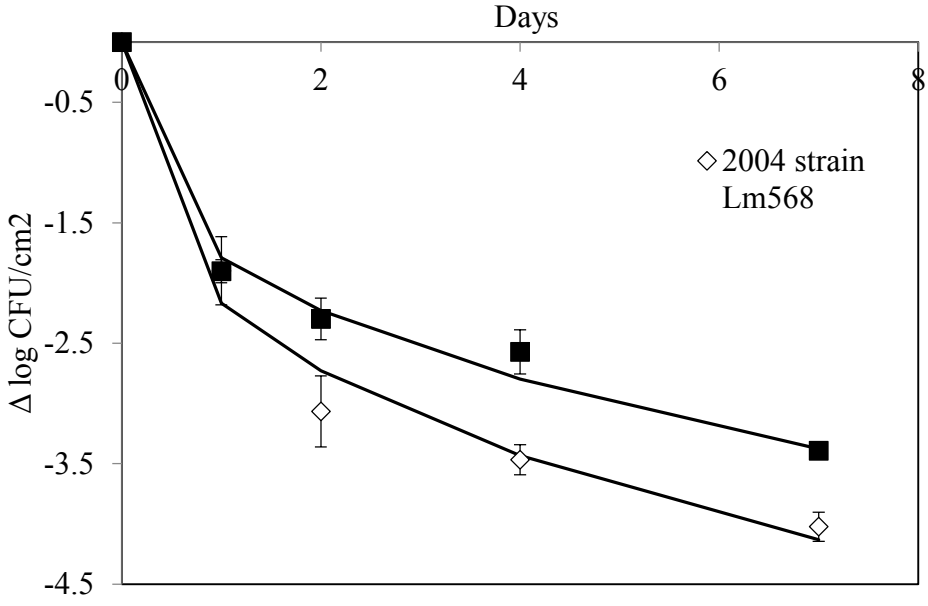

Figure 5-2. Desiccation survival of a 2004 and 2010 strain of *L. monocytogenes* on stainless steel coupons for 7 days at 43% RH. Strains were cultured and re-suspended in TSB-glu, spotted on stainless steel coupons at 7.5 log CFU/cm^2 and desiccated for 7 days at 43% RH, 15°C. Symbols are averages of replicates (n=3) and error bars indicate the standard deviation.

Table 5-1. Desiccation survival kinetics and model parameters of *Listeria monocytogenes* strain 568 cultured in 2004 and in 2010. Mutants were cultured and re-suspended in TSB-glu, spotted on stainless steel coupons and desiccated at 43% RH, 15°C for 7 days (n=3). F-values lower than 2.75 indicate that the fitting performance of the model is statistically accepted.

Strain year	Δ log CFU/cm^2 (day 7-day0)	Model parameter estimates		Statistical indices of model fit		
		Delta (days to 1 log reduction)	*p* (shape parameter)	MSE_{model}	r^2	*f-value*
2004	-4.08±0.02[a]	0.09±0.05[a]	0.33±0.04[a]	0.09	0.95	1.53
2010	-3.38±0.04[b]	0.33±0.11[b]	0.37±0.04[a]	0.04	0.97	2.04

Values in the same column followed by a different letter are statistically ($p<0.05$) different

Table 5-2. Thermotolerance of two Lm568 freezer cultures from 2004 and 2010. Cells were cultured in TSB-glu for 2 days at 15ºC, re-suspended in PS to 7.5 log CFU/ml and exposed to 55ºC for 30 min (n=3).

Lm568 Culture Year	Δ log CFU/ml
2004	-2.80 ± 0.07
2010	-2.15 ± 0.05

CHAPTER 6 CONCLUSIONS

Increasing consumer demand for fresh, minimally processed, ready-to-eat foods has led to larger production facilities, use of less preservatives, and elevated food safety concerns. The food-borne human pathogen *L. monocytogenes* is capable of colonizing food production facilities, with the same serotype being isolated for several years. The ability of this pathogen to resist the conditions commonly enforced to suppress bacterial growth makes *L. monocytogenes* a huge concern in the food industry. Such persistence leads to increased chances for cross-contamination of food products which when consumed by at risk groups, can be fatal. On average the US experiences 1,600 cases of listeriosis annually resulting from 2.4 outbreaks per year (Centers for Disease Control and Prevention, 2011) and Canada experiences an average of 100-140 cases per year (National Enteric Surveillance Program, 2011). To gain control of this pathogen in the food supply chain, understanding the mechanisms by which it survives is crucial. One such hypothesis is that long term persistence is linked to the bacterium's ability to survive in low-water activity environments.

In the first part of this study, the environmental factors contributing to *L. monocytogenes'* desiccation survival were investigated. Through the use of desiccation assays (43% RH, 15°C), the impact of initial contamination levels, osmoadaptation, biofilm maturity, and the presence of remaining fat and salt residues on *L. monocytogenes'* survival on stainless steel coupons was analyzed. The initial level of cells on the stainless steel surface had no impact on *L. monocytogenes'* desiccation survival kinetics. When the same three cell densities were used as starting points for two days of biofilm formation, three distinct stages of biofilm development resulted with the two highest initial

contamination levels forming more mature biofilm structures. Cells in these mature biofilms showed significant ($P<0.05$) increases in their desiccation survival in comparison to non-biofilm cells regardless of the initial contamination level. In contrast, cells in the lowest initial contamination level were unable to form a mature biofilm within the 48 h period and these cells consequently displayed desiccation kinetics similar to non-biofilm cells. Osmoadaptation, desiccation in the presence of salt, and increasing levels of saturated fat (20-60%) were shown to improve survival whereas low contents of unsaturated fat (5-10%) appeared to have no impact.

In the second phase of this study, to gain a deeper understanding of desiccation mechanisms in *L. monocytogenes*, a library of random insertional mutants were created in the *L. monocytogenes* 568 background using the *Himar1* transposon. In total, 11,700 mutants were screened for increased or decreased desiccation tolerance with respect to the wildtype. Fifteen desiccation tolerant and 16 desiccation sensitive strains were ultimately chosen for DNA sequencing based on displaying a $>\pm 0.50$ log CFU/cm^2 difference in survival from the wildtype after 4 days of desiccation on SS coupons. Using an arbitrary PCR protocol and Sanger sequencing, the site of the gene interruption was identified in all 15 of the desiccation tolerant mutants and 15 of the 16 desiccation sensitive mutants. Of these, interruption of genes encoding motility and FA membrane modification proteins were determined to play the largest role in desiccation tolerance whereas interruptions in energy and membrane transport related genes were the most recognized in desiccation sensitive mutants. Other contributing genes were categorized as being involved in protein metabolism, transcription regulating, and virulence. Analyzing the cross-protection of these gene interruptions against osmotic stress revealed a significant correlation although some genes were found to solely contribute to desiccation survival. This highlights the

activation of both general and specific stress responses during desiccation and demonstrates that different mechanisms are used by *L. monocytogenes* to survive dehydration imposed by solute and matric stress.

Lastly, the third part of this study attempted to isolate stable spontaneous desiccation resistant variants of *L. monocytogenes* 568 by subjecting survivors after 2, 7, and 20 days of desiccation on stainless steel coupons, to a second round of desiccation in a microplate assay. In total, 12 resistant and 16 sensitive variants out of the 1128 tested (376 for each of the 3 sampling days) were selected for desiccation on SS coupons to determine their survival kinetics in comparison to the wildtype. All variants showed survival similar to that of the wildtype. However, when a Lm568 freezer culture from 2004 was compared to a Lm568 2010 culture, the 2010 culture showed significantly enhanced desiccation tolerance demonstrating that resistant variants do exist and that an alternative approach might be more successful in their isolation.

Altogether, this work emphasizes the importance of understanding both the environmental and genetic factors contributing to desiccation survival in *L. monocytogenes*. Further characterization of the mechanisms behind FA membrane adaptation and motility in response to desiccation stress, will be useful in ultimately developing new and/or enhanced elimination strategies.

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APPENDIX A: SUPPLEMENTARY MATERIAL

Results from the ANOVA and MANOVA Analyses.

Table A1. Results from 4-way Analysis of Variance (ANOVA) of the effect of the main independent main factors with interactions for the response variable Δ log CFU/cm^2 (n=144)

Main Effects and Interactions	Df	SSE[a]	F-value	Pr (>F)[b]
Biofilm (Bf)	1	5.135	25.18	1.83×10^{-6} ***
Desiccation salt (Des-salt)	1	4.688	22.99	4.73×10^{-6} ***
Contamination level (Cont)	2	0.138	0.34	0.714^{NS}
Osmoadaptation (Osmo)	1	1.333	6.54	0.012 *
Bf×Des-salt	1	10.192	49.98	1.11×10^{-10} ***
Bf×Cont	2	6.709	16.45	4.87×10^{-7} ***
Des×Cont	2	0.243	0.59	0.553^{NS}
Bf×Osmo	1	1.505	7.38	0.008**
Des-salt×Osmo	1	0.510	2.50	0.116^{NS}
Cont×Osmo	2	2.997	7.35	0.001***
Bf×Des-salt×Cont	2	5.418	13.28	6.15×10^{-6} ***
Bf×Des-salt×Osmo	1	1.833	8.99	0.003**
Bf×Cont×Osmo	2	0.702	1.72	0.183^{NS}
Des×Cont×Osmo	2	5.530	13.56	4.91×10^{-6} ***
Bf×Des-salt×Cont×Osmo	2	1.519	3.72	0.027*

[a]Sums of Squares Error

[b]Probability of main effect or interaction being significant (* p≤0.05, **p≤0.01, ***p≤0.001, not significant [NS] p>0.05)

Table A2. Results from Multivariate Analysis of Variance (MANOVA) on main effects versus the combined response variables (Δ log CFU/cm^2, delta and p-shape parameters) (n=144)

Main Effect	Df	Wilks Λ	Approximate F-value	p-value[a]
Biofilm	1	0.83112	9.483	9.59×10^{-6} ***
Desiccation salt	1	0.78148	13.049	1.45×10^{-7} ***
Contamination level	2	0.94398	1.355	0.233^{NS}
Osmoadaptation	1	0.89879	5.255	0.002**

[a]Probability of main effect being significant (* p≤0.05, **p≤0.01, ***p≤0.001, not significant [NS] p>0.05)

Table A3. Results from one-way ANOVA on the effect of desiccation salt level on the desiccation survival of non-biofilm *L. monocytogenes* (n=72)

Main Effect (vs. Dependent)	Sums of Squares	Df	Mean Square	F-value	p-value
Desiccation salt (delta)	347.47	1	347.47	30.23	0.000***
Error	804.54	70	11.49		
Desiccation salt (Δ log CFU/cm^2)	16.72	1	16.72	39.95	0.000***
Error	29.30	70	0.42		

[a]Probability of main effect being significant (* p≤0.05, **p≤0.01, ***p≤0.001, not significant [NS] p>0.05)

Table A4. Results from one-way ANOVA on the effect of osmoadaption on the desiccation survival of non-biofilm *L. monocytogenes* (n=72)

Main Effect (vs. Dependent)	Sums of Squares	Df	Mean Square	F-value	p-value
Osmoadaption (delta)	84.39	1	84.39	5.533	0.021**
Error	1067.62	70			
Osmoadaption (Δ log CFU/cm^2)	3.19	1	3.19	5.217	0.025**
Error	42.83	70	0.61		

[a]Probability of main effect being significant (* p≤0.05, **p≤0.01, ***p≤0.001, not significant [NS] p>0.05)

Table A5. Results from one-way ANOVA on the effect of presence or absence of biofilm formation on the desiccation survival of *L. monocytogenes* initially applied to the stainless steel surface at the low contamination level of $10^{3.5}$ CFU/cm^2 (n=48)

Main Effect (vs. Dependent)	Sums of Squares	Df	Mean Square	F-value	p-value
Biofilm (delta)	7.45	1	7.45	0.624	0.624^{NS}
Error	549.59	46			
Biofilm (Δ log CFU/cm^2)	0.56	1	0.56	1.02	0.319^{NS}
Error	25.40	46	0.55		

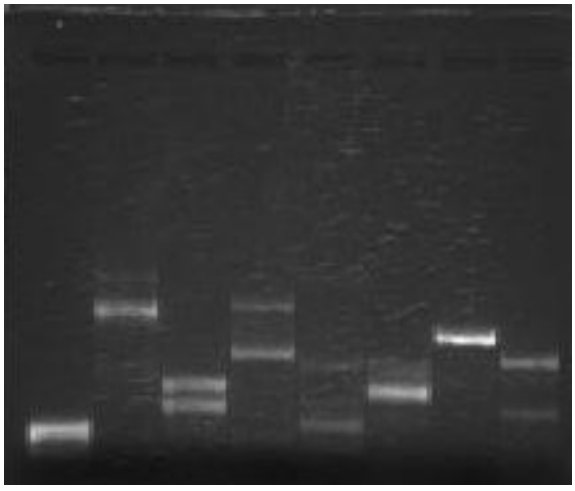
[a]Probability of main effect being significant (* p≤0.05, **p≤0.01, ***p≤0.001, not significant [NS] p>0.05)

Table A6. Results from one-way ANOVA on the effect of presence or absence of biofilm formation on the desiccation survival of *L. monocytogenes* initially applied to the stainless steel surface at the medium and high contamination levels of $10^{5.5}$ and $10^{7.5}$ CFU/cm^2 (n=96)

Main Effect	Sums of Squares	Df	Mean Square	F-value	p-value
Biofilm (Δ log CFU/cm^2)	19.49	1	19.49	26.272	0.000***
Error	69.72	94	0.74		

[a]Probability of main effect being significant (* p≤0.05, **p≤0.01, ***p≤0.001, not significant [NS] p>0.05)

Photograph of Gel Containing Round II Arbitrary PCR products



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