Factors that Influence Biofilm Formation, Desiccation Resistance, and Sanitizer Susceptibility in *Listeria monocytogenes* at a Simulated Food Processing Plant Temperature of 15°C

by

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Submitted in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

at

Dalhousie University

Halifax, Nova Scotia

December 2015

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Abstract

Listeria monocytogenes is a pathogenic foodborne bacterium whose persistence in food processing environments is in part attributed to its biofilm formation. A library of 11,024 *L. monocytogenes* 568 (serotype 1/2a) *Himar1* insertional mutants was created and screened for altered biofilm formation. Fourteen mutants expressed enhanced biofilm phenotypes, and harboured transposon insertions in genes encoding cell wall biosynthesis, motility, metabolism, stress response, and cell surface associated proteins. Deficient mutants contained interruptions in genes related to peptidoglycan, teichoic acid, or lipoproteins. Enhanced mutants produced significantly ($p<0.05$) higher cell densities in biofilm formed on stainless steel (SS) coupons at 15°C (48 h) than deficient mutants. Two enhanced biofilm formers were more resistant to enzymatic removal with DNase, proteinase K or pectinase than the parent strain. Scanning electron microscopy of individual biofilms showed formation of biofilm with dense zones by motility mutants, while deficient mutants exhibited sparse growth. In conclusion, biofilm formation of *L. monocytogenes* at 15°C involved 9 genes not previously linked to biofilm, as well as 10 genes previously associated with its formation at higher temperatures, indicating some temperature driven differences.

In the second research paper, the ability to form biofilm, resist desiccation, and sanitizer treatments was investigated among a panel of 14 *L. monocytogenes* strains, and in relation to the presence or absence of genetic markers including the stress survival islet (SSI-1), the *Listeria* genomic island (LGI1), and clonal complex 8, virulence type 59 (CC8 /VT59). SSI-1 alone did not correlate with desiccation survival. Benzalkonium chloride and desiccation resistance was greater in isolates that also carried the LGI1 and CC8/VT59 markers. In conclusion, LGI1 containing CC8/ VT59 isolates may pose a challenge for sanitation efforts and food safety.

The research presented in this thesis will help our understanding of biofilm formation in *L. monocytogenes,* its response to sanitizer application, and desiccation resistance. This information may lead to better strategies to remove or eradicate *L. monocytogenes* in food processing environments, and ultimately reduce the risk of foodborne illness.

List of abbreviations used

APB - acidified phosphate buffer

BAC - benzalkonium chloride

BHI - brain heart infusion, broth or agar.

bp - base pair

CC8/VT59 - clonal complex 8, virulence type 59

CFIA -Canadian Food Inspection Agency

CFU - colony forming unit

CV - crystal violet

d - day

eDNA - extracellular DNA

EPS - extracellular polymeric substances

erm - erythromycin

EU - European Union

FDA - Food and Drug Administration

h - hour

kan - kanamycin

LGI1 - *Listeria* genomic island 1

Lm - *Listeria monocytogenes*

Lm568 - *Listeria monocytogenes* strain 568

MIC - minimum inhibitory concentration

MBIC - minimum biofilm inhibitory concentration

MBEC - minimum biofilm eradication concentration

MLST – multi locus sequencing typing

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PFGE - pulsed-field gel electrophoresis

PS - peptone saline

RTE - ready to eat

RT PCR - reverse transcriptase polymerase chain reaction

Saf - safranin

SD - standard deviation

SEM - scanning electron microscopy

SS - stainless steel

SSI-1 - stress survival islet 1

TSB - tryptone soy broth

TSB-glu - tryptone soy broth with 1% glucose

USA- United States of America

Acknowledgements

First, I wish to thank my supervisor, Dr. Lisbeth Truelstrup Hansen, for this incredible opportunity, and her excellent support, guidance, inspiration and knowledge.

I would like to thank Dr. Timothy Ells and Dr. Andrew MacIntosh for serving on my committee, and giving their valuable advice, as well as their time. Dr. Alex Speers and Dr. Lori Burrows are thanked for their contributions, as well as Dr. Uyen Nguyen. I was also very fortunate to receive a Scotia Doctoral Scholarship from the Nova Scotia Health Research Foundation, and I am sincerely appreciative.

I gratefully acknowledge the technical support of Niamh Daly, Laura Purdue, Tasha Moideen, Patricia Scallion, and Dr. Nikhil Thomas. My labmates; Patricia Hingston, Céline d'Entremont, Yannan Huang, Amit Ross, Emma Stea, Dr. Kara Neudorf, Mandy McConnell, and Michael McDonald, have always been encouraging and made the lab a pleasant environment. Patricia Hingston deserves a round of applause for helping me plate the mutant library on selective agar that one very late evening.

I also thank my husband Adam for his incredible love, patience and never failing support during this process. My mother; Randi, and my wonderful in-laws; Ron, Christine, Amy, Irene, and Merrill are thanked as well, for their cheer and encouragement.

Chapter 1: Introduction

Listeria monocytogenes overview

Listeria monocytogenes is a Gram positive, rod shaped, saprophytic bacterium that is commonly found in the plant or soil environment in temperate climates (Hain et al., 2007). *L. monocytogenes* may also be found in fecal matter of wild mammals and birds, and in animal feed (Hain et al., 2007).

Other *Listeria* species occupy ecological niches similar to that of *L. monocytogenes*. *L. innocua*, *ivanovii* , *marthii*, *welshmerii*, *seeligeri*, and *grayi*, are generally thought to be non-pathogenic to humans (Cossart, 2011, Orsi et al., 2011). New species in the *Listeria* genus, including *L. rocourtiae*, , *L. weihenstephanensis*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. grandensis*, *L. riparia, L. booriae,* and *L. newyorkensis* have been recently described and are similarly thought to be harmless saprophytes (den Bekker et al., 2014, Weller et al., 2015a).

L monocytogenes is notable for its ability to grow in a wide range of temperatures (0 to 44°C) (Annous et al., 1997). This allows growth at refrigeration temperatures, in chilled food processing environments (often 10-20°C), and in the bloodstream of mammals and birds (Annous et al., 1997). *L. monocytogenes* is capable of growing at a relatively low water activity (a_w) of 0.92 (Beaufort, 2011). It is capable of growing in low pH environments (≥ pH 4.4) (Beaufort, 2011), and upwards of pH 8.5 (Ribeiro et al., 2006). It can survive for several minutes when challenged by very high pH (9-12)

(Mendonca et al., 1994). It can also grow at NaCl concentrations of 10% (w/vol) (Ribeiro et al., 2006).

Resistance to alternate stresses (cross adaptation) may occur after exposure to cold, pH change, detergents/sanitizers, or salt (reviewed in Hill et al., 2002). For example, relevant to the food industry, *L. moncytogenes* can be cultured in sublethal concentrations of a disinfectant, benzalkonium chloride, such that its resistance increases to the same disinfectant (To et al., 2002). Resistant cells have a higher efflux pump activity, a thicker peptidoglycan layer, and roughened cell morphology (To et al., 2002). Lundén et al. (2003) described progressively increasing disinfectant concentrations increased resistance to other disinfectants, and this cross resistance between unrelated disinfectants remained for 28 days. Rotating between different disinfectant classes to reduce resistance may not be a successful strategy in controlling *L. monocytogenes* in food processing environments (Lundén et al., 2003). Preconditioning cells with salt also influenced the rate of attachment to surfaces, but that varies between strains (Adrião et al., 2008).

1.1 Human illness caused by *L. monocytogenes*

L. monocytogenes rarely causes severe illness in healthy adults, though it has been linked to limited gastrointestinal illness in immunocompetent persons (Dalton et al., 1997; Aureli et al., 2000). The very young, elderly, immunocompromised persons, and pregnant women are thought to be susceptible to listeriosis. Underlying health conditions (e.g., diabetes, alcoholism, lupus, kidney disease, steroid therapy) may also be linked to increased susceptibility to the infection (Büla et al., 1995; Donnelly, 2001).

In the initial stages of infection, symptoms of gastrointestinal distress, or fever, may occur (Büla et al., 1995; Rocourt et al., 2000). In pregnant women, illness may be asymptomatic, or with gastrointestinal symptoms, fever, flu-like symptoms, headache, miscarriage, stillbirth, or birth of an infected infant (septicaemia or meningitis) (Rocourt et al., 2000). Fatality rates are often reported to range from 20-30%, but this is usually skewed to those with severe illness, and variation may occur between illness types (bacteremia, meningitis), and strains (Buzby et al., 1996; Rocourt et al., 2000).

After ingestion, *L. monocytogenes* infects gastrointestinal epithelial cells and replicates intracellularly. It then migrates successively into the lymph nodes, blood, liver and spleen, and then may move into fetal-placental tissues, and/or the central nervous system via infected monocytes (Cossart and Toledo-Arana, 2008). Systemic infections are marked by bacterial replication in the spleen and liver (de Bruijn et al., 1998; Drevets and Bronze, 2008). Infection of the central nervous system is thought to occur via infected bone marrow cells, after having first proliferated in the spleen and liver (Join-Lambert et al., 2005). Models of infection in pregnant animals point to infection of the liver, spleen, and placenta, within two days of infection (Williams et al., 2011). LD_{50} (lethal dose for 50% of the animals) values of ~10^7-10^{10} CFU have been calculated for several pathogenic strains in mice (reviewed in Liu, 2006). In humans, foods implicated in outbreaks have been reported to contain 10^5-10^9 CFU/g of *L. monocytogenes* (Aureli et al., 2000; Rocourt et al., 2000). Survivors of listeriosis may have long term neurological damage, including ocular nerve problems (Büla et al., 1995; Buzby et al., 1996).

This pathogen is grouped into 12 serotypes on the basis of reaction between O (somatic) and H (flagellar) antigens (Orsi et al., 2011).There are also phylogenetic divisions or lineages based on ribotyping and sequence variation (Meinersmann et al., 2004). Multi locus sequence typing (MLST) is a technique that examines single nucleotide polymorphisms in housekeeping genes. It allows rapid as well as interlaboratory comparisons, and the development of phylogenetic comparisons between *Listeria* strains (Reij and Den Aantrekker, 2004). While most outbreaks of listeriosis were previously investigated using Pulsed Field Gel Electrophoresis (PFGE) as in CDC's and the Canadian Pulsenet, development is moving toward the singular use of whole genome sequencing and ST-types outbreak investigations (Gilmour et al., 2011, Knabel et al., 2012, Althaus et al., 2014).

Serotypes 1/2b, 3b, 4b, 4d, and 4e, are considered to belong to Division (or Lineage) I; while 1/2a, 1/2c, 3a, and 3c, are found in Division/Lineage II (Graves et al., 1994; Aarts et al., 1999). A third group (Division/Lineage III) has been proposed to contain 4a, 4c, and some 4b strains (Meinersmann et al., 2004; Gray et al., 2004). This division is also associated with environmental, rather than human isolates (Wiedmann et al., 1997). Divisions III and IV are considered rare and contain serotypes 4a, 4b, and 4c (Orsi et al., 2011), though some group 4a, b and c serotypes are shared between lineage groups (Ward et al., 2008).

Three serotypes (4b, 1/2a and 1/2b) cause most of the clinical cases of human listeriosis (McLauchlin 1990; Jacquet et al., 2002). Serotype 4b is considered especially virulent, while 1/2a and 1/2b are less virulent, but are often associated with food

(Wiedmann et al., 1997, Jacquet et al., 2002). In surveys of foods and processing

facilities, serotypes 1/2a and 1/2b predominate (Ward et al., 2007, Gianfranceschi et al.,

2009).

1.2 Foodborne transmission of *L. monocytogenes*

Early evidence of foodborne transmission occurred in the 1980s, with outbreaks

associated with coleslaw (Schlech et al., 1983) and soft Mexican style cheese (Linnan et

al., 1988). Many ready to eat, refrigerated foods can support the growth *of L.

monocytogenes*. Consumption of foods such as soft cheeses (Fretz et al., 2010), and deli

meats, are commonly thought to be risky for those susceptible to listeriosis (Weatheril,

2009). Salad (Aureli et al., 2000), pasteurized milk (Fleming et al., 1985), and fresh fish

(Rocourt et al., 2000) have also been implicated in outbreaks. Monitoring data also show

the presence of *L. monocytogenes* in vegetables, cheeses, fruit, seafoods, and dairy

products (Gombas, et al., 2003, Little et al., 2009). Survey data 1988-2007 from

Australia, Canada, the European Union (EU), New Zealand and the United States (US)

reported *L. monocytogenes* outbreaks in foods (from most prevalent to least) in dairy

products, meats, seafood, multi-ingredient foods, and produce (Greig and Ravel, 2009).

Eggs, bakery products, and non-dairy beverages were not implicated in the 53 outbreaks

identified (Greig and Ravel, 2009). Examples of recent listeriosis outbreaks that have

occurred in Canada and USA are ready-to-eat meat products (Weatherill, 2009),

cantaloupe (anonymous, 2011), apples (CDC, 2015a) and ice cream (CDC 2015b).

The contamination of food commodities in agricultural settings can lead to

outbreaks of listeriosis, however, the contamination of foods during processing is thought

to lead to the majority of *L. monocytogenes* outbreaks (Oliver et al., 2007). *L.

monocytogenes may enter processing facilities via food commodities (Todd and Notermans, 2011). Water associated *L. monocytogenes* strains may also enter the food chain and food processing environments (Weller et al., 2015b), and be of serotypes associated with illness (Lyautey et al., 2007, Stea et al., 2015).

The incidence of listeriosis was thought to be trending downward since the mid 1990s in the USA (Tauxe et al., 2010). However, in the UK and some parts of the EU, an increase in the incidence listeriosis has been described (Gillespie et al., 2006, Goulet et al., 2008, Lomonaco et al., 2015).

1.3 Regulations for ready to eat (RTE) foods

Ready to eat (RTE) foods are of high concern with respect to listeriosis while *L. monocytogenes* is considered low risk in foods that are cooked or pasteurized (FDA, 2008).

In Canada, regulations for *L. monocytogenes* in RTE foods includes levels of risk categorization for different foods (depending on pH, a_w, the presence of preservatives, storage temperature), detailed instructions for environmental testing and end-product testing, and the encouragement to use post-lethality treatments and/or *L. monocytogenes* growth inhibitors. A policy documents states "a lower priority should be placed on products in which the organism cannot grow or, has a limited potential for growth whereby the levels do not exceed 100 CFU/g throughout the stated shelf-life" (Health Canada 2011).Trend analysis is also required when *Listeria* spp. on food and non-food contact surfaces are detected (CFIA, 2015), as part of determining the effectiveness of the controls in place.

In the United States, a zero tolerance policy has been adapted for RTE foods containing *L. monocytogenes* (($\leq$ 1 CFU in 25 g of sample, cited in Orsi et al., 2011), and surfaces contaminated with *L. monocytogenes* that have the potential of coming into contact with RTE foods (Warriner and Namvar, 2009). The reasoning behind the zero-tolerance includes the issue of the infectious dose of *L. monocytogenes* not being precisely known (Donnelley, 2001). However, that decision is also sometimes criticized, due the low overall incidence of listeriosis compared to the levels of *L. monocytogenes* that occurs in some RTE foods (Chen et al., 2003, Warriner and Namvar, 2009). FDA guidance (FDA, 2008) does not explicitly recommend trend analysis, when positive samples from food processing surfaces have occurred, or continue to occur. Other jurisdictions have a zero tolerance policy for some RTE foods, but exclude foods such as raw vegetables where they do not support *L. monocytogenes* growth and have a short (<4 day) shelf life (Warriner and Namvar, 2009).

1.4 Problem in perspective; cost of illness caused by *L. monocytogenes*

Many analyses find that despite a relatively low number of annual cases, when *Listeria monocytogenes* causes illness, the hospitalization rate and mortality rate are both high when compared to other bacterial pathogens (Mead et al., 1999). Other estimates place the cost of *L. monocytogenes* illness below that of non-typhoid *Salmonella*, but in the same range or higher than *Campylobacter* and *E. coli* O157:H7 (Buzby et al., 1996, Hoffmann et al., 2012). Overall, the costs of *L. monocytogenes* infections are relatively high, despite low numbers of annual cases.

Chapter 2: Literature Review: *Listeria monocytogenes* and Biofilms

2.1 Bacterial biofilms

Biofilms are defined as "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton, 2002), Alternative definitions include "surface associated bacterial communities" (Chavant et al., 2002) or "biologically active matrix of cells" (Kumar and Anand, 1998). Biofilms may form on a variety of surfaces; including foods (plant, animal tissue), drains, and food processing equipment and surfaces (glass, rubber, stainless steel) (Kumar and Anand, 1998). Biofilms are often described in terms of cells attached to a surface in an aqueous system, but aggregates or floccules within porous spaces in non-aqueous systems can be included (Costerton et al., 1995). Biofilms may form statically in fluids (e.g., in difficult to clean crevices in food processing equipment), or under flow conditions. Biofilms may also form on a surface with intermittent fluid deposition, with the biofilm sometimes exposed to air.

2.2 Steps in biofilm formation

The first step of biofilm formation is usually considered to be the attachment or adhesion of cells to a surface. This step is dependent on the surface properties, such as roughness, hydrophobicity and ionic charge (Geesey 2001), and cell surface properties (Davey and O'Toole, 2000). This step may be passive, involving free floating cells attaching to a surface by gravity or flow or active, involving bacterial motility (flagella or pili) (Kumar and Anand, 1998; Davey and O'Toole, 2000). While initial cell to surface

contact is easily reversible, irreversible adhesion of cells to the surface follows

attachment with interactions between the surface and flagella, pili, and later, polymeric

substances excreted by the cells (Kumar and Anand, 1998). What is termed irreversible

attachment does not mean the cells cannot be removed, but removal generally requires

scrubbing, scraping or other physical force (Kumar and Anand, 1998), or dispersal of

biofilm can occur due to enzymatic activity (Kumar and Anand, 1998, Boles and

Horswill, 2008, Nguyen and Burrows, 2014).

In the second step, cells proliferate to form a monolayer on the surface (Davey

and O'Toole, 2000; Costerton et al., 1995). The first and second steps in biofilm

formation are thought to be controlled by interactions between species (signals) and

environment (Stanley and Lazazzera, 2004).

Third, microcolonies form, usually mediated by pili expression (Davey and

O'Toole, 2000). These are small colonies of cells, held together by cell to cell

interactions and extracellular polymeric substances (EPS) (Stanley and Lazazzera, 2004).

EPS provides physical structure, sequestering metal ions and toxins, and protection

against rapid pH changes, antimicrobial agents, desiccation, and osmotic shock (Davey

and O'Toole, 2000).

The fourth and final step, biofilm maturation, involves growth in 3 dimensions

(including depth), and the formation of more complex structures (e.g., channels for liquid

flow, anaerobic pockets) (Davey and O'Toole, 2000; Stanley and Lazazzera, 2004).

Maturation may be controlled by cell to cell signalling (quorum sensing) or nutrient

availability (Stanley and Lazazzera, 2004). Mature biofilms may shed individual cells

(Kumar and Anand, 1998), or cells contained in EPS to colonize new, nutrient rich areas (Stanley and Lazazzera, 2004).

In practical terms, biofilms contain cells that may be resistant to, and/or protected from antimicrobial agents, nutrient depletion, mechanical removal, and are capable of shedding and spreading on a variety of surfaces (Kumar and Anand, 1998, Davey and O'Toole, 2000).

Listeria monocytogenes biofilms often do not resemble textbook diagrams of thick, 3 dimensional structures with large amounts of EPS. Monolayers of cells (Chavant et al., 2002, Borucki et al., 2003), microcolonies with sparse EPS (Daneshvar Alavi and Hansen, 2013), or structures that resemble knitted chains (Rieu et al., 2008) have been observed. Visible EPS may be sparse or absent in SEM images, but this could be due to sample preparation techniques.

2.4 Biofilms in the food processing environment and biofilm control

In a food processing environment, biofilm may have many unwanted effects. Aside from pathogens, biofilms may also contain spoilage organisms that spread to finished products, organisms that cause metal corrosion, or the biofilms can reduce heat transfer and processing efficiency (Simões et al., 2010).

Control processes for biofilms may include frequent cleaning and disinfection of food contact surfaces several times per day, while cleaning and disinfection of non-food contact surfaces may occur only once per day (Simões et al., 2010). Further, equipment design must minimize the presence of hard to clean crevices, gaskets, surfaces, and dead ends (Simões et al., 2010). Cleaning in place procedures (CIP) of food equipment

usually involves a pre-rinse step to thoroughly remove food residues, circulation of a detergent, rinsing to remove detergent, disinfection, and final rinse to remove disinfectant (Simões et al., 2010).

2.5 *L. monocytogenes* biofilms and sanitation

Many strains of *L. monocytogenes* will form biofilms on a variety of food processing surfaces such as rubber, or stainless steel (Smoot and Pierson, 1998a, Mafu et al., 1991), with or without the presence of other bacterial species. *Listeria* spp. can be present in food processing environments in drains, condensed or standing water, on floors, in food product residues, or in equipment (Cox et al., 1989). *L. monocytogenes* can be more resistant to sanitizers when co-cultured with other species (Lourenço et al., 2011). Further, biofilms formed at lower temperatures were more resistant to sanitizers than those formed at 37°C, and some strains of *L. monocytogenes* formed biofilms at 12°C that required more than the manufacturers recommended concentration of sanitizer to become eradicated (Lourenço et al., 2011). The repeated exposure of pre-formed biofilm to cleaning, starvation, and feeding cycles (simulating the conditions encountered by biofilm in a food processing facility) lead to not only sanitizer resistance, but also a final biofilm concentration of ~7 Log CFU/cm^2 of *Listeria monocytogenes* (Pan et al., 2006).

As with attachment or adherence, biofilm formation (usually measured by number of viable cells, or staining of the biofilm as a measure of biomass) is known to vary between *L. monocytogenes* strains, media types used, temperature, and surface materials (Moltz and Martin, 2005). In a survey of 80 isolates of *L. monocytogenes*, biofilm formation was found to be greater in Division II strains (serotypes 1/2a and 1/2c) and

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comparatively smaller in Division I strains (serotype 1/2b and 4b) (Borucki et al., 2003). However, the capability of *L. monocytogenes* cells to form biofilm varies greatly within the 1/2a, 1/2b and 4b serotypes (Lourenço et al., 2011).

Where *L. monocytogenes* biofilms provide protection against sanitizer activity, it is known that in suspension (planktonic cells), the minimal bactericidal concentration (MBC) may be an order of magnitude lower than the minimum concentration required to eradicate pre-formed biofilm (MBEC) (Cruz and Fletcher, 2012). Unfortunately, for chlorine, iodine, and quaternary ammonium compounds, manufacturers recommended concentrations may be too low to eradicate biofilm (Cruz and Fletcher, 2012). Ibusquiza et al. (2011) found *L. monocytogenes* biofilms that had matured for several days were resistant to several sanitizers, and that peracetic acid was the most effective sanitizer tested.

It is also possible that a sanitizer at an effective concentration could be applied to a biofilm, but the presence of organic matter (Ibusquiza et al., (2011), or insufficient contact time would allow some cells to survive. The sanitizer concentrations in the work by Cruz and Fletcher (2012) were also based on a 5 Log CFU reduction of viable counts, which is often lower than the viable counts for *L. monocytogenes* biofilm described elsewhere in the literature. Strategies for biofilm control should consider the amount of rinsing needed to remove organic matter and maximize sanitizer efficiency, as well as the importance of considering biofilm maturity, and the load of viable cells (Ibusquiza et al., 2011). Equipment design that minimizes areas that accumulate food residues, or areas difficult to clean and sanitize (Carpentier and Cerf, 2011) is also critical.

Thermal sanitation treatments have also been proposed to combat *L. monocytogenes* in food processing environments. Chmielewski and Frank (2006) determined treatments of 78-80°C water to for 10-15 minutes would have a lethal effect on *L. monocytogenes* biofilms in the presence of food soils. Mixed species biofilms required 15 minutes at 80°C to have the same effect (Chmielewski and Frank, 2006). Ban et al. (2012) determined low pressure steam resulted in ~ a 2 Log CFU reduction in 20 seconds. Belessi et al. (2011) found 60°C water applied for 20 minutes had no effect on attached *L. monocytogenes* cells. However, the application of heat is a practical challenge as it could be difficult to ensure that the correct temperature and exposure time is met, and the safety of workers is also key.

2.7 Persistence

Persistence is a phenomenon where a strain or clone of *L. monocytogenes* may survive in an environment for several years, despite cleaning and disinfection procedures (reviewed in Ferreira et al., 2014). Unnerstad et al. (1996) reported that in dairy processing facilities, one clone was found in the same plant for several years, despite the plant being renovated to improve hygiene. A strain may become part of the domestic microflora of an environment and become extremely difficult to eradicate, having adapted to the environment and possibly the sanitation programs (Unnerstad et al., 1996).

Persistent *L. monocytogenes* strains are noted to have better adherence (cells/cm^2) to stainless steel after short contact times when compared to non-persistent strains, and achieve higher levels of biofilm formation (Lundén et al., 2000). Large amounts of EPS and dense biofilms for persistent strains have been observed with scanning electron

microscopy (SEM), though persistence was not associated with specific serotypes in that study (Borucki et al. 2003). Persistent strains from retail food environments form more biofilm than transient strains initially, but that difference was not significant as the biofilms matured (Wang et al., 2015). Persistent strains were also not more sanitizer resistant, and variability in biofilm formation and sanitizer resistance was observed within each group (Wang et al., 2015). Other researchers have determined that persistent strains formed more biofilm, and that biofilm formed by some persistent strains have both high levels of EPS and are resistant to benzalkonium chloride (Nakamura et al., 2013).

However, not all studies point to differences in biofilm formation by persistent strains. Harvey et al. (2007) also found that weak biofilm formers formed similar levels of biofilm to strong biofilm formers, given sufficient incubation time, similar to the finding of Wang et al., (2015) for persistent and transient strains. Szlavik et al., (2012) found that persistent strains did not have a higher rate of initial attachment under flow conditions. Differences in sanitizer resistance between persistent and transient strains has been reported in planktonic cells (Aase et al., 2000), or no differences have been found (Holah et al., 2002, Harvey et al., 2007). Kostaki et al. (2012) found that in mixed strain *L. monocytogenes* biofilms, the strain with best survival after sanitizer exposure was not always a strain that formed more biofilm alone.

The concentrations of sanitizer needed to elicit a lethal effect on viable cells within a biofilm in many studies are below the recommended usage level (reviewed in Ferreira et al., 2014). In food processing facilities, that finding may point to a physical or other disconnect between sanitizer application and *L. monocytogenes* cells. Overall,

current findings indicate that many *L. monocytogenes* strains are capable of forming biofilm given sufficient time, or reaching a harborage site and remaining undisturbed. It is also possible that persistent strains have a survival advantage that does not always correlate to levels of biofilm formed. The literature does not often describe sanitizer concentrations required to cause a lethal effect on pre-formed biofim, or the characteristics of cells that survive sanitizer treatments which do not completely eradicate biofilm.

2.8 Desiccation

Solute stress or osmotic stress in a microbiology context occurs when bacteria in a liquid media experience a reduction in water activity (e.g., addition of salt to growth media). In matric stress, differences in water potential between an aqueous and gaseous environment cause the removal of water from bacteria, and intracellular solute concentrations increase (cited in van de Mortel et al., 2005).

L. monocytogenes can survive desiccation for weeks or months on stainless steel surfaces (Vogel et al., 2010, Takahashi et al., 2011). The resistance of pre-formed biofilm to desiccation also demonstrates another protective effect of biofilm formation (Hingston et al., 2013). The maturity of biofilms prior to desiccation also plays a role in survival, however, that was not directly relatable to the number of viable, surface attached cells exposed to desiccation (Hingston et al., 2013). EPS production is also known to increase in *Pseudomonas putida* under matric stress, and the biofilms formed are also thicker (Chang et al., 2007).

Food processing environments may expose *L. monocytogenes* planktonic cells or biofilm to desiccation through equipment design that traps soils, or through sanitation regimes that emphasize drying equipment and surfaces after disinfection (Carpentier and Cerf, 2011). *L. monocytogenes* remaining on processing equipment may also desiccate during periods where equipment is not in use, such as seasonal or holiday closures.

2.9 Mechanisms of biofilm formation

2.9.1 Attachment

The initiation of biofilm formation may occur due to factors such as oxidative stress (Geier et al., 2008), or as a result of high intracellular levels of c-di-GMP (Hickman et al., 2005, Lim et al., 2006). C-di-GMP (or bis-(3'-5)-cyclic dimeric guanosine monophosphate) is a well conserved intracellular signalling molecule, and c-di-GMP signalling pathways are known to affect motility, adhesion and EPS formation in numerous bacterial species (Boyd and O'Toole, 2012). Biofilm may form in nutrient rich environments despite the trade-off of a slower growth rate, because there is an advantage to having protective EPS, the ability to remain in that environmental niche, or having heterogeneity in physiological state (reviewed in Jefferson, 2004).

Initial attachment to surfaces can require protein synthesis, as in *Pseudomonas fluorescens* (O'Toole and Kolter, 1998). Attachment occurs through components such as pili (O'Toole and Kolter, 1998), flagella (Lemon et al., 2007, Houry et al., 2010), and cell surface adhesins, often glycosaminoglycans or similar polysaccharides (Mack et al., 1996, Wang et al., 2004).

Proteins are thought to play a key role in promoting initial attachment to surfaces in *L. monocytogenes*, as treatment with trypsin (Smoot and Pierson, 1998b), or protease

(Longhi et al., 2008) reduces biofilm formation. Protease treatment reduces the presence of ami 4b, an actin assembly protein (actA), and internalin B on the cell surface (Longhi et al., 2008). In some *L. monocytogenes* strains, BapL is present and may contribute to initial attachment (Jordan et al., 2008). This protein has homology to Staphylococcal Bap, a cell wall associated protein which promotes attachment and greater biofilm formation (Cucarella et al., 2001).

Motility by flagella has been reported as necessary for *L. monocytogenes* biofilm formation in static systems (Lemon et al., 2007), with the lack of motility causing a defect in biofilm formation occurring in initial attachment, and ongoing biofilm maturation (Lemon et al., 2007). Centrifugation was found to restore initial attachment, which may indicate the flagella mediate surface contact and adhesion can occur via other factors (Lemon et al., 2007). Residual attachment ability also has been demonstrated in mutants lacking flagella (Tresse et al., 2009). Todhanakasem and Young (2008) described hyperbiofilm formation in *L. monocytogenes* motility mutants under laminar flow conditions after allowing the initial attachment to occur statically. *Bacillus cereus* motility mutants with similar defects in static biofilm formation were not biofilm defective under flow conditions (Houry et al., 2010). Interestingly, initial adhesion to glass under flow conditions was found to be increased for an immotile *flaA* mutant (Houry et al., 2010).

2.9.2 Extracellular polymeric substances and extracellular DNA
Well studied biofilm forming bacteria such as *S. aureus* are known to form EPS that has a polysaccharide component, (reviewed by O'Gara, 2007). Recently, (Chen et al.,

2014, Köseoğlu et al., 2015) described a c-di-GMP regulated genetic locus for EPS production in *L. monocytogenes*, with the polymer composed of β-1,4-linked N-acetylmannosamine with α-1,6-linked galactose. Deletion of a gene that downregulates EPS production were found to cause visible increases in EPS in SEM images (Köseoğlu et al., 2015).

Harmsen et al. (2010) described the importance of extracellular DNA (eDNA) on the attachment of *L. monocytogenes* to surfaces, and the need for N-acetyl- glucosamine as a cofactor for DNA mediated attachment. Initial attachment may require EPS similar to *Staphylococcal* poly-*N* acetylglucosamine (PNAG). *L. monocytogenes* eDNA is proposed to be a polymer of N-acetyl-glucosamine and high molecular weight DNA (Harmsen et al., 2010).

DNA may be externalized through peptidoglycan turnover and autolysins (Bose et al., 2012), or the action of a DNA translocase (*fstk*) (Mata et al., 2015). In *Bacillus cereus,* eDNA is important for attachment and biofilm formation, and purine biosynethsis genes have involvement (Vilain et al. 2009). In *S. mutans*, the presence of glucans combined with chromosomal DNA enhances adhesion (Liao et al., 2014), which is similar to the findings of Harmsen et al. (2010). Sortase A (*srtA*) is thought to be necessary in anchoring glucan binding proteins to the cell surface in *S. mutans* (Liao et al., 2014).Experimental data describing *L. monocytogenes* biofilm removal with pectinase also points to EPS having a polysaccharide component (Xue and Blais, 2012). Pectinase primarily acts with polygalacturonase activity (cited in Xue and Blaise, 2012), and that is consistent with galactose being described as a major component of *L. monocytogenes*

EPS by Köseoğlu et al., (2015). Nguyen and Burrows (2014) found DNase I and proteinase K were effective in dispersing pre-formed biofilm, which also demonstrates the importance of DNA and protein in the biofilm matrix. Overall, *Listeria monocytogenes* EPS is likely composed of both eDNA, protein and polysaccharide components.

2.9.3 Quorum sensing

Quorum sensing (QS), commonly thought of as cell-to-cell communication, is employed by many bacterial species (Ng and Bassler, 2009), though the production of small, diffusible signalling molecules, called autoinducers (Bai and Rai, 2011). As a bacterial population reaches a threshold (or quorum), the concentration of autoinducer in the local environment will reach a level that has an effect on genetic regulation. Gram positive QS molecules are typically peptide derivatives (Ng and Bassler, 2009). Gram positive autoinducer receptors are usually two-component regulatory systems, with a membrane bound histidine kinase that is activated by a high concentration of autoinducer (Ng and Bassler, 2009). The cytosolic histidine is then autophosphorylated, with the phosphate group then transferred to a cytosolic response regulator, which binds DNA and functions as a transcriptional regulator (Ng and Bassler, 2009). In this manner, bacterial population density (Bai and Rai, 2011) may have a role in regulating virulence, biofilm formation, antimicrobial peptide production, or the development of genetic competence (Bai and Rai, 2011). While homology may exist between Gram positive bacterial receptor kinase systems, their ligand sensing domains may be diverse and specific (Ng and Bassler, 2009).

In the well -studied *agr* (accessory gene regulator) locus in *Staphylococcus aureus*, QS is activated by increasing cell density (Ji et al., 1995, Dunman et al., 2001), leading to repression of some surface proteins, but increased expression of certain virulence factors (including hla, alpha toxin) (Ji et al., 1995, Dunman et al., 2001). The *agr* P2 operon encodes its own activator, which activates its own promoter, and upregulates signal transduction proteins (Ji et al., 1995). In the context of *S. aureus* biofilm, *agr* is thought to mediate biofilm dispersal, though it is not the only mechanism of dispersal (Boles and Horswill, 2008). Deletion of *agr* in *S. aureus* has varying effects on biofilm formation, depending on conditions (Yarwood et al., 2004), which may indicate different sets of genes under *agr* control affect biofilm under certain growth conditions (Yarwood et al., 2004). Biofilm formation and maturation in *S. aureus* is complex, with *agr* regulation affected by sigma factor B, and extracellular proteases *aur* and *spl* (Lauderdale et al., 2009).

L. monocytogenes has an *agr* system that also affects the transcription of many genes, and deletion of *agrA* also impacts transcription depending on temperature (Garmyn et al., 2012). At 25°C, ABC type transporters, cell wall associated proteins, and internalins A and B, had reduced transcription levels with the deletion of *agrA*. At 37°C, deletion of *agrA* caused overexpression of genes including many involved in ABC type transport, PTS transport, lipoproteins, purine and pyrimidine biosynthesis, motility, chemotaxis, while genes including regulators *prfA*, and *comK* had reduced transcription (Garmyn et al., 2012). Interestingly, biofilm formation was enhanced in the *agr* deletion mutant at 37°C, but reduced at 25°C (Garmyn et al., 2012). Other workers have found reduced *L. monocytogenes* biofilm formation at 30°C with an *agrD* deletion mutant, and

reduced inlA in the cell wall (Riedel et al., 2009). Genes affected by *agrD* deletion were more numerous in stationary phase culture than during exponential phase growth, and included genes related to the cell envelope, DNA metabolism, protein synthesis, and transport and binding proteins (Riedel et al., 2009). Mixed cultures of EGD-e and *agrD* deletion mutant form biofilm normally, as the agrD protein diffuses to the mutant (Riedel et al., 2009). Deletion of *agrD* also has been found to reduce initial attachment to glass (Rieu et al., 2007). Other QS systems in *L. monocytogenes* include the AI-2 system (*Vibrio harveyi* homologue). Deletions of *LuxS* shows reduced AI-2 activity and increased biofilm formation on stainless steel and glass (reviewed in Garmyn et al. 2009).

2.9.4 Additional regulators of biofilm formation

The *L. monocytogenes* virulence regulator *prfA* controls the expression of many proteins that are important in intracellular invasion, including actA (actin assembly protein), internalins, and Listeriolysin O (Milohanic et al., 2003). *PrfA* is temperature controlled, with higher activity at temperatures of ~37°C (cited in Milohanic et al., 2003). Stress, such as acid shock, or oxygen depletion can also increase *prfA* expression (Anderson et al., 2007, Neuhaus et al., 2013), and this increase can occur at lower temperatures (Neuhaus et al., 2013). *PrfA* positively regulates biofilm formation at ~20, 30 and 36°C, but does not impact the initial attachment process (Lemon et al., 2010). At elevated temperatures, a mutant that produced a non-functional prfA protein and cannot transition to intracellular activity produced more biofilm than the wild type (Lemon et al., 2010). In minimal media, *prfA* also alters gene expression in cells within a biofilm, including genes important in peptidoglycan teichoic acid biosynthesis, cell division, and

DNA repair (Luo et al., 2013). These findings may draw attention to genes that have a role in biofilm formation, or those that are affected by temperature.

The response regulator DegU, is considered an orphan as it lacks the histidine kinase DegS, that acts as a sensor in *B. subtilis* (cited in Gueriri et al., 2008a). DegU is thought to control motility in *L. monocytogenes* (Gueriri et al., 2008), and have an effect on the transition from planktonic to sessile cells, depending on its phosphorylation level, and perhaps the metabolic state of the cells (reviewed in Abee et al., 2011). In *L. monocytogenes*, DegU is important for growth at high temperatures, and also has a positive role in biofilm formation (Gueriri et al., 2008b).

Other genes, such as *relA*, *hpt* (Taylor et al., 2002), and the SigB regulator (van der Veen and Abee, 2010), play a role in *L. monocytogenes* biofilm formation.

Chapter 3: Research Objectives and Rationale

The overall objective of this research was to better understand the genetic factors that influence *L. monocytogenes* biofilm formation at a temperature common to food processing environments, and the characteristics of the biofilm formed with respect to sanitizer treatment, desiccation, and biofilm removal.

The first section of experimental work in this thesis focused on generating a library of >11,000 *L. monocytogenes* transposon mutants, screening those mutants for altered biofilm formation (15°C), followed by identification of the genes disrupted, and characterization of the biofilm. Treatment with peroxide and benzalkonium chloride (BAC) early in the biofilm maturation process was examined, as well as the response of a panel of biofilm mutants to hydrolytic enzymes. The biofilms formed by a selection of mutants were also examined with scanning electron microscopy, and reverse transcriptase PCR experiments were performed to determine if polar effects occurred as a result of the transposon insertion. The results of the experiments are described in Chapter 4.

The second section of experiment work focused on the role of genetic elements that are thought to influence *L. monocytogenes* survival; with respect to biofilm formation on stainless steel and peg lids, sanitizer and desiccation susceptibility. A panel of Canadian *L. monocytogenes* strains was examined to better understand the variation between isolates of clinical, food processing, and watershed origin in terms of their biofilm formation, sensitivity to benzalkonium chloride to inhibit planktonic growth and biofilm formation, sensitivity to desiccation treatment, and the occurrence of genetic markers that may influence those outcomes. The results of these experiments are described in Chapter 5.

Thesis hypotheses investigated included:

Chapter 4:

1.1 The biofilm mutants identified will have some overlap with previous research,
however, some mutants identified may differ with respect to phenotype, or they may
have disruptions in genes not previously identified in mutant libraries that were
screened for biofilm formation at higher temperatures.

1.2 Biofilm mutants (identified by staining protocols) will also show a similar phenotype
(deficient or enhanced) on stainless steel coupons.

1.3 A model sanitizing assay will determine which biofilm mutants survive well on
stainless steel when treated with an oxidizing sanitizer and a quaternary ammonium
compound. Deficient mutants are expected to exhibit reduced survival compared to
mutants that produce excess biofilm.

1.4 Mutants that are biofilm deficient, and also those with genes thought to influence
attachment, motility or EPS composition are predicted to form low levels of biofilm
in the peg lid assay, and/or they may retain little biofilm following treatment with
enzymes.

Chapter 5:

1.1 The watershed strains are not expected to have markers associated with clinical
strains while any of the strains may have the SSI-1 marker. Isolates from food or
food processing environments that were not previously screened may have the LGI1
marker, or belong to the CC8/VT59 group.

1.2 The presence of LGI1 or CC8/VT59 markers will increase the minimum
concentration of benzalkonium chloride required to inhibit biofilm formation.

1.3 Desiccation resistance will correlate to the genetic markers for SSI-1 and LGI1.

1.4 There may be some strains that produce high levels of EPS (including desiccation

resistant strains), and they will be BAC resistant and resistant to the action of

hydrolytic enzymes.

Findings from this research will provide a better understanding of the genetics of

biofilm formation, relevant to sanitation and food processing safety, and the contributions

of SSI-1, LGI1, and CC8/VT59 to sanitizer resistance and desiccation response. Such

knowledge is needed to develop effective *Listeria* control strategies, highlight differences

between *L. monocytogenes* isolates that may pose a challenge to sanitation efforts, and to

aid the development of biofilm prevention and removal regimes.

Chapter 4: Genes Involved in *Listeria monocytogenes* Biofilm formation at a Simulated Food Processing Plant Temperature of 15°C

A portion of this research was included in the manuscript "Genes involved in *Listeria monocytogenes* biofilm formation at a simulated food processing plant temperature of 15°C " authored by Marta Piercey, Patricia Hingston, and Lisbeth Truelstrup Hansen, and submitted to the International Journal of Food Microbiology in October, 2015.

Abstract

Listeria monocytogenes is a pathogenic foodborne bacterium whose persistence in food processing environments is in part attributed to its biofilm formation. Most biofilm studies have been carried out at 30-37°C rather than at temperatures found in the food processing plants (i.e., 10-20°C). The objective of the present study was to mine for novel genes that contribute to biofilm formation at 15°C in *L. monocytogenes* using the random insertional mutagenesis approach. A library of 11,024 *L. monocytogenes* 568 (serotype 1/2a) *Himar1* insertional mutants was created. Mutants with reduced or enhanced biofilm formation at 15°C were detected in microtiter plate assays with crystal violet and safranin staining. Fourteen mutants expressed enhanced biofilm phenotypes, and harboured transposon insertions in genes encoding cell wall biosynthesis, motility, metabolism, stress response, and cell surface associated proteins. Deficient mutants (n=5) contained interruptions in genes related to peptidoglycan, teichoic acid, or lipoproteins. Enhanced mutants produced significantly ($p<0.05$) higher cell densities in biofilm formed on stainless steel (SS) coupons at 15°C (48 h) than deficient mutants, which were also more sensitive to benzalkonium chloride. All biofilm deficient mutants and four

enhanced mutants in the microtiter plate assay (*flaA*, *cheR*, *lmo2563* and *lmo2488*) formed no biofilm in a peg lid assay while insertions in *lmo1224* and *lmo0543* led to excess biofilm in all assays. Two enhanced biofilm formers were more resistant to enzymatic removal with DNase, proteinase K or pectinase than the parent strain. Scanning electron microscopy of individual biofilms made by five mutants and the parent on SS surfaces showed formation of heterogeneous biofilm with dense zones by immotile mutants, while deficient mutants exhibited sparse growth. In conclusion, interruptions of 9 genes not previously linked to biofilm formation in *L. monocytogenes* (*lmo2572*, *lmo2488* (*uvrA*), *lmo1224*, *lmo0434* (*inlB*), *lmo0263* (*inlH*), *lmo0543*, *lmo0057* (*esaA*), *lmo2563*, *lmo0453*), caused enhanced biofilm formation in the bacterium at 15°C. The remaining mutants harboured interruptions in 10 genetic loci previously associated with biofilm formation at higher temperatures, indicating some temperature driven differences in the formation of biofilm by *L. monocytogenes*.

4.1 Introduction

Listeria monocytogenes is a pathogenic bacterium that causes foodborne illness, often in pregnant women, the elderly, and immunocompromised persons (Donnelly, 2001). Recent outbreaks have occurred due to contaminated foods such as ready-to-eat meat products (Weatherill, 2009), cantaloupe (anonymous, 2011), apples (CDC, 2015a) and ice cream (CDC 2015b). *L. monocytogenes* can survive desiccation (Vogel et al., 2010), grows at refrigeration temperatures, and forms biofilm; communities of cells attached to surfaces, as reviewed by Chavant and colleagues (2002). Biofilms are difficult to remove from food processing equipment, and bacteria in biofilms are often more resistant to sanitizers than planktonic cells (Carpantier and Cerf, 2011; Lourenço et al.,

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2011). Many outbreaks have been linked to *L. monocytogenes* biofilms in food processing facilities, including the luncheon meat outbreak in Canada in 2008 (Weatherill, 2009; Gilmour et al., 2010, Knabel et al., 2012).

Previous studies have identified numerous genes associated with *L. monocytogenes* biofilm formation at 30-37°C; rather than temperatures more commonly associated with food processing (10-20°C). It is unknown if the genes involved in biofilm formation differ at food processing temperatures, but it may be likely, considering that the environmental temperature alters cell surface hydrophobicity (Di Bonaventura et al., 2008), motility (Mauder et al., 2008), and the expression of other genetic factors (Liu et al., 2002, McGann et al., 2007) in *L. monocytogenes*.

Transposon mutagenesis is a tool commonly used in bacterial genetic analysis for gene discovery and investigation. Previous transposon mutant libraries have been generated with the mobile genetic element Tn*917*, which inserts itself at TATAA sequences, but does so with a low transposition efficiency (Garsin et al., 2004). In contrast, the mariner based *Himar1* transposon vector pMC39 designed by Cao et al. (2007), was found to exhibit a higher rate of transposition at the frequently occurring TA sites, leading to better chromosomal coverage.

The goal of the present study was to investigate novel genes that contribute to biofilm formation at 15°C in *L. monocytogenes*, using the random insertional mutagenesis approach with the mariner *Himar1* transposon. A library of 11,000 transposon mutants was screened for increased or decreased biofilm formation, as well as susceptibility to benzalkonium chloride (BAC), a sanitizer commonly used in the food

industry. The ability of selected hydrolytic enzymes to remove biofilms made by mutants with altered phenotypes was also examined to probe for differences in biofilm composition, as were the susceptibility of the sessile cells to BAC and H_2O_2. Ultimately, 19 genetic loci associated with biofilm formation were identified in this study, 9 of which have previously been associated with biofilm formation at higher temperatures (30-37°C) by other researchers, and 10 of which have not been previously associated with biofilm formation.

4.2. Materials and methods

4.2.1 Bacterial strains and media

Insertional mutagenesis was performed in *L. monocytogenes* 568 (Lm568), which is a serotype 1/2a food processing isolate (Kalmokoff et al., 2001). Routine culturing was carried out on Brain Heart Infusion agar (BHIA, BHI broth [Difco, Mississauga, ON, Canada] with 1.5% (w/w) technical agar [Difco]) or in Tryptic Soy Broth (TSB, Oxoid, Nepean, ON, Canada). Media was supplemented with 10 µg/mL erythromycin (erm, Sigma Aldrich, Oakville, ON, Canada) and/or kanamycin (kan, Sigma).

4.2.2 Generation of transposon mutant library

To transform Lm568 with the *Himar1* delivery plasmid (pMC39, generously donated by Dr. H. Marquis, Cornell University (Ithaca, NY, USA), the bacterium was grown overnight in Luria-Bertani broth (LH, EMD, Mississauga, ON, Canada) broth at 37°C. One mL was transferred into fresh LH broth and grown with shaking at 37°C for ~ 6 hours. Cells were chilled for 30 min on ice and then washed three times in ice cold 10% (v/v) glycerol followed by centrifugation at 5400 x *g* for 15 min and resuspension of the pellet in 1 mL of glycerol solution. Cell suspension aliquots of 40 µL were together with

1 µL of the *Himar1* delivery plasmid placed in chilled 0.1 cm gap cuvettes (Biorad Laboratories, Inc., Mississauga, Ontario, Canada), gently mixed and then pulsed for ~5 µs with a field strength of 1.8 kV (Biorad). SOC media (20 g/L Tryptone [Oxoid], 5 g/L Yeast Extract [Oxoid], 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose]) was then added (1 mL) and cuvette contents gently mixed. Cells were then incubated at 28°C without shaking for 1 hour and plated on BHIA with 5 µg/mL erm followed by incubation at 30°C for 24 hours to yield colonies with the transformed Lm568 (Lm568:pMC39).

For transposon mutagenesis, following the method outlined by Cao et al. (2007), one colony of Lm568:pMC39 was inoculated into BHI containing 10 µg/mL erm (BHI-erm) and incubated with shaking at 30°C overnight. Subsequently, cells were diluted 1:200 in pre-heated BHI-erm, and incubated with shaking at 30°C for 1 hour. The temperature was then shifted to 40°C and cultures grown until reaching an absorbance at 600 nm of ~0.4 (NanoPhotometer P330, Implen, Westlake Village, CA, USA). This culture was then diluted in peptone saline (PS, 8.5 g/L sodium chloride, 1 g/L peptone, Oxoid) and plated on BHIA-erm at 40°C.

More than 11,000 colonies of temperature shifted Lm568:pMC39 were picked from agar plates and grown in TSB supplemented with 20% (v/v) glycerol at 22°C for 48 hours in 96 well microtiter polystyrene plates (Costar, #3370, Fisher Scientific, Whitby, ON, Canada). These master plates were then frozen and stored at -80°C.

4.2.3 Screening mutants for altered biofilm formation and benzalkonium chloride resistance

Ten µL of thawed mutant cultures were transferred into 190 µL of TSB. Cultures were grown for 24 hours at 22°C before being transferred (10 µL) into microtiter plates containing fresh TSB (190 µL). Plates were sealed with parafilm (Parafilm M®, Bermis NA, Neenah WI, USA), and incubated at 15°C for 6 days at 100% relative humidity (RH) to allow for biofilm formation. The ability of each mutant to form biofilm was determined using both the Crystal Violet (CV) and Safranin (Saf) staining methods. Both stains are commonly used to assess biofilm formation in Gram-positive bacteria (see for example Nguyen and Burrows, 2014). The susceptibility of mutant biofilm to BAC was also assessed.

For the CV staining, planktonic cells were removed by tapping the microtiter plates, followed by rinsing wells twice with distilled water, and heat-fixing (60°C for 40 min) of remaining adhered cells. One hundred µL of CV stain (10 g/L, BDH Chemicals, Toronto, ON, Canada) was added to each well and left for 40 min. Each well was then rinsed twice with distilled water, and remaining stain solubilized in 150 µL of 95% (v/v) ethanol. Plates were then shaken for 10 s before measuring the absorbance at 570 nm (A_{570nm}) (Biotek EL 808 Absorbance Microplate reader, Fisher Scientific). Mutants with absorbance readings that differed from the mean absorbance value of the parent Lm568 strain by ±2 standard deviations were selected as potential biofilm mutants.

The Saf staining procedure was similar to the CV staining, except that 7.5 g/L Saf (Sigma Aldrich) and absorbance measurements at 490 nm were used. While most deficient and enhanced biofilm formers were identified as described above for the CV

protocol, potential biofilm deficient mutants were also identified as those that were highlighted to have low absorbance by the Gen 5 software (v. 2.0, Biotek, Fisher Scientific).

The BAC susceptibility of the sessile cells was determined after removal of the planktonic cells by gentle pipetting, and addition of 70 µL of BAC (0.05 mg/mL, Acros Organics, Fairlawn, NJ, USA) to each well. After 30 min, wells were then rinsed with PS and 70 µL fresh TSB containing Resazurin (0.1 g/L, Fisher Scientific) was then added before incubating plates at 15°C for 24 h. Mutants that did not regrow (redox dye remained blue) were considered susceptible to the sanitizer (and potential biofilm mutants). The sessile parent Lm568 strain cells were not susceptible to this concentration of BAC.

4.2.4 Selection of a panel of potential biofilm mutants
Mutants (n=46) selected for further study were those that showed altered phenotype in 2 (or 3) of the biofilm and BAC assays. To confirm the absence of pMC39, mutant colonies were streaked on BHIA containing kan (25 µg/mL) and incubated at room temperature for 72 h. Mutant showing no growth were considered free of the plasmid.

To eliminate growth deviant mutants, each transposon mutant confirmed not to retain the pMC39 plasmid (n= 36), and Lm568 were grown for 48 h in TSB at 15°C, standardized to an absorbance ($A_{490\ nm}$) of ~ 1.0, and diluted 10-fold in PS before inoculation in TSB in microtiter plates. Two mutants were found to have poor growth ($A_{490\ nm}$ <0.5) and were discarded from further experiments.

Microtiter plates were then incubated at 15°C for 48 h, with plates shaken and $A_{490 nm}$ readings measured every 3 h. Each mutant (n=38) and control were analyzed in triplicate in two independent experiments (n=6). Data from each strain was analyzed using MicroSoft Excel and the Solver Add-In with the 4 parameter logistic model (Dalgaard and Koutsoumanis, 2001). The model was then evaluated for goodness of fit with the MSE model, F value, and adjusted R^2 (Ells et al., 2009).

Each mutant growth curve was pooled with the control (Lm568). The F statistic was generated from the ratio of [(SSpooled-SSseparate)/(DFpooled-DFseparate)]/(SSseparate/DFseparate) (Motulsky and Ransnas, 1987). A large F statistic would indicate that separate fits are significantly different (mutant growth differs from the control). Additionally, 95% confidence bands for the mutants, and 95% probability bands for Lm568 were generated in Sigmaplot software (Systat Software Inc., San Jose, CA, USA) to visually compare the growth curves between the control strain and mutants.

4.2.5 Determination of the site of transposon insertions

To determine the site of transposon insertion, the arbitrary PCR method (Garsin et al., 2004; Cao et al., 2007) was used to amplify genetic sequences flanking the transposon. This method uses two successive PCR reactions (see Table S1 for primers), carried out for both the left and right side regions surrounding the transposon insert.

The first PCR reaction (total volume 25 µL) was performed with 1 mutant colony added to 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 (IDTaq DNA Polymerase Kit, ID labs Manufacturing Inc., London, ON, CA), 18.25 µL RNA free dH_2O, 0.5 µL of each primer stock. The

following PCR cycle was conducted using a Tgradient thermocycler (Whatman Biometra, Göttingen, Germany): initial denaturation step 5 min at 95°C, 30 cycles of 30 s at 95°C, 45 s at 34°C, and 1 min 72°C, followed by a 10 min final elongation cycle (72°C).

For the second PCR reaction (total volume 25 µL), PCR products from the first reaction were diluted (1/20) in RNA free distilled water and 0.5 µL of that mixture was amplified with 0.5 µL high fidelity Taq polymerase (HotStar Taq DNA Polymerase, Qiagen, Toronto, ON, CA), 5 µL of 5x HotStar PCR buffer, 14 µL RNA free dH$_2$O, 2.5 µL of 10 µM primer stock using the following temperature program: An initial denaturation step of 5 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 34°C, 1 min at 72°C, followed by a 10 min final elongation step at 72°C. Gel electrophoresis in an agarose gel [1.5% agarose, 1% TBE (Tris-borate EDTA)] was used to confirm the presence of amplicons prior to sequencing.

PCR products were purified with a PCR cleanup kit (Qiagen), and sequenced commercially by the Sanger method (Genome Québec, Montréal, QC, CA). Sequences were aligned to *L. monocytogenes* strain EGD-e (GenBank ID NC_003210) using BLASTn online software (National Center for Biotechnology Information, Bethesda, MD, USA). Genbank annotation records for EGD-e also included UniProtKB/Swiss-Prot protein function descriptions.

Where 11 mutants did not have both left and right side regions of the transposon insert identified, primers (Table S2) were designed to prime upstream from the missing

side and used in a supplemental PCR assay together with the transposon-specific left or right side primer.

4.2.6 Confirmation of altered biofilm and benzalkonium chloride susceptibility phenotype

A secondary screening was performed for the mutants that deviated in at least 2 of the 3 first round screening assays (n=46), did not retain the plasmid (n=38), exhibited normal planktonic growth (n=29), and had the transposon insertion site identified in the arbitrary PCR assay (n=22).

Cultures were grown in TSB at 15°C for 48 h, and inocula were standardized ($A_{450 \text{ nm}} \sim 0.8$) for the mutants and Lm568. Cultures were then diluted in TSB to the desired density. The BAC assay was run in triplicate and repeated in two independent experiments. The CV and SAF assays were run with triplicate samples in three independent experiments.

4.2.7 Response of pre-formed biofilm to sanitizers on stainless steel

Cultures were grown in TSB (48 h) at 15°C and standardized to $A_{450 \text{ nm}} \sim 1.0$ in TSB, from which a suitable dilution was used to inoculated food grade stainless steel coupons (SS, 314, type 4 finish) to an initial concentration of ~ 4 Log CFU/cm^2 by placing 10 μL on the surface of each coupon. The static biofilm was allowed to form by incubating the coupons at 100% RH for up to 48 h.

Coupons (n=3 for each strain and sanitizer) were sampled after 6, 24, and 48 h and treated with PS (control), 0.15 mg/mL BAC (rinsing in neutralizing buffer (Difco, Mississauga, ON, Canada)), or 0.375% (w/v) H_2O_2 (Fisher Scientific) (rinsing in PS) by submerging the coupons in the solutions for 1 min followed by rinsing. Coupons were

placed in 1 mL PS and sonicated at room temperature (22°C) to remove biofilm cells (Elmo S120H sonicating bath, Fisher Scientific) using the method of Leriche and Carpentier (1995), followed by vigorous vortexing and removal of the SS coupons. Biofilm cells were enumerated after samples were serially diluted in PS, spot plated (5 spots of 20 μL each) on Tryptone Soy Agar (TSA, Oxoid) and incubated for 48 h at 37°C. Counts were converted to $Log_{10}(CFU/cm^2)$.

4.2.8 Removal of pre-formed biofilm on polystyrene peg lids by enzymatic treatments

Two hundred microliters of suspensions of Lm568 and mutant strains, which had been standardized in TSB-glucose to a concentration of 10^8 CFU/mL, were added to three wells each in 96-well microtiter plate bottoms. Microtiter plate polystyrene peg lids (Thermo Fisher Scientific, Roskilde, Denmark) were then added. Plates were then wrapped with parafilm and incubated at 15°C with refreshment of the media every 48 h, achieved by placing the peg lids in a 96-well microtiter plate bottom with fresh TSB-glucose.

After 6 days of incubation, the peg lids were then treated with phosphate buffered saline (PBS, pH 7.4, control), PBS containing DNase (50 μg/mL, Sigma Aldrich Canada), proteinase K (0.1 μg/mL, Sigma Aldrich Canada), or acidified phosphate buffer (APB, pH 5.0), with or without (control) pectinase (1.9 U/mL, Sigma Aldrich Canada) for 1 h at 22°C. Samples were then stained with CV (0.1% w/v) for 20 min. and then the pegs were briefly rinsed with PBS, and the stain solubilized in 33% (v/v) acetic acid (Nguyen and Burrows, 2014). Absorbance was measured as previously described (section 4.2.6).

4.2.9 Scanning electron microscopy of mature biofilm on stainless steel surfaces

Mature biofilms made by Lm568 and selected mutants were prepared on SS coupons as described previously, and allowed to grow for 6 days at 15°C and 100% RH. Following the fixation protocol of Austin and Bergeron (1995), coupons were submerged in 0.1 M sodium cacodylate (Fisher Scientific) solution with 2% (w/v) glutaraldehyde (50% w/w, Fisher Scientific, pH=7.2) for 2 h. The coupons were rinsed in 0.1 M cacodylate buffer supplemented with 3% (w/v) glucose (pH=7.2) three times for 10 min each followed by submersion in 1% (v/v) osmium tetroxide solution (4% aqueous solution, Electron Microscopy Science (EMS), Cedarlane, Burlington, ON, Canada) in 0.1 M cacodylate buffer (pH=7.2) for 4 h. The coupons were submerged in three fresh suspensions of 0.1 M cacodylate buffer for 10 min each. They were then dehydrated in an ascending ethanol (Fisher Scientific) gradient series (35, 50, 70, 90 and 100% v/v, for 15 min each, with the last step repeated thrice). The coupons were then dehydrated in a HMDS/ethanol (hexamethyldisilazane, EMS) mixture series (25:75, 50:50, 75:25 and 100:0, 15 min each, with the last repeated twice). The coupons were air dried for 1 h, mounted onto the aluminum mounts, with carbon adhesive tabs (9 mm diameter, EMS) and sputter coated with Au/Pd nanoparticles (SC502-314B gold/palladium sputter target, 0.1 mm thick, Quorum Technologies LTD Canada, Montréal, QC, Canada).

Biofilms were viewed under the field-emission surface scanning electron microscope (Hitachi S-4700 FE-SEM), with 20 µA emissions current, 10 kV acceleration voltage (V_{acc}), and UHR-A lens mode. Micrographs were taken at different magnification powers from several views for duplicate coupons.

4.2.10 Impact of the transposon insertion on the transcription of affected, and neighbouring genes in selected mutants

In this experiment, end-point RT-PCR was used to determine expression of interrupted genes, and downstream genes, in eight biofilm mutants. Mutants and Lm568 were grown statically in TSB-glu at 15°C until reaching $A_{450\ nm} \sim 0.8$. Two volumes of RNA stabilizer (RNAprotect bacterial reagent, Qiagen) was added to 1 volume of cell culture. Cells were treated with 20 mg/mL lysozyme (Roche, Mississauga, ON, Canada) and 20 µg/mL proteinase K (Roche) for lysis. RNA was extracted with the RNAeasy Minikit (Qiagen).

Prior to cDNA conversion, the genomic DNA was eliminated, using genomic DNA wipeout buffer (Qiagen), with two min incubation at 42°C. RNA was then converted to cDNA using a reverse transcriptase kit (Qiagen), with the reverse transcriptase reaction incubating for 25 min at 42°C, followed by inactivation of the reverse transcriptase enzyme (3 min at 95°C).

PCR was performed on the cDNA using primer sets (Table S3) designed to amplify (~200 bp) both the affected and adjacent downstream gene in each of the biofilm mutants and Lm568 strain. The primers were first confirmed to work on genomic DNA. The negative control was RNA after genomic DNA removal. Two positive primer controls were used; 16S rRNA and the motility gene *lmo0676* (*fliP* not impacted in any of the mutants) with the RT-PCR products.

The PCR (25 µL total volume) was performed using 0.5 µL 1.5 mM Mg^{2+}, 12.5 µL OneTaq Hot Start 2× master mix (New England Biolabs, Whitby, ON, Canada), 0.5 µL of each 10 µM primer, 10 µL H_2O and 1 µL of 1/10 diluted cDNA mixture (or

equivalent amount of RNA following genomic DNA removal that was not converted to cDNA). Temperature conditions for PCR were 5 min at 95°C for an initial denaturation step, followed by 30 cycles of 95°C for 30 s, 60°C for 45 s (54°C for 16S rRNA), 72°C for 60 s, and final extension for 5 min at 72°C.

4.2.11 Other phenotypic assays: motility and Congo red biofilm/slime formation

Biofilm mutants (section 4.2.5) and Lm568 (motile positive control) were stabbed in semi-solid TSA media (0.3% agar, Difco). Plates were examined after 72 hours growth at 15°C. Growth in a small zone surrounding the initial point of inoculation indicated reduced or absent motility.

To test the formation of biofilm/slime formation, all strains were streaked on Congo red agar, Brain Heart Infusion (Difco), 80 µg/mL Congo red (Sigma Aldrich, Canada), with or without 5% w/v sucrose, incubated at 15°C for 72 h. Strains with a production of slime (i.e. extracellular polysaccharides) yield red colonies (Chen et al., 2014, Kaiser et al., 2013).

4.2.13 Statistical analysis

In the secondary screening of mutants (section 4.2.6), statistical differences in stain absorbance values among strains were calculated using one way ANOVA (Dunnett's t-test) or t-tests, in Sigmaplot (Systat Software Inc.), at a significance level of 5%.

To compare the response of pre-formed biofilm to sanitizers among strains (section 4.2.7), differences between viable counts before and after sanitizer application were calculated for all strains (e.g., the viable count of the 24 h biofilm (control) minus the viable count after H_2O_2 treatment of the 24 h biofilm made by the same strain = Δ

Log CFU/cm^2 reduced). The Δ Log CFU/cm^2 reduced values were analyzed for significant differences among strains using the one-way ANOVAs with Tukey-Kramer post-hoc comparison ($p<0.05$).

The response of pre-formed biofilm to enzymes on polystyrene peg lids (section 4.2.8) was analyzed using the same method as for sanitizer application; significant differences in absolute biofilm formation were considered, as well as relative retention of biofilm following enzyme application.

4.3.0 Results

4.3.1 Initial selection of putative biofilm mutants

Screening of 11,024 transposon insertion mutants in the three assays identified 637 biofilm mutant candidates. The majority of mutants were identified by CV and/or Saf staining (281 and 346 mutants identified by those assays respectively), while 58 mutants were susceptible to BAC (Figure 4.1).

Of the 46 potential mutants that deviated from the parent strain in 2 (or 3) of the primary screening assays (Figure 4.1), 8 were eliminated from further study due to plasmid retention. The library of mutants overall had < 2.5% plasmid retention (data not shown), indicating that this much higher plasmid retention (17.4%) in putative biofilm/BAC mutants may be due to the pMC39 plasmid affecting biofilm formation, BAC sensitivity and/or reducing planktonic growth.
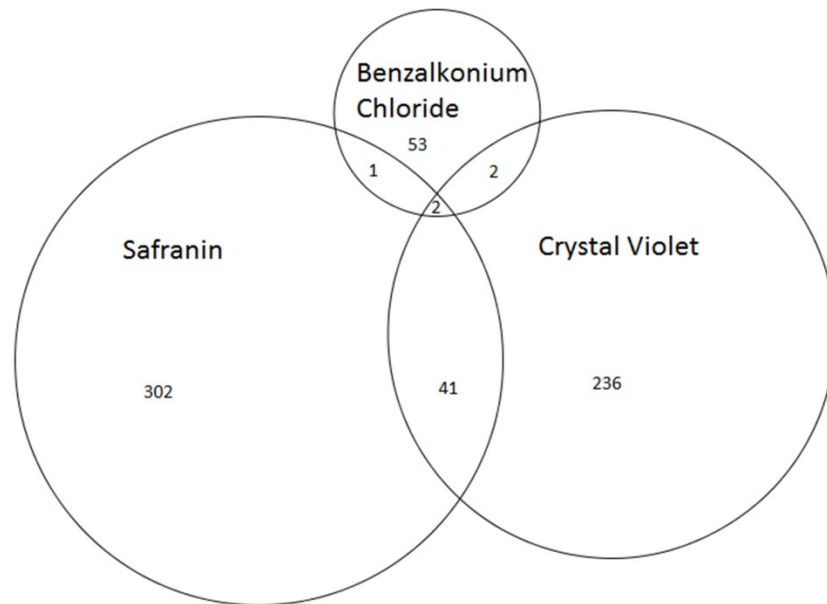
Figure 4.1. Potential biofilm mutants (637 total out of 11,024 screened insertional mutants) identified in crystal violet, safranin and benzalkonium chloride susceptibility microtiter plate assays, represented by proportional circles. Safranin staining identified 346 biofilm mutants, 281 biofilm mutants were identified in crystal violet staining, and benzalkonium chloride susceptibility was identified in 58 mutants. Overlap indicates the mutants whose phenotype deviated from the wild-type in two or three of the assays. (The figure was created in EulerAPE software, v3.0, Micallef and Rogers, 2014)

Fitting of the 4 parameter logistic model to the planktonic growth curves, which had been obtained for the putative biofilm mutants and the parent strain, resulted in an overall mean adjusted R^2 of 0.97 ± 0.02, MSE_{model} of 0.089 ± 0.002, and acceptable F values ($p<0.05$) for all strains. Eight mutants deviated significantly ($p<0.05$) from the growth kinetics of the parent strain (data not shown) and were omitted from further work.

Among the remaining mutants that were free of the plasmid, exhibited normal planktonic growth (n =30), and had the transposon insertion site identified (n =21), the secondary phenotypic screening with standardized cultures revealed 19 mutants which continued to express deviating phenotypes in regards to biofilm formation (CV and/or Saf assays) and/or BAC susceptibility.

4.3.2 Location of transposon insertion sites

DNA sequences flanking both the left and right transposon insertion sites were identified by arbitrary PCR in 10 mutants, while this technique only produced sequence data for one side of the transposon insertion site in the remaining 9 mutants. Use of alternate primers designed for the missing side of the affected gene (Table S2) in the 9 mutants together with the transposon specific primer succeeded in identifying the transposon insertion site. It should be noted that while unlikely, this approach does not rule out that two transposon insertions did not occur in these mutants.

The interrupted genes in enhanced biofilm forming mutants (111-135% of biofilm formed by Lm568, p<0.05, Table 4.1) included those related to motility (*flaA* and *cheR*), cell signalling (*lmo2563, lmo0531*), sorbitol and lipoprotein transport (*lmo0543, lmo1224*), DNA processing (*lmo2488*), and folate metabolism (*lmo2572*). Mutant lmo2526:Himar1 had an insertion downstream of *lmo2527* (hypothetical membrane protein) and 168 bp before the start codon of *lmo2526* (peptidoglycan synthesis).

Genes interrupted in enhanced biofilm forming mutants also included three internalins (*inlA*, *inlB*, *inlH*), an adhesion related gene (*lmo0453*), and a type VII secretion protein (*lmo0057*).

The deficient biofilm mutants (59-88% of biofilm formed by Lm568, Table 4.2) had insertions in genes responsible for teichoic acid metabolism (*lmo1080, lmo973*), peptidoglycan synthesis (*ami*), and lipoprotein assembly (*lmo2482*). A transposon insertion was found ~30 bp before the start codon of *lmo1068* (attachment related lipoprotein).

Table 4.1. Transposon insertion sites and gene functions for enhanced biofilm forming *L. monocytogenes* mutants.

Mutant Strain[a]	Predicted or known function of the interrupted gene	Insertion site mapped to *L. monocytogenes* EGD-e	% Biofilm formed in Safranin assay (Lm568 = 100%)
lmo2526:Himar1	peptidoglycan biosynthesis and shape function (*murA*)	2,605,323-324[b]	135*[c]
lmo2563:Himar1	zinc metalloprotease – signal and gene expression	2,643,318-322	129*
lmo0690:Himar1	flagellin A (*flaA*)	725,179-180	124*
lmo0683:Himar1	similar to chemotactic methyltransferase (*cheR*)	719,014-016	123*
lmo1224:Himar1	ABC-type transport system, involved in lipoprotein release	1,245,982-984	123*
lmo2488:Himar1	excinuclease ABC subunit A processing of DNA lesions (*uvrA*)	2,563,192-193	119
lmo2572:Himar1	similar to Chain A, Dihydrofolate Reductase	2,650,618-619	119*
lmo0543:Himar1	PTS sorbitol transporter subunit IIBC	581,827-829	117*
lmo0531:Himar1	c-di-GMP synthetase (diguanylate cyclase, GGDEF domain)	569,312-314	117*
lmo0433:Himar1	Internalin A. Bacterial adhesion/invasion protein (*inlA*)	456,789-790	112*
lmo0263:Himar1	Internalin H. Bacterial adhesion/invasion protein (*inlH*)	285,840-841	112
lmo0057:Himar1	type VII secretion protein N-terminal domain (*EsaA*)	62,118-119	111*
lmo0453:Himar1	uncharacterized conserved protein, possibly adhesion related	487,807-808	111
lmo0434:Himar1	Internalin B. Bacterial adhesion/invasion protein (*inlB*)	457,605-457,658	111

[a] Mutants are named according to the location of the Himar1 transposon mapped to the sequenced *L. monocytogenes* EGD-e (GenBank, accession number NC_003210). [b] insertion in the upstream region 168 bp from the lmo2526 start codon

[c] indicates a significant difference ($p<0.05$) from the wildtype in the safranin assay only.

Table 4.2. Transposon insertion sites and gene functions for deficient biofilm *L. monocytogenes* mutants.

Mutant Strain[a]	Predicted or known function of the interrupted gene	Insertion site mapped to *L. monocytogenes* EGD-e	% Biofilm formed in the CV assay (Lm568 = 100%)	BAC susceptible
lmo1080:Himar1	teichoic acid biosynthesis (*GgaB*)	1,114,681-720	88	No
lmo0973:Himar1	esterification of lipoteichoic acid /teichoic acid.	1,004,352-353	75*[b]	Yes
lmo2482:Himar1	prolipoprotein diacylglyceryl transferase (*lgt*)	2,557,759-760	67*	Yes
lmo2558:Himar1	autolysin amidase, peptidoglycan synthesis (*ami*)	2,635,298-300	65*	Yes
lmo1068:Himar1	hypothetical protein. May be an attachment factor (Tongkhao, 2011)	1,095,942-943[c]	59*	Yes

[a] Mutants are named according to the location of the Himar1 transposon mapped to the sequenced *L. monocytogenes* EGD-e (GenBank, accession number NC_003210).

[b] * indicates a significant difference ($p<0.05$) from the wildtype with crystal violet staining. Only mutants that were coded as deficient biofilm formers were also benzalkonium chloride susceptible.

[c] Transposon insertion ~30 bp before start codon of 1068.

4.3.3 Sanitizer susceptibility on stainless steel surfaces

Biofilms of different maturities were formed on stainless steel. While there were no differences in viable counts after 6 h, significant differences ($p<0.05$) occurred between the enhanced and deficient biofilm formers after 24 and 48 h (Table 4.3).

Treatment with H_2O_2 did not affect the enhanced and deficient biofilm formers and the parent strain differently ($p>0.05$). Generally, the strains became less susceptible to the sanitizers as the biofilm matured. This trend was significant ($p<0.05$) for the parent strain.

Cells in mature biofilms (48 h) made by the deficient mutants were significantly ($p<0.05$) more susceptible to treatments with BAC than Lm568, while the enhanced mutants did not significantly ($p>0.05$) differ from the parent strain. Increasing biofilm maturity reduced in all cases the lethal effect of the sanitizer significantly ($p<0.05$).

Table 4.3. Biofilm formed by *L. monocytogenes* and its biofilm insertion mutants on stainless steel coupons, and biofilm sensitivity to treatment with H_2O_2 and benzalkonium chloride.

	Lm568	Enhanced mutants (14)	Deficient mutants (5)
Biofilm formation (Log CFU/cm^2)[a]			
6 h	4.1± 0.1 A[b]	4.1± 0.4 A	3.9± 0.6 A
24 h	5.4± 0.1 ab B	5.9± 0.4 a B	5.3± 0.6 b B
48 h	6.8± 0.0 ab C	7.1± 0.4 a C	6.7± 0.4 b C
Effect of H_2O_2 (Δ Log CFU/cm^2)[c]			
6 h	1.1± 0.3 (91.3[d]) A	0.5± 0.5 (68.6) A	0.6± 0.8 (74.8) A
24 h	0.4± 0.2 (58.3) AB	1.1± 0.9 (92.4) A	0.8± 0.7 (85.7) A
48 h	0.1± 0.1 (25.7) B	0.6± 0.4 (72.4) A	0.3± 0.5 (54.2) A
Effect of BAC (Δ Log CFU/cm^2)[c]			
24 h	2.8± 0.0 (99.8[d]) A	2.3± 1.0 (99.5) A	2.4± 0.5 (99.6) A
48 h	0.1± 0.0(21.3) b B	0.6± 0.4(76.2) ab B	0.8± 0.5 (83.5) a B

[a] Average Log CFU/cm^2 ± standard deviation (n-1).

[b] Significant (p<0.05) differences by the Tukey Kramer test among the Log CFU/cm^2 results are indicated by different small letters to compare across rows and capital letters to compare within the columns for each treatment.

[c] The reduction in Log CFU/cm^2 ± standard deviation (n-1) following sanitizer treatment of the biofilm as compared to the untreated biofilm of the same maturity (i.e., 6, 24 and 48 h).

[d] Numbers in parentheses indicate the percentage reduction of viable cells between the untreated and treated biofilm of the same maturity.

4.3.4 Enzymatic removal of biofilms pre-formed on polystyrene peg lids

Biofilm deficient mutants formed no detectable levels of biofilm on polystyrene peg lids (Table 4.4). Interestingly four of the enhanced mutants (mutants lmo0690:Himar1, lmo0683:Himar1, lmo2563:Himar1 and lmo2488:Himar1) were also incapable of forming biofilm on peg lids. Therefore the effect of hydrolytic enzymes on pre-formed biofilms could not be determined for these mutants.

Among the enhanced biofilm forming mutants, determined in the microtiter assay, lmo2526:Himar1, lmo0453:Himar1, and lmo0433:Himar1 formed significantly ($p<0.05$) less biofilm than Lm568, while the biofilm formation by mutants lmo1224:Himar1 and lmo0543:Himar1 remained significantly ($p<0.05$) increased compared to Lm568 in the polystyrene peg lid assay with CV staining.

Washing of the biofilms on the peg lids with the acidic APB instead of PBS generally reduced the amount of remaining biofilm. Mutants lmo1224:Himar1, and lmo0543:Himar1 continued to retain a greater ($p<0.05$) biofilm mass on the pegs in comparison to the parent strain (Table 4.4).

Using low concentrations of the hydrolytic enzymes as a tool to probe the composition of the *L. monocytogenes* mutant biofilms, it was observed that mutants lmo2526:Himar1, and lmo0433:Himar1, retained significantly ($p<0.05$) more biofilm than Lm568 after a proteinase K treatment (Table 4.4). Mutants lmo2526:Himar1, lmo0057:Himar1, lmo0453:Himar1, and lmo0531:Himar1 were resistant to removal with

DNase. Mutants whose biofilms could be removed by pectinase included lmo2572:Himar1, lmo0057:Himar1, lmo0453:Himar1, and lmo0434:Himar1.

The parent strain's biofilm was significantly ($p<0.05$) more resistant to proteinase K and DNase, than to pectinase activity, which resulted in the removal of 28% of the biofilm mass (Table 4.4). Significantly ($p<0.05$) more mutant lmo0433:Himar1 biofilm was removed by DNase than by proteinase K. Biofilms made by mutants lmo0057:Himar1, lmo0453:Himar1, and lmo0434:Himar1 were all significantly ($p<0.05$) more sensitive to the pectinase treatment than to the other enzymes.

Despite its formation of excess biofilm, mutant lmo1224:Himar1 demonstrated poor overall biofilm retention following exposure to the hydrolytic enzymes compared to Lm568. Mutant lmo0434:Himar1 formed similar levels of biofilm relative to Lm568, however, it showed decreased ($p<0.05$) overall resistance to the panel of enzymes. In contrast, biofilms made by mutants lmo2526:Himar1 and lmo0531:Himar1 were significantly ($p<0.05$) more resistant to enzymatic removal compared to Lm568, with less than 10% of pre-formed biofilm being removed by any of the enzymes.

Table 4.4. Biofilm formation of *L. monocytogenes* 568 (Lm568) and its biofilm insertional mutants on polystyrene peg lids, and biofilm removal following treatments with buffers containing Proteinase K, DNase I, or Pectinase (in acidified phosphate buffer).

Strain	Relative biofilm formation [a]	Biofilm with APB [a]	Biofilm retained after enzyme treatment [b]			Overall Biofilm Retention [c]
			Proteinase K	DNase	Pectinase	
lmo2526:Himar1	69* de[d]	70 cd	122* ab	99* ab	124 a	345* a
lmo2563:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a
lmo0690:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a
lmo0683:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a
lmo1224:Himar1	210* a	157* a	74 cd	73 bc	70 bce	217* de
lmo2488:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a
lmo2572:Himar1	75 ce	77 c	57 d	69 bc	41* e	167* f
lmo0543:Himar1	149* b	119* b	84 bcd	66 c	89 acd	239 cd
lmo0531:Himar1	93 cd	73 cd	94ad	108* a	93 ac	295* b
lmo0433:Himar1	44* ef	37* de	128*A[e] a	58 B c	80 AB ae	266 bc
lmo0263:Himar1	93 cd	70 cd	63 d	79 ac	89 ae	232 cd
lmo0057:Himar1	111 bc	85 bc	108 A ac	98*A ab	60*B ce	265 bc
lmo0453:Himar1	71* de	91* bc	98 A ad	99*A ab	48*B ae	244 cd
lmo0434:Himar1	96 cd	86 bc	76 A cd	74 A bc	43*B de	193* ef
Lm568	100 cd	64 cd	82 AB cd	72 A c	103 B ab	257 c
lmo1080:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a
lmo0973:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a
lmo2482:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a
lmo2558:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a
lmo1068:Himar1	<13* f	<13* e	n/a	n/a	n/a	n/a

[a] The absorbance of stain recovered from Lm568 biofilm treated with PBS was used as the reference (= 100%) for the biofilm formed by all strains.

[b] For each enzyme treatment, the percentage of biofilm remaining was calculated relative to the individual strain's biofilm formation in the buffer without enzyme.

[c] Overall biofilm retention is the sum of the percentage biofilm remaining after enzyme treatments for each strain.

< 13% = below the detection limit in PBS or APB, equivalent to 2 times the average standard deviation of the negative control measurements.

[d] Significant differences ($p<0.05$) between mutant strains and Lm568 are marked with a * within each column, while lower case letters indicate significant differences between strains ($p<0.05$).

[e] Significant differences ($p<0.05$) in biofilm remaining between enzyme treatments for each strain are marked with upper case letters within a row.

4.3.5 Scanning electron microscopy of mature biofilm on stainless steel

Electron microscopy was used to visualize the biofilms formed by seven select biofilm mutants on SS coupons after incubation for 6 days at 15°C. The parent strain was observed to form a thick monolayer of biofilm after 6 days with visible microcolonies as well as threadlike attachments to the SS surface (Figure 4.2d).

The immotile mutant lmo0690:Himar1 formed a thick monolayer of cells, without large microcolonies and bare spots on the SS surface (Figure 4.2a). Unlike Lm568, the bacterium lacked threadlike appendages or flagella. Close-up images of another immotile mutant lmo0683:Himar1 revealed presence of appendages or flagella attached to the surface (Figure 4.2b). Biofilm coverage was heterogeneous with large microcolonies, areas with thick monolayers of cells and well defined empty zones on the coupons.

In contrast, the enhanced biofilm mutant lmo0531:Himar1 covered the SS surface with a heterogeneous monolayer of cells and appeared normally attached to the SS surface with threadlike appendages (Figure 4.2c).

Among the biofilm deficient mutants, Lmo0973:Himar1 formed a sparse biofilm monolayer after 6 days (Figure 4.3b), as did mutant lmo1080:Himar1 (Figure 4.3a), and Lmo2482:Himar1 (Figure 4.3c), where, however, some large microcolonies were visible. Mutant lmo1068:Himar1 formed an irregular and sparse monolayer of cells with some large microcolonies interdispersed (Figure 4.3d).
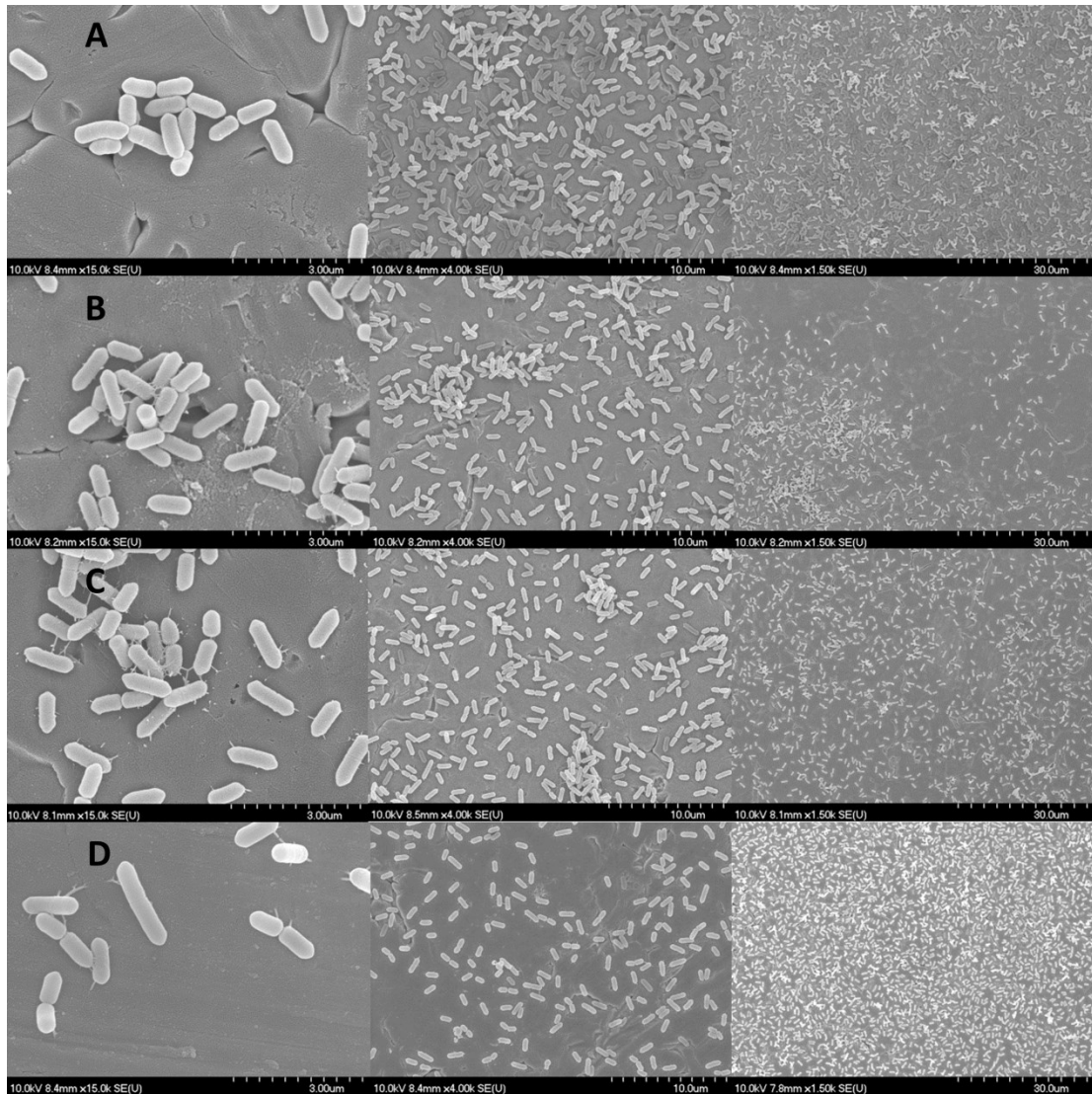
Figure 4.2. SEM images of biofilm formed on SS coupons (6 d, 15°C) by mutants lmo0690:Himar1, lmo0683:Himar1, lmo0531:Himar1, and Lm568, rows A-D respectively.
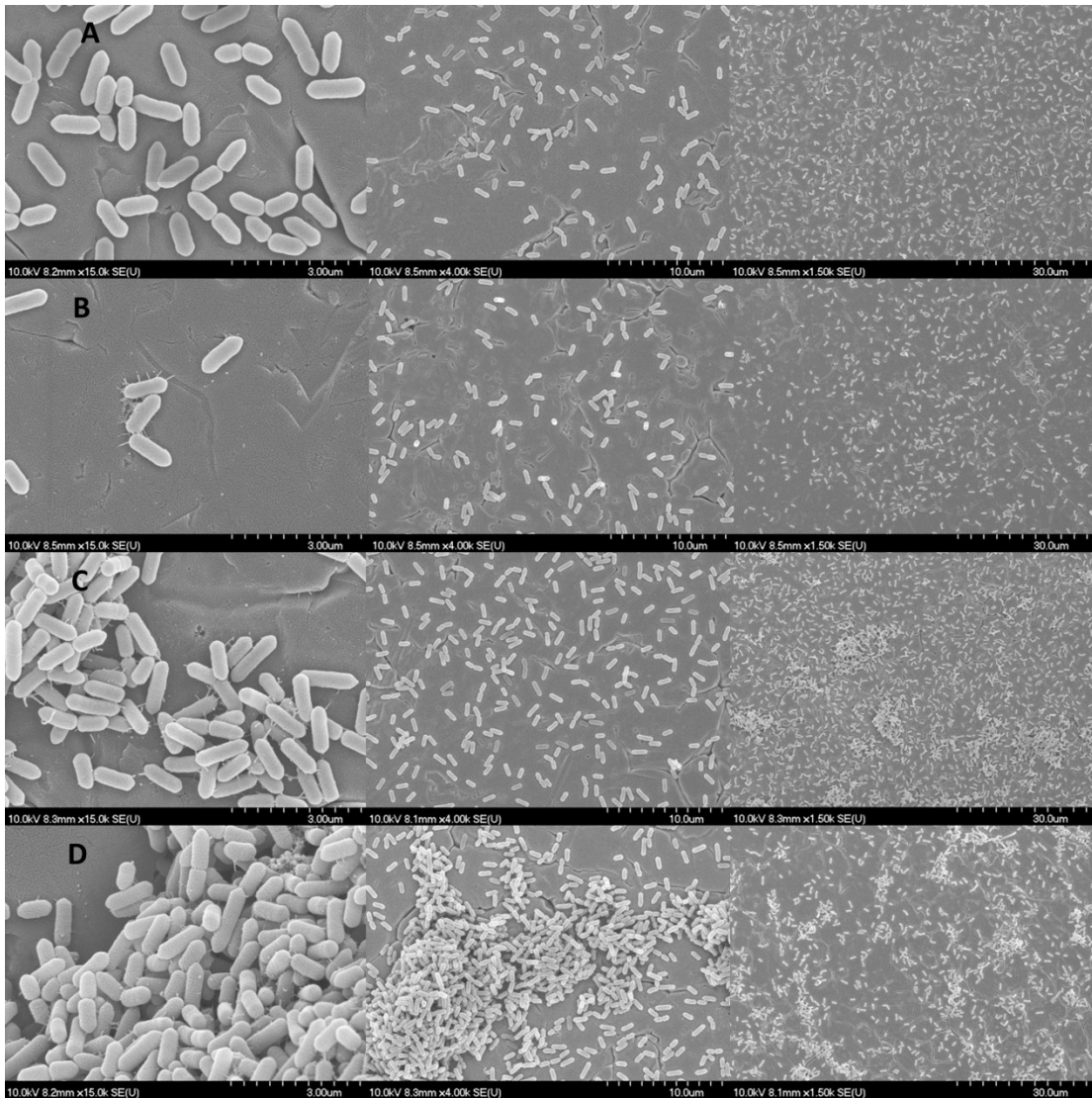
Figure 4.3. SEM images of biofilm formed on SS coupons (6 d, 15°C) formed by mutants lmo1080:Himar1, lmo0973:Himar1, lmo2482:Himar1, and lmo1068:Himar1, rows A-D respectively.

4.3.6 Transcriptional analysis to determine polar effects of the transposon insertion

Downstream genes were expressed in 7 of 8 selected mutants (Table 4.5), with only mutant lmo2482:Himar1 showing disruption of downstream gene expression (*lmo2481,* pyrophosphatase *PpaX*).

In mutant lmo0531:Himar1, the upstream genes (lmo527-0530) encoded in the operon containing lmo0531 were all expressed (Table S3).

Mutant lmo0433:Himar1 expressed an N terminal RNA segment upstream of its transposon insertion, while lmo0263:Himar1 did not. Neither the parent strain nor mutant lmo0434:Himar1 expressed the N terminal region of *lmo0434* during planktonic growth (data not shown).

Table 4.5. Bands representing RNA expression in selected transposon interrupted and neighbouring genes.

Strain	Affected Gene	Downstream Gene	Control genes		Affected gene expression		Downstream gene expression	
			16S rRNA	*fliP*	mutant	Lm568	mutant	Lm568
lmo0690:Himar1	lmo0690	lmo0691			-			
lmo0683:Himar1	lmo0683	lmo0684			-			
lmo1224:Himar1	lmo1224	lmo1225			-			
lmo0531:Himar1	lmo0531	lmo0532			-			
lmo1080:Himar1	lmo1080	lmo1081			-			
lmo0973:Himar1	lmo0973	lmo0972			-			
lmo2482:Himar1	lmo2482	lmo2481			-		-	
lmo1068:Himar1	lmo1068	lmo1069			-			

4.3.7 Motility and Congo Red agar assay

Only mutants lmo0690:Himar1 and lmo0683:Himar1, were found to have impaired motility in semi-solid media at 15°C.

Red colonies characteristic of slime or increased biofilm formation (Chen et al., 2014) were not observed for any of the biofilm forming mutants nor the parent strain.

4.4 Discussion

In this study, insertional mutagenesis of Lm568 led to the identification of 19 loci with putative roles in biofilm formation.

4.4.1 Impaired motility is associated with enhanced biofilm formation under static conditions

Disruptions in flagella related genes (lmo0690 (*flaA*) and lmo0683 (*cheR*)), resulted in non-motility and increased biofilm formation in the static microtiter (safranin staining) and SS coupon assays, while no biofilm formed in the peg lid assay. This suggests motility is required for biofilm formation on surfaces hanging into a liquid medium. In contrast, Alonso et al. (2014) and Chang et al. (2012) found *L. monocytogenes* motility mutants formed less biofilm than the parent strain in microtiter plates with CV staining at 35 and 32°C, respectively. This discrepancy could be explained by differences in staining (CV vs. safranin), assay temperature, strains, or inadvertent removal of poorly attached thick biofilms by vigorous rinsing prior to staining. Todhanakasem and Young (2008) found hyperbiofilm formation in motility mutants after an initial attachment under static conditions and subsequent biofilm formation under flow conditions which according to Klausen et al., (2003) would be classified as laminar. Interestingly, it was also observed that immotile *Staphylococcus aureus* did not form biofilm in the peg lid assay at 15°C (d'Entremont, 2015). The current

study demonstrates that motility is not critical for biofilm formation on top of a flat surface but may be necessary for formation of biofilms on "hanging" surfaces.

4.4.2 Disruptions in DNA related genes led to enhanced biofilm formation under static conditions

Transposon insertions in genes *lmo2572*, and *lmo2488* created mutants that produce excess biofilm in the microtiter plate assay (safranin staining), and decreased biofilm in the peg lid assay. The product of the lmo2572 gene (dihydrofolate reductase) is important in folate metabolism, and subsequent purine and pyrimidine biosynthesis. In *Streptococcus mutans*, dihydrofolate reductase inhibitors have been found to inhibit biofilm formation in a microplate assay with CV staining (Zhang et al., 2015). Using a similar assay, Alonso et al. (2014) found disruptions in several purine biosynthesis genes caused reduced biofilm formation in *L. monocytogenes*. Interestingly, *Streptococcus sanguis* biofilm cells were found to bind antibodies raised to an enzyme (dihydrofolate synthetase) from the same pathway, indicating its possible presence on the cell and/biofilm surface (Black et al., 2004). Harmsen et al. (2010) described the role of extracellular DNA (eDNA) in *L. monocytogenes* in the early stages of cell attachment to surfaces, and reported that N-acetylglucosamine acted as a co-factor and/or scaffolding in eDNA formation. In this context, it should be noted that safranin binds to glycosaminoglycans (Kiviranta et al., 1985). One may hypothesise that this mutant has reduced eDNA secretion due to the disrupted folate metabolism, leading to increased safranin staining of the N-acetylglucosamine that would normally be bound to eDNA. An altered EPS profile was also indicated by the mutant biofilm being more sensitive to the panel of hydrolytic enzymes, especially pectinase, than its parent strain.

Lmo2488 (*uvrA*) is involved in DNA excision and repair. In terms of related SOS

response system genes, a *recA* mutation in *Pseudomonas aeruginosa* was found to

increase the number of dead cells within the biofilm (Boles and Singh, 2008). It may be

that disruption of *uvrA* similarly caused presence of excess dead cells in lmo2488:Himar1

biofilms to increase staining in the microtiter plate assay and poor biofilm formation in

the peg lid assay. Other workers (Chang et al., 2013) found disruptions in a putative

DNA translocase (lmo1368) reduced biofilm formation in *L. monocytogenes* in a CV

microtiter plate assay at 32°C indicating genes involved in DNA metabolism affect

biofilm formation differently.

4.4.3 Disruption of biofilm regulatory genes may enhance biofilm formation

Mutant lmo0531:Himar1 formed excess biofilm in the microtiter plate assay, with

the biofilm formed on the peg lids being resistant overall to the panel of enzymes. SEM

data shows heterogeneous, but not sparse biofilm on SS. The transposon insertion in this

mutant was mapped to a gene predicted to being involved in regulating EPS formation

(Köseoğlu et al., 2015). Chen and colleagues (2014) determined that the absence of

lmo0531 in *L. monocytogenes* EGD-e resulted in reduced biofilm formation at 30°C,

while the current study found the opposite effect at 15°C. RNA expression experiments

confirmed that the genes both upstream (lmo0527-0530, responsible for EPS biosynthesis

according to Chen et al., 2014) and downstream of lmo0531 were not affected by the

transposon insertion, indicating the absence of polar effects in the mutant. In *Yersinia*

pestis, a similar biofilm activator, hmsT (25% protein homology to lmo0531, Chen et al.,

2014), is degraded at 37°C, but not at 25°C (Perry et al., 2004). At 37°C, the hmsD

regulator in *Y. pestis* ensures biofilm forms in the absence of hmsT (Bobrov et al., 2015).

It seems possible that in the absence of lmo0531 expression, a currently unidentified homologue of hmsD or another activator could be present at 15°C, causing the formation of excess biofilm.

The permease component of an ABC type transporter system was interrupted in mutant lmo1224:Himar1. Disruption of another ABC transporter permease (lm.G_1771) has similarly been found to enhance biofilm formation in *L. monocytogenes* (Zhu et al., 2008), and both of these genes share an FstX-like permease family domain. Later it was shown that absence of the lm.G_1771 transport gene increased levels of superoxide dismutase (SOD), leading to cells in denser biofilms surviving greater oxidative stress (Suo et al., 2012). The mutant in the current study may have altered biofilm phenotype due to a change in gene regulation, or altered transport.

4.4.4 Internalins affect biofilm formation

Mutant lmo0433:Himar1, which formed excess biofilm in the microtiter plate assay with safranin staining, but reduced biofilm formation in the peg lid assay, harboured an insertion in the internalin A gene, one of several internalins involved in adhesion and internalization into eukaryotic cells (Cabanes et al., 2002). Interestingly, Franciosa et al. (2009) found increased biofilm formation by strains of *L. monocytogenes* with truncated *inlA* proteins. Truncated inlA proteins are common in food related *L. monocytogenes* strains, although these tend to have attenuated virulence (Jacquet et al., 2004, Nightingale et al., 2005, Kovacevic et al., 2013). Orsi et al. (2010) suggest that 'generalist' *L. monocytogenes* strains have evolved an *inlA* sequence that is more likely to become reversibly truncated compared to disease associated strains, which may lead to an advantage in adapting to certain environmental niches.

Mutants lmo0263:Himar1 and lmo0434:Himar1 contained the transposon disruptions in internalin H and internalin B, respectively. Like internalin A, internalins H and B have a leucine rich repeat (LRR) domain, and an LPXTG domain that covalently anchors the protein to the cell wall. Both lmo0434:Himar1 and lmo0263:Himar1 formed excess biofilm in the microtiter plate assay, but similar amounts of biofilm to the parent strain in the peg lid assay. The *inlB* mutant produced biofilm that is pectinase sensitive, while the *inlH* mutant is very similar to the parent strain with respect to its enzymatic removal. The absence of internalin J (another non secreted internalin) in *Porphyromonas gingivalis* reduces or enhances biofilm formation, depending on the conditions (Capestany et al., 2006). Proteins with LRR domains can have multifunctional roles including signal transduction, which may also influence biofilm growth (Capestany et al., 2006). That research is consistent with the present study that found disruptions in internalins A, B or H altered biofilm formation in Lm568.

4.4.5 Alterations in other genes may increase biofilm formation

Mutant lmo0543:Himar1 had an insertion in a sorbitol PTS transport gene, but sorbitol is not thought to be an EPS component in *L. monocytogenes* (Köseoğlu et al. 2015). In *Streptococcus mutans*, knock-outs of PTS system genes cause widespread changes in expression of many genes (Abranches et al., 2006), and PTS gene interruptions have been found to cause biofilm overproduction in *Streptococcus pneumoniae* (Muñoz-Elías et al., 2008). *L. monocytogenes* appears to respond in a similar manner as the PTS mutant exhibited excess biofilm formation on the peg lids. Mutant lmo0057:Himar1 had the disruption in a type VII secretion gene; with homology to *EsaA*; part of an extracellular protein secretion pathway in *S. aureus* that similarly to

the internalins contributes to virulence and intracellular infection (Anderson et al., 2011; Korea et al, 2014). Mutant lmo0057:Himar1 forms biofilm in the peg lid assay, which is difficult to remove with proteinase K and DNase indicating disruption of lmo0057 does not abolish EPS production.

Mutant lmo2563:Himar1 harboured the transposon disruption in a gene which has been hypothesized to encode a membrane bound peptidase responsible for signalling and stress response (Bierne and Cossart, 2007). This family of S2P M50 metalloproteases is known to regulate sporulation, stress response and cell division in prokaryotes (NCBI, 2015). In *S. aureus*, the deletion of the metalloprotease *aur* was found to disrupt biofilm detachment function and result in high levels of biofilm (Boles and Horswill, 2008).

The disruption in mutant lmo0453:Himar1 was located in a gene that encodes a protein with a Von Willebrand protein domain commonly associated with the formation of multiprotein complexes, and potentially adhesion (Whittaker and Hynes, 2002). This gene is not well characterized with respect to its role in biofilm.

The gene lmo2526 (*murA*), is controlled by the SecA2 pathway, and its absence is known to alter *L. monocytogenes* biofilm architecture (Machata et al., 2005, Renier et al., 2014). Similar to our observations at 15°C (data not shown), disruptions in *murA* caused long chains of cells to form at 25°C , and cells to sediment in planktonic culture (Renier et al., 2014), which could be consistent with the excess biofilm formation observed in our microtiter plate assay. In the peg lid assay, lmo2526:Himar1 biofilm is relatively proteinase K and DNase resistant, despite forming low levels of biofilm, indicating it has altered EPS production. Cell aggregation may also be responsible for poor biofilm formation on the pegs.

4.4.6 Deficient biofilm formation was associated with disruptions in peptidoglycan and teichoic acid biosynthesis genes

The interrupted gene in mutant lmo1080:Himar1 is teichoic acid biosynthesis related. Eugster et al. (2015) noted that disruptions in lmo1080 abolished teichoic acid rhamnosylation. In *S. aureus*, disruptions in teichoic acid biosynthesis genes have been found to reduce biofilm formation (Vergara-Irigaray et al., 2008). Increased hydrophobicity and poor adhesion in teichoic acid mutants have also been reported in *Staphylococcus epidermidis* (Holland et al., 2011). SEM images showed mutant lmo1080:Himar1 formed sparse biofilm on SS, which is consistent with the literature. The interruption in mutant lmo2558:Himar1 was located in an autolysin amidase, which is involved in peptidoglycan turnover, interaction with lipoteichoic acid on the bacterial surface and adhesion to eukaryotic cells (Milohanic, et al., 2001; Cabanes et al., 2002). Kumar et al. (2009) also determined that a mutation in lmo2558 reduced *L. monotyogenes* biofilm in a microtiter assay, as well as caused sparse biofilm formation on glass. A *S. aureus* strain with a deletion in the lmo2558 homologue *atl* was also reported to be a poor biofilm former (Biswas et al., 2006). Overall, the literature supports that poor attachment may contribute to reduced biofilm formation in this mutant.

Mutant lmo0973:Himar1, which shows sparse biofilm in SEM and is a poor biofilm former in both assays and sensitive to BAC, contained the insertion in one of two genes in *L. monocytogenes* responsible for the esterification of D-alanine in teichoic acid biosynthesis. Ouyang et al. (2012) noted that a mutation in *dltD* (the other D-alanine esterification gene) also negatively affected biofilm formation. In *Staphylococcus aureus*, the absence of D-alanine esters in teichoic acid reduced the ability to colonize

polystyrene or glass, possibly due to altered surface charge affecting adhesion, or due to

surface charge affecting the folding of exoproteins (Gross et al., 2001).

4.4.7 Changes in lipoproteins were associated with poor biofilm formation

Gene lmo2482 encodes a prolipoprotein diacylglyceryl transferase (*lgt*). The

deletion of *lgt* in *L. monocytogenes* caused numerous unlipidated proteins to be

extracellularly secreted, including several ABC transport system proteins (Baumgärtner

et al., 2007), which could affect biofilm formation. Disruption of lipoprotein *Nlpl* in *E.*

coli is associated with poor biofilm formation due to reduced eDNA production

(Sanchez-Torres et al., 2010). Under flow conditions, *Streptococcus gordonii* mutants

that lack lipoprotein *adcA* are biofilm deficient, but that was not observed under static

conditions (Mitrakul et al., 2005).

The interrupted gene in mutant lmo1068:Himar1 encodes a lipoprotein (Baumgärtner et

al., 2007) considered important in attachment and biofilm formation (Tongkhao, 2011).

Consistent with that research, the mutant is notably the most deficient biofilm former in

the microtiter plate assay, and did not form biofilm on pegs.

4.5 Conclusions

This study, carried out at a temperature common in food production environments

revealed the involvement of nine genetic loci not previously linked to biofilm formation

in *L. monocytogenes*, as well as 10 genes also known to be associated with *L.*

monocytogenes biofilm formation at higher temperatures. The 9 novel biofilm genes

(*lmo2572, lmo2488 (uvrA), lmo1224, lmo0434 (inlB), lmo0263 (inlH), lmo0543,*

lmo0057 (EsaA), lmo2563, lmo0453) all led to enhanced biofilm formation in the

microtiter plate assay using safranin.Future studies to characterize the affected genes will

help identify biofilm formation mechanisms, and lead to improved processes to control

Listeria in food processing environments.

Chapter 5: Variations in Biofilm formation, Desiccation Resistance, and Sanitizer Susceptibility Among a Panel of Canadian *Listeria monocytogenes* Strains of Environmental, Food Processing, and Outbreak Origin

Abstract

Listeria monocytogenes is a pathogenic foodborne microorganism noted for its ability to persist for long periods in the environment and food processing facilities. Persistence has been attributed to the bacterium's ability to form biofilms but may also be related to desiccation resistance.

The objective of this study was to investigate if the ability to form biofilm and resist desiccation and sanitizer treatments varied among a panel of 14 *L. monocytogenes* strains isolated from diverse environmental niches.

Pre-formed biofilms of 14 outbreak and environmental *L. monocytogenes* strains (serogroups IIa and IIb) were desiccated on stainless steel coupons at ~23% relative humidity at 15°C. Viable counts were determined over the course of 7 days. The presence of genetic markers indicative of strains harbouring the stress survival islet (SSI-1), the 50 kbp *Listeria* genomic island (LGI1), and clonal complex 8, virulence type 59 (CC8/VT59) associated with previous Canadian outbreaks, were determined by PCR.

The most desiccation susceptible strains contained none of the SSI-1 and LGI1 markers, while the majority of strains exhibiting desiccation resistance were harboured at least one of the LGI1 and SSI-1 markers. All CC8/VT59 strains contained both the LGI1 and SSI-1 markers, and several demonstrated no decrease in survivors over the entire desiccation period. Benzalkonium chloride resistance was greater in planktonic cells of CC8/VT59 isolates compared to non CC8/VT59 isolates, and higher concentrations of benzalkonium chloride were needed to inhibit the formation of biofilm in CC8/VT59 strains after 48 h and 6 days compared to non CC8/VT59 isolates. In conclusion, LGI1 containing CC8/VT59 isolates may pose a challenge for sanitation efforts.

5.1 Introduction

Listeria monocytogenes is a pathogenic bacterium that causes the foodborne illness listeriosis. It mostly affects pregnant women, the elderly, and immunocompromised persons (Donnelly, 2001). Many *L. monocytogenes* strains are capable of adhering to and subsequently forming biofilms on a variety of surfaces, which is known to enhance the resistance of cells to sanitizers, or hinders physical removal (Carpentier and Cerf, 2011; Lourenço et al., 2011). *L. monocytogenes* may enter food processing facilities via agricultural commodities (Todd and Notermans, 2011), or water (Weller et al., 2015b), however the contamination of foods during processing is thought to lead to the majority of *L. monocytogenes* outbreaks (Oliver et al., 2007).

An individual strain of *L. monocytogenes* can be considered persistent if it is isolated from the same food processing facility over a long period of time (Ferreira et al., 2015). Persistent strains have been found to have better initial attachment to surfaces (cells/cm^2) when compared to transient strains (Lundén et al., 2000), and persistent strains have been shown to form higher levels of biofilm initially, but not to exhibit significant differences in biofilm formation with longer incubation periods (Wang et al., 2015). Some non-persistent (i.e., transient) strains also show high levels of initial attachment, and with time, differences in initial attachment between persistent and transient strains become less pronounced (Lundén et al., 2000).

Differences in sanitizer susceptibility have also been reported between persistent and transient *L. monocytogenes* strains for planktonic cells (Aase et al., 2002), while in contrast other researchers found no differences (Holah et al., 2002, Harvey et al., 2007).

The literature similarly contains opposing observations when it comes to biofilms, as Kastbjerg and Gram (2010), and Wang et al. (2015) both found no differences in sanitizer susceptibility of biofilms made by persistent and transient *L. monocytogenes* strains, respectively, while Nakamura et al., (2013) reported finding significant differences.

Where persistent strains may not always have an advantage in biofilm formation, or sanitizer resistance, other mechanisms that influence survival and growth should be considered. *L. monocytogenes* can survive desiccation for weeks or months on stainless steel surfaces (Vogel et al., 2010, Takahashi et al., 2011). The resistance of pre-formed biofilm to desiccation also demonstrates another protective effect of biofilm formation (Hingston et al., 2013). The presence of the stress survival islet (SSI-1) is known to influence growth at temperatures common to food processing facilities ($<20°C$) and survival under conditions of acid and osmotic stress (Ryan et al., 2010). The SSI-1 cluster of genes contains a bile tolerance locus, part of a glutamate decarboxylase acid resistance system, and a transcriptional regulator. The *Listeria* Genomic Island 1 (LGI1) is a 50 kbp horizontally acquired genetic feature that has been isolated from clinical and food processing *L. monocytogenes* strains (Gilmour et al., 2010). It encodes a putative peptidoglycan hydrolase, an adhesin, which may influence biofilm formation, as well as type IV secretion system genes, a multidrug efflux pump, a MarR transcription regulator, and a signal transduction system.

With respect to Canadian listeriosis cases, a recent study has implicated the clonal complex 8 virulence type 59 (CC8/VT59) group in many outbreaks, covering a nearly 30 year timeframe (Knabel et al., 2012). Verghese et al. (2011) has also found a CC8/VT59 strain of Canadian origin formed more biofilm than others isolated from meat and poultry

processing facilities. Knabel et al. (2012) suggests that the CC8 group shares a common ancestor, and that it is both virulent and environmentally successful.

The objectives of this research were to compare a panel of 14 Canadian *L. monocytogenes* strains with respect to biofilm formation, benzalkonium chloride sensitivity, and desiccation resistance in relation to the presence or absence of the Stress Survival Islet (SSI-1) and *Listeria* Genomic Island 1 (LGI1) genetic markers. Strains selected for this study included three clinical isolates, six isolates from the meat and fish processing industry and five isolates from fresh water, where five of the 14 isolates belonged to the CC8/VT59 group (Knabel et al., 2012).

5.2 Materials and methods

5.2.1 Bacterial strains and culture conditions

Canadian *L. monocytogenes* strains 08-5578, 01-7210, 08-7376, 08-6040, 06-6956, and 06-6837 were generously donated by Dr. Gilmour of the National Microbiology Laboratory, Public Health Agency of Canada (Winnipeg, MB, Canada), and are fully described in Knabel et al. (2012), and Gilmour et al. (2010). Meat and seafood processing associated strains (collected 1999- 2002 by the Canadian Food Inspection Agency, Dartmouth, NS, Canada, described by Loder, 2006) LmG, NB1, and Lm568 (described in Kalmokoff et al., 2001) were also selected. Five urban watershed *L. monocytogenes* strains were collected in the province of Nova Scotia between 2012-2013 as described by Stea et al. (2015), and designated 'CP' (Table 5.1). Routine culturing was carried out in Tryptic Soy Broth (TSB, Oxoid, Nepean, ON, Canada), or TSB with 1%

glucose (Fisher Scientific, Whitby, ON, Canada) (TSB-glu), or TSB with 1.5% (w/w) technical agar (Difco).

5.2.2 PCR based method to determine to determine serogroup

A multiplex PCR method based on the work of Doumith et al. (2004) and Kérouanton et al. (2010) was performed determine the serogroup of the watershed *L. monocytogenes* isolates (Table 5.1). The serogroup (and serotype) of all other isolates had previously been determined. PCR was performed in a Biometra T-Gradient thermocycler (Biometra, Goettingen, Germany) using the following temperature program: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 53°C for 45 s and extension at 72°C for 75 s, with a final extension at 72°C for 7 min. Each colony PCR reaction (25 μL) consisted of 0.5 μL of colony mass, 1 U taq DNA polymerase (IDTaqtm Taq Polymerase kit, London, ON, Canada), 1× $MgCl_2$ free buffer, 3 mM $MgCl_2$, 1 mM dNTPs, 0.4 μM of each of the lmo737 F/R, lmo1118F/R, orf 2819 F/R and orf2110 F/R; and 0.2 μM of the LIP1/LIP2a (*prfA* F/R) primer sets (Table S6) and DPEC water.

The amplified PCR products were visualized on 2% agarose (Fisher Scientific, Oakville, ON, Canada) gels, containing 0.016% (v/v) GelRed (Biotium, Hayward, CA, USA) for visualization. The gel band patterns were then used to confirm the identity and assign the *L. monocytogenes* isolates to their respective serogroups.

5.2.3 PCR based method to determine the presence of markers for the Stress Survival Islet (SSI-1), *Listeria* Genomic Island 1 (LGI1), and CC8/VT59 clonal complex

DNA was extracted using the Triton X-100 method. A mixture of 5 mM

Tris/EDTA with 1% Triton X100 (Sigma Aldrich) was added to overnight cultures grown

in TSB. Samples were vortexed, boiled at 95°C for 30 minutes, and cooled on ice. The

supernatant recovered after centrifuging (10 min, 10,000 x *g*) was then frozen (-20°C) for

later use.

Primer sets lmo0448, representing the SSI-1 (Ryan et al., 2010), ECV 2229,

representing the CC8/VT59 group (Knabel et al., 2012), and 1886, representing the 50

kbp LGI1 island, (Knabel et al., 2012) were run in separate PCR reactions (primers

described in Table S1). The temperature program for the PCR reactions was as above,

with the following modifications: 35 cycles, with an annealing temperature of 55°C for

primer set lmo0448, an annealing temperature of 58°C for primer sets 1886 and ECV

2229 , and final extension time of 5 minutes. Each PCR reaction mixture consisted of 1

μL (1/10) diluted DNA, 0.5 μM of each primer, with the rest of the reaction mixture as

above. PCR products were visualized in agarose gels as described previously.

5.2.4 Desiccation survival in pre-formed biofilms exposed to 23% relative humidity (RH)

Cultures were grown in TSB-glu at 15°C for 48 h and standardized to an

absorbance (450 nm, $A_{450\,nm}$) of ~1.0 in TSB-glu. After diluting the standardized culture

in TSB-glu, 10 μL was spotted on stainless steel coupons (SS, 314, type 4 finish, 0.5 x

0.5 cm size) to an initial concentration of ~4 Log CFU/cm^2. Coupons were placed in a

humidity chamber (100% RH) for 48 h to form biofilm at 15°C. To desiccate the pre-

formed biofilms, the coupons were then moved to a desiccation chamber, where the RH

had been conditioned to 23% by the placement of petri dishes containing saturated solutions of potassium acetate (BDH Chemicals) in the bottom of the desiccator, and left to incubate for up to 7 days at 15°C.

Coupons (n=3) were sampled after 0, 1, 2, 3, 5 and 7 d, and were rinsed in peptone saline (PS, 8.5 g/L sodium chloride, 1 g/L peptone, Oxoid) to remove loosely adhered cells. Coupons were placed in 1 mL PS and sonicated at room temperature (~22°C) to remove viable biofilm cells (Elmo S120H sonicating bath, Fisher Scientific) using the method of Leriche and Carpentier (1995), followed by vigorous vortexing. Biofilm cells were enumerated after samples were serially diluted in PS, spot plated (100 µL) on Tryptone Soy Agar (TSA, Oxoid) and incubated for 48 h at 37°C. Counts were converted to $Log_{10}(CFU/cm^2)$.

5.2.5 Response of strains to benzalkonium chloride
The panel of 14 strains was grown in TSB-glu at 15°C for 48 h, before standardizing the concentration as above. Cultures were then inoculated to a final concentration of 10^7 CFU/cm^2 with TSB-glu, or TSB-glu with benzalkonium chloride (BAC, Fisher Scientific) in microtiter plates (Costar #3370, Fisher Scientific). Plates were sealed with parafilm (Parafilm M®, Neenah WI, US), and incubated at 15°C. Concentrations of BAC used to determine inhibition ranged from 0.313 to 40 µg/mL. Controls included uninoculated media, inoculated media, and uninoculated media with BAC (0.03 µg/mL).

The minimum inhibitory concentration (MIC) of benzalkonium chloride for each strain was determined after 48 h of growth, by pipetting 75 µL of well contents into a

new 96 well plate and measuring $A_{490\,nm}$ (Biotek EL 808 Absorbance Microplate reader, Fisher Scientific)

The minimum biofilm inhibitory concentration (MBIC) of benzalkonium chloride for each strain was determined after 48 h and 6 days of biofilm formation at 15°C. Spent media was discarded followed by addition of 100 μL fresh TSB-glu and 10 μL 12 mM MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Sigma Aldrich, Canada) to each well. Plates were incubated at 37°C for 2 h. Subsequently, 85 μL of media was removed, 50 μL dimethyl sulfoxide (Sigma Aldrich) was added to solubilize the stain with gentle mixing. The plates were incubated for a further 10 min (37°C) before measurement of the absorbance ($A_{540\,nm}$).

The minimum biofilm eradication concentration (MBEC) was determined after pre-forming biofilm in microtiter plates at 15 °C for 6 days (TSB-glu with no BAC). After removing the liquid well contents, aqueous BAC solutions ranging in concentrations from 0 (control) and 0.625 to 80 μg/mL were applied for 1 h at room temperature (22°C). The BAC solutions were removed, and the MTT staining protocol (above) was performed. Each MIC, MBIC, MBEC determination was performed in triplicates, with the entire experiment repeated once (n = 6).

5.2.6 Formation of biofilm on polystyrene peg lids, and removal by enzymatic treatments

The panel of 14 strains was grown in TSB-glu at 15°C for 48 h, before standardizing the cell concentration in TSB-glu as described above. Two hundred microliters of each strain, at a concentration of 10^8 CFU/mL, were added to 96-well microtiter plate bottoms (n= 3 for each treatment). Microtiter plate polystyrene peg lids

(Thermo Fisher Scientific, Roskilde, Denmark) were placed in the culture media. Plates were then wrapped with parafilm and incubated at 15°C (100% RH), with refreshment of the media every 48 h, achieved by placing the peg lids in a 96-well microtiter plate bottom with fresh TSB-glu.

After 6 days of incubation, the peg lids were then treated with phosphate buffered saline (PBS, pH 7.4, control), or PBS containing DNase (50 μg/mL, Sigma Aldrich, Oakville, ON, Canada), or proteinase K (0.1 μg/mL, Sigma Aldrich Canada), or acidified phosphate buffer (APB, pH 5.0), without (control) or with pectinase (1.9 U/mL, Sigma Aldrich Canada) for 1 h at 22°C.

Peg lids were then stained with 200 μL crystal violet, (10 g/L, BDH Chemicals, Toronto, ON, Canada) for 20 min. Stained pegs were briefly rinsed with PBS, and stain was then solubilized in 200 μL 33% (v/v) acetic acid (Nguyen and Burrows, 2014). Absorbance was measured at 570 nm ($A_{570\ nm}$). Negative controls (PBS, APB, or enzyme in buffer), were subtracted from sample data. Biofilm retention after enzymatic treatment was calculated as a percentage based on the biofilm that was obtained in well treated with PBS or APB alone (Biofilm retention (%) = ($A_{570\ nm}$ enzyme treatment)/($A_{570\ nm}$ no enzyme treatment) x 100).

5.2.7 Statistical analysis
To calculate percent inhibition for MIC, MBIC, or % reduction in survival (i.e., metabolic activity) for the MBEC:

$$\% \ inhibition = \frac{(positive \ control - blank) - (test - blank)}{(positive \ control - blank)} x100$$

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A simple T-test (Sigmaplot software, Systat Software Inc., San Jose, CA, USA) was used to determine the lowest concentration that significantly inhibited growth or inhibited biofilm formation compared to the positive control. For eradication, the lowest concentration that reduced absorbance by >80% was considered to be the MBEC.

Total biofilm formed on stainless steel prior to desiccation (Log CFU/cm^2), or the reduction in viable counts during desiccation (Δ Log CFU/cm^2) was compared between strains at each time point with a one way ANOVA and Tukey-Kramer post-hoc test at significance level of 0.05% (R based applet, Assaad et al., 2014)

Biofilm formed in the peg lid assay (with PBS, or APB), % biofilm remaining after each enzyme treatment, and total biofilm retained across the three enzyme treatments, was compared between strains with a one way ANOVA and Tukey-Kramer post-hoc test at significance level of 0.05%.

Where some data was coded as ordinal (presence/absence of genetic markers), or was not continuous (MIC/MBIC data), a Spearman rank-order correlation coefficients test was used to determine correlations between factors at a significance level of 0.05% (Sigmaplot software) .

5.3 Results

5.3.1 SSI-1, LGI1, CC8/VT59 marker distribution among the panel of strains

The SSI-1 marker was found in 10 strains and was widely distributed among the panel of strains, including watershed samples (3/5), but was not present in all food associated strains (5/6). In contrast, the LGI1 marker was only found in strains belonging

to CC8/VT59 to which none of the Atlantic Canada food and fresh water associated

strains belonged (Table 5.1).

Table 5.1 *Listeria monocytogenes* strain origin, and presence or absence of genetic markers.

Listeria monocytogenes strain	Origin	Serotype	Serogroup [a]	SSI-1	LGI1	CC8/ VT59	Biofilm formed 48 h (Log CFU/cm^2) [b]
01-7210	Liverwurst Sausage	1/2a	IIa [c]	+	+	+	7.22 a
08-7376	Environment-Food Processing	1/2a	IIa	+	+	+	7.35a
08-5578	Blood- 2008 RTE meat outbreak	1/2a	IIa	+	+	+	7.42 a
NB1	Smoked Salmon	1/2a	IIa	+	-	-	7.39 a
06-6837	Blood	1/2a	IIa	+	+	+	7.12 a
06-6956	Blood	1/2a	IIa	-	-	-	7.33 a
Lm568	Food processing environment	1/2a	IIa	-	-	-	7.30 a
08-6040	Food – 2008 RTE meat outbreak	1/2a	IIa	+	+	+	7.34 a
LmG	Food processing environment	1/2b	IIb	+	-	-	7.16 a
CP5 2-3	watershed		IIa	+	-	-	7.17 a
CP5 2-2	watershed		IIa	+	-	-	7.30 a
CP5 2-1	watershed		IIa	+	-	-	7.02 a
CP4 5-1	watershed		IIa	-	-	-	7.06 a
CP4 5-2	watershed		IIa	-	-	-	7.18 a

[a] Serogroup IIa is most often serotype1/2a or 3a
[b] Different letters within the column indicate a significant difference between strains (p<0.05).

5.3.2 Biofilm formation on stainless steel and desiccation survival

Biofilm formation on SS prior to desiccation did not differ significantly ($p>0.05$) between the strains (Table 5.1). All strains reached a cell density of ~7 Log CFU/cm^2 attached to the coupons at 48 h. When desiccated, strains 01-7210, 08-7376, 08-5578 did not have a reduction in viable counts. Strain 06-6836 had a slight initial increase in viable counts, and then a reduction in viable count on days 5 and 7. Strains that were moderately desiccation sensitive exhibited a ~0.5-1 Log CFU/cm^2 reduction by day 7, and these strains tended to be clinical or food associated strains. In contrast, all watershed strains experienced a ~ 1 Log CFU/cm^2 reduction on the third day of desiccation, and had viable counts that were 1.4-2.2 Log CFU/cm^2 lower than the most desiccation resistant strain on day 7 ($p<0.05$) . Desiccation susceptibility correlated negatively with the presence of the LGI1 and /CC8/VT59 markers ($p<0.05$). Desiccation susceptibility also correlated negatively with biofilm formation at 48 h on SS, MIC at 48 h, and MBIC at 48 h ($p<0.05$). Overall, the isolates, which formed more biofilm prior to desiccation, were more desiccation resistant. The desiccation resistant isolates also required higher concentrations of BAC both to inhibit planktonic growth and biofilm formation at 48 h (Table 5.5).

Biofilm formation for 48 h on SS also correlated significantly ($p<0.05$) with increases in MIC at 48 h, and MBIC at 6 days, despite the lack of significant differences between strains. Isolates that formed more biofilm on SS (48 h) also required a higher concentration of BAC to inhibit the growth of planktonic cells, or inhibit biofilm formation over 48 h (Table 5.4).
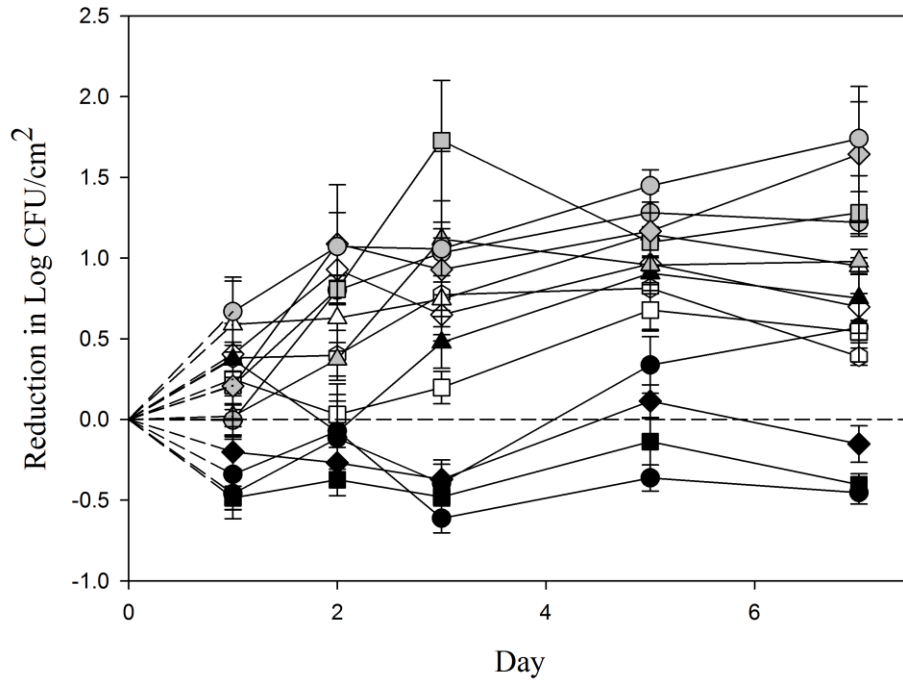
Figure 5.1. Reduction in Log CFU/cm^2 of *L. monocytogenes* during desiccation (23% RH, 15°C) after prior biofilm formation (48 h, 15 °C, 100% RH, n = 3). Open symbols; non CC8/VT59 clinical and food processing strains, grey symbols; watershed isolates, black symbols; LGI1/CC8/VT59 isolates.

5.3.3 Benzalkonium chloride resistance and susceptibility

For the panel of strains, the MIC for BAC ranged from 1.25-10 µg/mL (Table 5.2). The strains' MBIC for biofilm formation over 48 h were mostly recorded as the same value as the MIC. However, the MBIC for biofilm formation over 6 days was typically 2-4 fold higher than the MBIC at 48 h. For all strains, the MBEC, which was measured on a 6-day old biofilm, was 80, or >80 µg/mL. Overall, the BAC concentration required to inhibit planktonic growth was lower than the concentration needed to inhibit biofilm formation, while the concentration needed to inhibit biofilm formation over 6 days was higher than the concentration needed to inhibit biofilm over a 48 h period. The concentration needed to elicit a lethal effect on a mature (6 day) biofilm was 8-64 times higher than the concentration needed to inhibit planktonic growth. The watershed strains typically yielded lower MIC and MBIC-48 h values as compared to clinical and food associated strains, but that trend was less clear for the 6 day MBIC values. The MIC values positively correlated with MBIC (48 h and 6 days), where the latter also positively correlated with each other ($p < 0.05$). This meant that strains, which were more sensitive to BAC as planktonic cells, were also more sensitive to BAC as sessile cells (Table 5.4).

Table 5.2. Concentrations of benzalkonium chloride (µg/mL) required to inhibit planktonic growth (MIC), inhibit biofilm formation (MBIC) at 48 h or 6 days , or eradicate pre-formed biofilm (MBEC), formed with 6 days incubation.

Strain	MIC 48 h	MBIC 48 h	MBIC 6 day	MBEC 6 day
01-7210	10	5	10	80
08-7376	10	5	10	>80
08-5578	5	5	10	80
NB1	10	10	10	80
06-6837	5	5	10	>80
06-6956	2.5	1.25	10	80
Lm568	2.5	1.25	5	80
08-6040	5	10	10	80
LmG	2.5	1.25	5	80
CP 5 2-3	1.25	1.25	5	80
CP 5 2-2	1.25	1.25	10	80
CP5 2-1	1.25	1.25	5	80
CP4 5-1	1.25	1.25	5	80
CP4 5-2	1.25	1.25	10	80

5.3.4 Biofilm formation on peg lids

The level of biofilm that formed on peg lids varied between strains (Table 5.3). Generally, watershed strains formed significantly (p<0.05) less biofilm (in PBS), than the food associated or clinical strains, however, there were variations within each group.

Many strains were not removable with hydrolytic enzymes at the concentrations tested (>100% retention). The reason for this remains unclear, and the non-removability occurred both in strains that formed high or low levels of biofilm. Strains 01-7210, 08-5578, and 06-6837 can be considered highly resistant to enzymatic removal, while strains CP5 2-1, CP5 2-2, CP4 5-1 and CP4 5-2 have a similar phenotype, though not all of the watershed strains were resistant to all three enzymes tested. Where proteinase K, DNase, or pectinase removed biofilm, many strains did not show significant differences in their response to different enzymes, though 08-7376 was significantly (p<0.05) more sensitive to pectinase. In comparing overall response to the enzymes, the non CC8/VT59 watershed CP5 2-3 and food LmG strains retained very little biofilm overall (p<0.05), while the CC8/VT59 06-6956, and 08-6040 were very resistant overall (p<0.05), and also formed a high level of biofilm initially.

Table 5.3. Biofilm formation of the panel of *L. monocytogenes* strains on polystyrene peg lids, and biofilm removal following treatments with buffers containing Proteinase K, DNase I, or Pectinase (in acidified phosphate buffer).

Strain	Biofilm formation [a]	Biofilm with APB [a]	Biofilm retained after enzyme treatment [b]			Overall Biofilm Retention [c]
			Proteinase K	DNase	Pectinase	
01-7210	0.038 e	0.074bd	>100	>100	>100	>300
08-7376	0.149 b	0.152 b	95 A	98 A	14 B	207b
08-5578	0.101 bd	0.123 bc	>100	>100	>100	>300
NB1	0.069 cde	0.031 cd	82 A	81 A	56 A	219b
06-6837	0.12 6bc	0.159b	>100	>100	>100	>300
06-6956	0.381 a	0.322 a	94 A	90 A	88 A	272 ab
Lm568	0.069 cde	0.044 cd	82 AB	72 A	103 B	257 ab
08-6040	0.383 a	0.354 a	105 A	91 A	94 A	289 a
LmG	0.044 de	0.019 d	82 A	43 A	<LOD[f]	125 c
CP5 2-3	0.048 de	0.058cd	19 A	28 A	64 A	111 c
CP5 2-2	0.054 de	0.034 cd	>100	>100	>100	>300
CP5 2-1	0.07 cde	0.108 bd	>100	96 A	67 A	>263
CP4 5-1	0.055 de	0.09 bd	>100	>100	85	>285
CP4 5-2	0.054 de	0.096 bd	>100	>100	53	>253

[a] The absorbance of crystal violet stain recovered from biofilm treated with PBS or APB.
[b] For each enzyme treatment, the percentage of biofilm remaining was calculated relative to the individual strain's biofilm formation in the buffer without enzyme. Significant differences in the same row are marked with capital letters.
[c] Overall biofilm retention is the sum of the percentage biofilm remaining after enzyme treatments for each strain.
[d] Significant differences (p<0.05) in biofilm formed, or biofilm remaining after enzyme treatments for each strain are marked with lower case letters within a column.
[f] = below limit of detection, equivalent to 2 times the average standard deviation of the negative control measurements.

5.3.5 Correlation matrix

All correlations reported in Table 5.4 are significant at 5% or better, with the strength of association between parameters considered moderate to strong (an effect of ≤ -0.5 or ≥ 0.5). Experimental factors that did not have any significant correlations were omitted from the table. The SSI-1 marker did not correlate with the presence of LGI1 or CC8/VT59 (p>0.05).

All LGI1 positive samples were also contained in the CC8/VT59 group, and their correlations are identical (data not shown). The SSI-1 marker correlated (p<0.05) positively with only one parameter tested; MBIC at 48 h. The CC8/VT59 and LGI1 strains correlate negatively with desiccation susceptibility, but positively with biofilm formed at 48 h on SS, and correlate with MIC, MBIC (48 h and 6 day) data (i.e., these strains require a higher concentration of BAC to inhibit planktonic growth or inhibit biofilm formation).

Table 5.4 Spearman rank order correlations between the LGI1, CC8/VT59, SSI-1 markers, biofilm formed at 48 h, desiccation susceptibility, MIC, and MBIC at 48 h and 6 day. Significance values (p<0.05) are indicated in brackets below each correlation coefficient.

	SSI-1	LGI1 and CC8/VT59	MIC 48 h	MBIC 48 h	MBIC 6 day	Desiccation susceptibility [a]
MIC 48		0.710 (p<0.01)				
MBIC 48	0.529 (p<0.05)	0.748 (p<0.01)	0.861 (p<0.001)			
MBIC 6 day		0.556 (p<0.05)	0.861 (p<0.001)	0.624 (p<0.05)		
Desiccation susceptibility [a]		-0.647 (p<0.05)	-0.909 (p<0.001)	-0.667 (p<0.01)		
Biofilm Formed on SS (48 h)[b]		0.549 (p<0.05)	0.559 (p<0.05)		0.555 (p<0.05)	-0.605 (p<0.05)

[a] Based on the reduction in viable counts (Δ Log CFU/cm^2)at 7 days.
[b] Log CFU/cm^2.

5.4 Discussion

Strains which tested positive for LGI1 and CC8/VT59 markers, formed more biofilm on SS, were more desiccation resistant, and exhibited higher BAC resistance than other strains tested. Knabel et al. (2012) found LGI1 in 90% of all Canadian CC8/VT59 strains tested, however, not all CC8 strains contain the LGI1 marker, and that prevalence may vary geographically (Althaus et al., 2014). The SSI-1 marker was found in strains of various origins in the current study, in half of the isolates used in the original study by Ryan et al., (2010), and it was also found in CC8 isolates in Switzerland, as well as in other 1/2a, 1/2b and 1/2 c serotypes of clinical origin (Althaus et al., 2014). The prevalence of SSI-1 was also found to be high (~50%) in a study of both Canadian and Swiss food associated strains, however, SSI-1 was not found to positively influence cold growth adaptation (Arguedas-Villa et al., 2014). In line with this finding, the current study found the presence of SSI-1 did not correlate with desiccation survival, biofilm formation, and did not correspond to BAC resistance at 48 h for planktonic cells, or the MBIC at 6 days (p>0.05). Taken together, SSI-1 does not appear to be a major determinant of survival during exposure to the food processing sanitation stresses tested in the current study.

Ziegler (2011) determined there were few differences in planktonic susceptibility of LGI1 containing isolates to numerous antibiotics compared to other strains, but LGI1 positive isolates had higher MIC values for BAC and a related sanitizer benzethonium chloride. The current study similarly found that LGI1 positive *L. monocytogenes* strains elicited greater resistance to BAC both as planktonic cells and biofilms as compared to LGI1 negative *L. monocytogenes* strains.

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LGI1 contains a multidrug efflux pump that may impact sanitizer resistance, and genes that code for type II and type IV secretion systems, and pili-like surface structures may influence biofilm formation (Gilmour et al. 2010). It may therefore not be surprising that the biofilm formed by LGI1 positive *L. monocytogenes* strains is BAC and desiccation resistant. However, due to the co-occurrence of the two markers, it is also possible the SSI-1 has an effect combined with LGI1. Further studies should elucidate the precise function of LGI1 in the presence and absence of SSI-1 in the persistence of *L. monocytogenes* in food processing plants.

There was also a positive correlation ($p < 0.05$) between biofilm formed prior to desiccation, and desiccation resistance. Initial attachment in biofilm formation did not differ significantly ($p > 0.05$) between strains (data not shown), and did not correlate with other parameters tested. Differences in extracellular polymeric substance (EPS) production or rather biofilm maturity have previously been shown to influence desiccation survival even if the initial level of viable cells exposed to desiccation were similar (Hingston et al. 2013). Differences in biofilm maturity between strains may also impact sanitizer resistance. Nakamura et al. (2013) observed high levels of EPS in the biofilm of a *L. monocytogenes* strain that was both persistent and BAC resistant. Fox et al. (2011) found increased expression of many peptidoglycan related genes in a persistent *L. monocytogenes* strain when exposed to a quaternary ammonium chloride sanitizer, benzethonium chloride. Where LGI1 encodes a peptidoglycan hydrolase, with a possible positive role in biofilm formation (Mercier et al., 2002), and an adhesin, which combined could lead to increases in the biofilm formation ability, as well as an efflux pump, it is likely the genetic island plays a role in BAC survival.

Poor biofilm formation in the peg lid assay was observed for some strains, which was in contrast to all strains forming mature biofilms with ~7 Log CFU/cm^2 on SS coupons. Variation among strains in this assay could be explained by differences in attachment, differing responses to media being refreshed, or motility. Our laboratory has found that *L. monocytogenes* motility mutants (see Chapter 4) and *Staphylococcus aureus* (data not shown) do not form biofilm in the peg lid system.

Application of higher concentrations of BAC for 1 h was needed to eradicate mature (6 day) biofilms, compared to the concentrations needed to inhibit the growth of planktonic cells or biofilms forming over 48 h or 6 days. The MBEC does not vary greatly among strains, and the lack of variation may relate to biofilm maturity (i.e., initial variation between strains in the amount of biofilm formed diminishes with sufficient incubation time) as was described by Harvey et al. (2007), and Wang et al. **(**2015), or due to the range of BAC concentrations tested.

Watershed samples tended to have lower MICs and MBICs at 48 h compared to clinical and food associated strains, and they were also more susceptible to desiccation. Recent research points to water associated *L. monocytogenes* strains having the capability to enter the food chain and food processing environments (Weller et al., 2015b), and water associated strains are often similar to ones (serotypes or pulsotypes) known to cause illness (Lyautey et al., 2007; Stea et al., 2015). Strains with similar phenotypes to this study's watershed samples may enter food processing facilities but would be less difficult to manage from a sanitation standpoint, based on this study's findings with respect to sanitizer and desiccation response. In contrast, the finding of the enhanced

survival potential for strains belonging to the CC8/VT59 group when exposed to stresses

commonly occurring in the food industry may help to explain the long term

predominance of this group in clinical cases in Canada over a 30 year period.

5.5 Conclusions

The LGI1/CC8/VT59 strains may pose a serious challenge to sanitation efforts, as

their established biofilms may be both relatively resistant to desiccation and a commonly

used sanitizer. Future work should include surveying a larger numbers of strains, and

examining alternative treatments to eradicate mature biofilms, or the removal of

desiccated biofilms on stainless steel.

Chapter 6: Conclusions

The capability of *L. monocytogenes* to form biofilm and survive sanitation regimes in food processing facilities remains of great concern. Foodborne outbreaks of listeriosis continue to occur, and it is clear that better understanding of the biology of *L. monocytogenes* is needed for progress to be made. Research to determine the genetic factors associated with biofilm formation, and examine factors relating to the pathogen's interaction with sanitizers and survival of desiccation stress, may lead to better understanding of phenomena such as persistence, the predominance of CC8/VT59 isolates in Canadian clinical cases, and may ultimately help prevent future outbreaks.

Using the insertional mutagenesis approach, a large *himar1* transposon mutant library (>11,000) was mined for the purpose of identifying genes that impact biofilm formation in *L. monocytogenes* at the food processing environment temperature of 15°C. The findings from this study revealed some overlap with previous research as genes such as *flaA*, *inlA*, *lmo0683*, an EPS related gene (*lmo0531*), several genes involved in peptidoglycan biosynthesis and teichoic acid metabolism, and a lipoprotein (*lmo1068*) had been previously identified as having a role in biofilm formation at higher temperatures (30-37°C). However, the current study has newly identified two internalins, a metalloprotease (*lmo2563*), a gene containing a von Willebrand protein domain (*lmo0453*), a PTS system gene (*lmo0543*), a type VII secretion protein (*lmo0057*), a folate metabolism gene (*lmo2572*), an ABC transport system gene (*lmo1224*), and a DNA repair gene (*lmo2488*), as affecting *L. monocytogenes* biofilm at 15°C. The phenotype of these biofilm mutants was also sometimes unexpected, as a disruption of *lmo0531* had been found to reduce biofilm formation at 30°C by other researchers, while in contrast,

we found this mutant to exhibit an enhanced biofilm phenotype. The role of motility in biofilm formation now seems more complicated, as the literature describes reduced biofilm formation in static systems for motility mutants, but hyperbiofilm formation for immotile cells under flow conditions. The current study found motility mutants formed excess biofilm on stainless steel at 15°C, but no biofilm in the peg lid assay.

The deficient or enhanced biofilm mutants did not show large differences in cell surface densities from the parent strain (<1 Log CFU/cm^2) when biofilms were formed on stainless steel for 6, 24, and 48 h, though differences in viable counts between groups were significant at 24 and 48 h ($p<0.05$). The differences in biofilm formation, which were observed in the microtiter plate assay, were less pronounced when biofilm was formed on stainless steel.

In examining the application of sanitizers to pre-formed biofilm on SS, deficient biofilm mutants were significantly ($p<0.05$) more sensitive to benzalkonium chloride at 48 h compared to the parent strain. There were no other significant differences in sanitizer susceptibility between groups. This may due to the fact that although the enhanced biofilm mutants formed more biofilm than the parent strain, reductions in viable counts following H_2O_2 or benzalkonium chloride treatment were similar to the parent strain and/or deficient group (differences ~ 0.5 Log CFU/cm^2 or less).

SEM experiments found motility mutants (*Himar1* insertions in *lmo0690*, *lmo0683* or *lmo0531*) formed a heterogeneous biofilm with mixture of dense microcolonies and empty areas on the SS surface, while deficient mutants (*lmo1068*, *lmo0973*, *lmo2482*, *lmo1080*) were found to produce sparse biofilm, confirming the results of the microtiter plate assay.

Significant differences (p<0.05) were observed in the biofilm formation of nearly all mutants when compared to the parent strain in the peg lid assay. The ability of treatments with hydrolytic enzymes to remove biofilm formed on peg lids also varied significantly (p<0.05) among mutants and the parent. Along with deficient mutants, several mutants, which produced enhanced biofilms in the microtiter plate assay, including motility mutants, did not form biofilm on pegs, or formed significantly less biofilm than the parent strain (p<0.05). Many mutants appeared to differ significantly (p<0.05) from the parent strain in terms of the composition of their biofilm, as measured by its removability by DNase, proteinase k, and pectinase. Two mutants, *lmo0543:Himar1*, and *lmo1224:Himar1*, were shown to form high levels of biofilm in the peg lid assay compared to the parent strain (p<0.05), and in absolute terms also retained more biofilm after enzyme treatments than the parent strain.

Overall, the screening process was successful. Several genes that have a role in biofilm formation at food processing temperatures were newly identified, and the importance of genes known to affect biofilm formation at higher temperatures was confirmed at 15°C. The different impact of numerous genes in biofilm formation in the peg lid assay as compared to the microtiter plate assay was unexpected and indicates the importance of using a multifaceted approach to assaying the biofilm formation potential of strains.

The results of the enzyme assays suggest that protein, polysaccharide, and eDNA have roles in biofilm formation in many mutants, and it may be possible that combinations of enzymatic treatments could form the basis of approach for biofilm removal, even among strains of *L. monocytogenes* that differ in EPS composition.

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In examining variation between *L. monocytogenes* isolates of diverse origin, it was found that SSI-1 was widespread in its distribution, as has been described by other researchers, and in a collection of Canadian *L. monocytogenes* strains (Arguedas-Villa et al., 2014). However, SSI-1 was not found to be associated with desiccation survival, and was not found to correlate with benzalkonium chloride resistance in most experiments (p>0.05). LGI1 was found in all of the CC8/VT59 strains in the current study, but none of the non-CC8/VT59 isolates in the panel. Interestingly, LGI1 was not found in any CC8/VT59 strains in a recent study of Swiss clinical strains, indicating that LGI1 varies in its geographic distribution. The LGI1/CC8/VT59 strains were associated with higher levels of biofilm formed on SS at 48 h (p<0.05), desiccation resistance (p<0.05), and required higher concentrations of BAC to inhibit planktonic and sessile cell growth (p<0.05). While it is difficult to precisely determine the factors that contribute to that phenotype, strains with those genetic features may be of greater concern and pose a challenge in sanitation or removal efforts. These results may help explain the long term occurrence and predominance of the CC8/VT59 group in Canadian clinical cases, as described by Knabel et al. (2012). However, it is not clear why LGI1 or CC8/VT59 strains are not frequently isolated from foods or food processing facilities within Canada (Kovacevic, 2014), given their survival advantage.

In terms of future research, the importance of peptidoglycan and teichoic acid in biofilm formation at a wide range of temperatures may be an area to consider. Treatments applied to planktonic cells, or some method of treatment that has a continuous or longer term effect on those targets may be an avenue to prevent biofilm formation. The use of enzymes to remove biofilm, or disrupt EPS, may also be of interest to industry. The

importance of desiccation survival and the issue of cross adaptation may mean that treatments designed to remove desiccated biofilms is very useful, especially when considering persistent strains or harbourage sites. With respect to variability among *L. monocytogenes* strains, future studies that examine desiccation survival or sanitizer response could include LGI1 strains that do not have the SSI-1 marker, and CC8/VT59 strains that do not have LGI1.

References:

Aarts, H.J.M., Hakemulder,L.E , Van Hoef, A.M.A. 1999.Genomic typing of *Listeria monocytogenes* strains by automated laser fluorescence analysis of amplified fragment length polymorphism fingerprint patterns. International Journal of Food Microbiology 49, 95–102.

Aase, B., Sundheim, G., Langsrud, S., Rørvika, L. M. 2000. Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*. International Journal of Food Microbiology 62,57–63.

Abee, T., Kovács, A., Kuipers, O.P., van der Veen, S.2011. Biofilm formation and dispersal in Gram-positive bacteria. Current Opinion in Microbiology, 22, 172–179.

Abranches, J., Candella, M.M., Wen, Z.T., Baker, H.V., Burne, R. A. 2006. Different roles of EIIABMan and EIIGlc in regulation of energy metabolism, biofilm development, and competence in *Streptococcus mutans*. Journal of Bacteriology 188, 3748-3756.

Adrião, A., Vieira, M.,Fernandes, I.,Barbosa M., Sol,M., Tenreiro, R.P, Chambel, L.,Barata, B., Zilhao, I., Shama, G., Perni, S., Jordan, S.J.,Andrew, P.W., Faleiro, M.L. 2008. Marked intra-strain variation in response of *Listeria monocytogenes* dairy isolates to acid or salt stress and the effect of acid or salt adaptation on adherence to abiotic surfaces. International Journal of Food Microbiology 123, 142–150

Alonso, A. N., Perry, K. J., Regeimbal, J. M., Regan, P. M., Higgins, D. E. 2014. Identification of *Listeria monocytogenes* determinants required for biofilm formation. PLoS ONE 9(12), e113696. http://doi.org/10.1371/journal.pone.0113696

Althaus,D.,Lehner,A., Brisse,S., Maury,M. Tasara, T., Stephan, R. 2014. Characterization of *Listeria monocytogenes* strains isolated during 2011–2013 from human infections in Switzerland. Foodborne Pathogens and Disease 11, 753-758.

Andersen ,J.B., Roldgaard, B.B., Christensen, B.B., Licht, T.R. 2007.Oxygen restriction increases the infective potential of *Listeria monocytogenes in vitro* in Caco-2 cells and *in vivo* in guinea pigs. BMC Microbiol ogy 7, 55.

Anderson, M., Chen, Y.H., Butler, E.K., Missiakas, D.M. 2011. EsaD, a secretion factor for the Ess pathway in *Staphylococcus aureus*. Journal of Bacteriology 93, 1583-1589.

Annous, B.A., Becker, L.A., Bayles, D.O., Labeda, D.P. 1997. Critical role of anteiso-C15:0 fatty acid in the growth of *Listeria monocytogenes* at low temperatures. Applied and Environmental Microbiology 63, 3887–3894.

Anonymous, 2011. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe-United States, August–September 2011. Morbidity and Mortality Weekly Report 60, 1357–1358

Arguedas-Villa, C., Kovacevic, J.,Allen, K.J., Stephan, R., Tasara, T. 2014. Cold growth behaviour and genetic comparison of Canadian and Swiss *Listeria monocytogenes* strains associated with the food supply chain and human listeriosis cases. Food Microbiology 40, 81-87.

Assaad, H.I., Zhou, L., Carroll, R.J., Wu, G. 2014. Rapid publication-ready MS-Word tables for one-way ANOVA. SpringerPlus 3, 474. doi:10.1186/2193-1801-3-474.

Aureli, P., Fiorucci, G.C., Caroli, D., Marchiaro, G., Novara, O., Leone, L., Salmaso, S. 2000. An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. New England Journal of Medicine 342, 1236-41.

Austin, J.W., Bergeron, G. 1995. Development of bacterial biofilms in dairy processing lines. Journal of Dairy Research 62, 509-519.

Bai, A.J., Rai, V.R. 2011. Bacterial quorum sensing and food industry. Comprehensive Reviews in Food Science and Food Safety 10, 183-193.

Ban, G., Park,S., Kim, S., Ryu, S., Kang D. 2012. Synergistic effect of steam and lactic acid against *Escherichia coli* O157:H7, *Salmonella Typhimurium,* and *Listeria monocytogenes* biofilms on polyvinyl chloride and stainless steel. International Journal of Food Microbiology 157, 218-223.

Baumgärtner, M., Kärst, U., Gerstel, B., Loessner, M., Wehland, J., Jänsch, L. 2007. Inactivation of Lgt allows systematic characterization of lipoproteins from *Listeria monocytogenes* . Journal of Bacteriology 189 313–324. http://doi.org/10.1128/JB.00976-06

Beaufort, A. 2011. The determination of ready-to-eat foods into *Listeria monocytogenes* growth and no growth categories by challenge tests. Food Control 22, 1498–1502.

Belessi, C.A., Gounadaki, A.S., Psomas, A.N., Skandamis, P.N. 2011. Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. International Journal of Food Microbiology 145, S46-S52.

Bierne, H., Cossart, P. 2007. *Listeria monocytogenes* surface proteins: from genome predictions to function. Microbiology and Molecular Biology Reviews 71(2), 377–397. http://doi.org/10.1128/MMBR.00039-06

Biswas, R., Voggu, L., Simon, U.K., Hentschel, P., Thumm, G., Götz, F. 2006. Activity of the major staphylococcal autolysin Atl. FEMS Microbiology Letters 259, 260-268.

Black,C., Allan,I., Ford, S.K., Wilson, M., McNab, R. 2004. Biofilm-specific surface properties and protein expression in oral *Streptococcus sanguis*. Archives of Oral Biology 49, 295-304.

Bobrov, A. G., Kirillina, O., Forman, S., Mack, D. Perry, R. D. 2015. The *Yersinia pestis* HmsCDE regulatory system is essential for blockage of the oriental rat flea (*Xenopsylla cheopis*), a classic plague vector. Environmental Microbiology 10, 1419–1432.

Boles, B.R., Horswill, A.R. 2008. *Agr*-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Pathogens 4(4), e1000052. doi:10.1371/journal.ppat.1000052.

Boles, B.R., Singh, P.K. 2008. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. Proceedings of the National Academy of Sciences of the United States of America 105, 12503–12508.

Borucki,M.K., Peppin,J.D., White,D., Loge,F.,Call, D.R. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. Applied and Environmental Microbiology 69, 7336-7362.

Bose, J.L., Lehman, M.K., Fey, P.D., Bayles, K.W. 2012. Contribution of the *Staphylococcus aureus* Atl AM and GL murein hydrolase activities in cell division, autolysis, and biofilm formation. PLoS ONE 7:e42244.

Boyd, C.D., O'Toole, G.A. 2012. Second messenger regulation of biofilm formation: breakthroughs in understanding c-di-GMP effector systems. Annual Review of Cell and Developmental Biology 28, 439-462.

Büla, C.J., Bille, J., Glauser, M.P. 1995. An epidemic of food-borne listeriosis in western Switzerland: description of 57 cases involving adults. Clinical Infectious Diseases 20, 66-72.

Buzby, J.C., Roberts, T., Lin, C.T.J., MacDonald, J.M. 1996. Bacterial foodborne disease: medical costs and productivity losses. Food and Consumer Economics Division, Economic Research Service, U.S. Department of Agriculture. Agricultural Economic Report No. 741.

Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L., Cossart,P., 2002. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends in Microbiology 10, 238-245.

Cao, M., Bitar, A.P., Marquis, H., 2007. A *mariner*-based transposition system for *Listeria monocytogenes*. Applied and Environmental Microbiology 73, 2758–2761.

Capestany, C. A., Kuboniwa, M., Jung, I. Y., Park, Y., Tribble, G. D., Lamont, R. J. 2006. Role of the *Porphyromonas gingivalis* InlJ protein in homotypic and heterotypic biofilm development . Infection and Immunity 74(5), 3002–3005.

Carpentier, B., Cerf, O. 2011. Review — Persistence of *Listeria monocytogenes* in food industry equipment and premises. International Journal of Food Microbiology 31, 1-8.

CDC 2015a. Multistate outbreak of listeriosis linked to commercially produced, prepackaged caramel apples made from Bidart Bros. apples (Final Update). Accessed August 2015. http://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html

CDC 2015b. Multistate outbreak of listeriosis linked to Blue Bell Creameries products (Final Update). Accessed August 2015. http://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/index.html

CFIA, 2011. Policy on *Listeria monocytogenes* in Ready-to-Eat Foods. http://www.hc-sc.gc.ca/fn-an/legislation/pol/policy_listeria_monocytogenes_2011-eng.php#summary. Accessed September 20, 2015.

CFIA, 2015.Annex H: Policy on the control of *Listeria monocytogenes* in Ready-to-Eat (RTE) Meat and Poultry Products. http://www.inspection.gc.ca/food/meat-and-poultry-products/manual-of-procedures/chapter-4/annex-h/eng/1370541840583/1370541911699#app1. Accessed October 1, 2015.

Chang , Y., Gu, W., Zhang, F., McLandsborough, .L . 2013. Disruption of *lmo1386*, a putative DNA translocase gene, affects biofilm formation of *Listeria monocytogenes* on abiotic surfaces. International Journal of Food Microbiology 161, 158–163.

Chang, Y., Gu, W., Fischer, N., Mclandsborough, L. 2012. Identification of genes involved in *Listeria monocytogenes* biofilm formation by *mariner*-based transposon mutagenesis. Applied Genetics and Molecular Biotechnology 93, 2051-2062.

Chang,W., van de Mortel, M., Nielsen, L.,   de Guzman,G.N., Li,X., Halverson, L.J., 2007. Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions. Journal Of Bacteriology 189, 8290–8299.

Chavant, P., Martinie, B., Meylheuc, T., Bellon-Fontaine, M., and Hebraud, M. 2002. *Listeria monocytogenes* LO28: surface physicochemical properties and ability to form biofilms at different temperatures and growth phases. Applied and Environmental Microbiology 68, 728–737.

Chen, L.H., Köseoğlu, V.K., Güvener. Z.T., Myers-Morales. T., Reed. J.M., D'Orazio, S.E., Miller, K.W., Gomelsky, M. 2014. Cyclic di-GMP-dependent signaling pathways in the pathogenic firmicute *Listeria monocytogenes*. PLoS Pathogens 10(8), e1004301. doi: 10.1371/journal.ppat.1004301.

Chen, Y., Ross, W.H., Scott, V.N., Gombas, D. E.. 2003. *Listeria monocytogenes*: low levels equal low risk. Journal of Food Protection. 4, 570-578.

Chmielewski, R.A.N., Frank, J.F. 2006.A predictive model for heat inactivation of *Listeria monocytogenes* biofilm on buna-N rubber. LWT- Food Science and Technology, 39, 11-19.

Cossart, P., Toledo-Arana, A. 2008. *Listeria monocytogenes*, a unique model in infection biology: an overview. Microbes and Infection 10, 1041-1050.

Cossart, P. 2011. Illuminating the landscape of host–pathogen interactions with the bacterium *Listeria monocytogenes*. Proceedings of the National Academy of Sciences of the United States of America 108, 19484–19491

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M. 1995. Microbial biofilms. Annual Review of Microbiology 49, 711-745.

Cox, L.J., Kleiss, T., Cordier, J.L., Cordellana, C., Konkel, P., Pedrazzini, C., Beumer, R., Siebenga, A. 1989. *Listeria* spp. in food processing, non-food, and domestic environments. Food Microbiology 6, 49-61.

Cruz, C.D. Fletcher, G.C. 2012. Assessing manufacturers' recommended concentrations of commercial sanitizers on inactivation of *Listeria monocytogenes*. Food Control 26, 194-199.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penades, J.R. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. Journal of Bacteriology 183, 2888–2896.

d'Entremont, C. 2015. Inhibition of single and mixed species biofilms formed by *Listeria monocytogenes* and food related bacteria by natural compounds. M.Sc. Thesis, Dalhousie University, Halifax, NS, Canada.

Dalgaard, P., Koutsoumanis, K. 2001. Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. Journal of Microbiological Methods 43, 183-196.

Dalton, C..B, Austin, C.C., Sobel, J., Hayes, P.S., Bibb, W.F., Lewis, M.S., Graves, L.M.,Swaminathan, B., Proctor, M.W., Griffin, P.M. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. New England Journal of Medicine,336,100-105

Daneshvar Alavi, H.E., Truelstrup Hansen, L. 2013. Kinetics of biofilm formation and desiccation survival of *Listeria monocytogenes* in single and dual species biofilms with *Pseudomonas fluorescens*, *Serratia proteamaculans* or *Shewanella baltica* on food-grade stainless steel surfaces. Biofouling 29,1253-1268.

Davey, M.E., O'Toole, G.A. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiology and Molecular Biology Reviews 64, 847–867.

de Bruijn, M., van Vianen, W.,Ploemacher, R.E., Bakker-Woudenberg, I., Campbell, P.A., van Ewijk, W., Leenen, P. 1998. Bone marrow cellular composition in *Listeria monocytogenes* infected mice detected using ER-MP12 and ER-MP20 antibodies: a flow cytometric alternative to differential counting. Journal of Immunological Methods 217, 27–39.

den Bakker ,H.C., Warchocki, S., Wright, E.M., Allred,A.F., Ahlstrom,C., Manuel,C.S., Stasiewicz, M.S., Burrell, A., Roof, S., Strawn, L.K., Fortes, E., Nightingale, K.K., Kephart, D., Wiedmann, M. 2014. *Listeria floridensis* sp. nov., *Listeria aquatica* sp.nov., *Listeria cornellensis* sp. nov., *Listeria riparia* sp. nov. and *Listeria grandensis* sp. nov., from agricultural and natural environments. International Journal of Systematic and Evolutionary Microbiology 64, 1882-1889.

DiBonaventura, G., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, A., Conter, M., Ianieri, A., 2008. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. Journal of Applied Microbiology 104, 1552–1561.

Donlan, R.M., Costerton, J.W. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews 15, 167–193.

Donnelly, C.W. 2001. *Listeria monocytogenes*: a continuing challenge. Nutrition Reviews 59, 183–194.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P. 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. Journal of Clinical Microbiology 42, 3819–3822. http://dx.doi.org/10.1128/JCM.42.8.3819.

Drevets, D.A., Bronze, M.S. 2008. *Listeria monocytogenes*: epidemiology, human disease, and mechanisms of brain invasion. FEMS Immunolology Medical Microbiology 53, 151–165

Dunman P.M, Murphy, E., S. Haney, D. Palacios, Tucker-Kellogg, G., Wu, S., Brown, E. L., Zagursky,R. J. , Shlaes, D. , Projan, S.J. 2001. Transcription profiling-based identification of *Staphylococcus aureus genes* regulated by the *agr* and/or *sarA* loci. Journal of Bacteriology 183, 7341–7353.

Ells, T.C., Speers, R.A., Truelstrup Hansen, L. 2009. Insertional mutagenesis of *Listeria monocytogenes* 568 reveals genes that contribute to enhanced thermotolerance. International Journal of Food Microbiology 136, 1-9.

Eugster, M.R., Morax, L.S., Hüls, V.J., Huwiler, S.G., Leclercq, A., Lecuit, M., Loessner, M.J. 2015. Bacteriophage predation promotes serovar diversification in *Listeria monocytogenes*. Molecular Microbiology 97, 1365-2958.

FDA.2008. Guidance for Industry: Control of *Listeria monocytogenes* in refrigerated or frozen ready-to-Eat foods; draft guidance. http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/FoodProcessingHACCP/ucm073110.htm#measure (Accessed September 14, 2015).

Ferreira,V.,Wiedmann,M.,Teixeira,P. Stasiewicz, M. J. 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. Journal of Food Protection 77, 150-170.

Fleming, D.W., Cochi,S.L. , MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D. , Holmes, M.B., Audurier, A. , Broome, C.V. , Reingold, A.L. 1985. Pasteurised milk as a vehicle of infection in an outbreak of listeriosis. New England Journal of Medicine 312, 404–407.

Fox, E. M., Leonard, N. , Jordan, K. 2011. Physiological and transcriptional characterization of persistent and nonpersistent *Listeria monocytogenes* isolates. Applied and Environmental Microbiology 77, 6559–6569.

Franciosa, G., Maugliani, A., Scalfaro, C., Floridi, F., Aureli, P., 2009. Expression of internalin A and biofilm formation among *Listeria monocytogenes* clinical isolates. International Journal of Immunopathology and Pharmacology 22, 183–193.

Fretz, R., Pichler, J., Sagel, U., Much, P., Ruppitsch, W., Pietzka, A.T., Stöger, A.,Huhulescu, S., Heuberger, S., Appl,G., Werber, D., Stark, K., Prager, R., Flieger, A., Karpíšková, R., Pfaff, G., , Allerberger, F. 2010. Update: Multinational listeriosis outbreak due to 'Quargel', a sour milk curd cheese, caused by two different *L. monocytogenes* serotype 1/2a strains, 2009-2010. Eurosurveillance 15, 2-3.

Garmyn, D., Gal,L., Lemaitre,J., Hartmann, A., Piveteau, P.2009. Communication and autoinduction in the species *Listeria monocytogenes*. Communicative and Integrative Biology 2, 371-374.

Garmyn, D., Augagneur, Y.,Gal, L., Vivant, A., Piveteau, P. 2012. *Listeria monocytogenes* differential transcriptome analysis reveals temperature-dependent Agr regulation and suggests overlaps with other regulons. PLOS One 7, e43154.

Garsin, D.A., Urbach, J., Huguet-Tapia, J.C., Peters, J.E., Ausubel, F.M. 2004. Construction of an *Enterococcus faecalis* Tn917-mediated gene disruption library offers insight into Tn917 insertion patterns. Journal of Bacteriology 186, 7280-7289.

Geesey, G.G. 2001. Bacterial behavior at surfaces. Current Opinion in Microbiology 4, 296–300

Geier, H.m Mostowy, S., Cangelosi,G.A., Behr,M.A., Ford,T.E. 2008. Autoinducer-2 triggers the oxidative stress response in *Mycobacterium avium*, leading to biofilm formation. Applied And Environmental Microbiology 74, 1798–1804

Gianfranceschi, M.V., D'Ottavio, M.C.,Gattuso, A., Bella, A., Aureli, P. 2009.Distribution of serotypes and pulsotypes of *Listeria monocytogenes* from human, food and environmental isolates (Italy 2002–2005).Food Microbiology 26, 520–526

Gillespie,I.A., McLauchlin, J., Grant, K.A., Little, C.L., Mithani,V., Penman,C., Lane,C., Regan, M. 2006. Changing pattern of human listeriosis, England and Wales, 2001–2004. Emerging Infectious Diseases 12, 1361-1366.

Gilmour, M.W., Graham, M., Van Domselaar, G., Tyler, S., Kent,H., Trout-Yakel,K.M., Larios, O., Allen, V., Lee, B., Nadon, C. 2010. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. BMC Genomics 1, 120. doi:10.1186/1471-2164-11-120

Gombas, D.E., Chen, Y., Clavero, R.S. and Scott, V.N. 2003. Survey of *Listeria monocytogenes* in ready-to-eat foods. Journal of Food Protection 66, 559-569

Goulet, V., Hedberg, C., Le Monnier, A., de Valk, H. 2008. Increasing incidence of Listeriosis in France and other European countries. Emerging Infectious Diseases 14, 734-740. doi:10.3201/eid1405.071395.

Graves, L.M., Swaminathan, B., Reeves, M.W., Hunter, S.B., Weaver, R.E., Plikaytis, B.D., Schuchat, A., 1994. Comparison of ribotyping and multilocus enzyme electrophoresis for subtyping of *Listeria monocytogenes* isolates. Journal of Clinical Microbiology 32, 2936–2943.

Gray, M.J., Zadoks, R.N., Fortes, E.D., Dogan, B., Cai, S., Chen, Y., Scott, V.N., Gombas, D.E., Boor, K.J., Wiedmann, M., 2004. *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. Applied and Environmental Microbiology 70, 5833–5841.

Greig, J.D., Ravel,A. 2009.Analysis of foodborne outbreak data reported internationally for source attribution. International Journal of Food Microbiology 130, 77–87.

Gross, M., Cramton, S.E., Götz, F. Peschel, A. 2001. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. Infection and Immunity 69 3423-3426.

Gueriri, I., Cyncynatus,C., Dubrac, S., Toledo Arana, A., Dussurget, O., Msadek, T. 2008a. The DegU orphan response regulator of *Listeria monocytogenes* autorepresses its own synthesis and is required for bacterial motility, virulence and biofilm formation. Microbiology 154, 2251–2264

Gueriri, I., Bay, S., Dubrac, S.,Cyncynatus, C., Msadek, T. 2008b. The Pta–AckA pathway controlling acetyl phosphate levels and the phosphorylation state of the DegU orphan response regulator both play a role in regulating *Listeria monocytogenes* motility and chemotaxis. Molecular Microbiology 70, 1324-1357.

Hain,T., Chatterjee, S.S, Ghai, R., Kuenne,C.T., Billion, A., Steinweg, C., Domann, E., Kärst, U., Jünsch, L., Wehland, J., Eisenreich, W., Bacher, A., Joseph, B., Schär, J., Kreft, J., Klumpp, J., Loessner, M.J., Dorscht, J., Neuhaus, K., Fuchs, T.M. Scherer, S., Doumith, M., Jacquet, C., Martin, P., Cossart, P., Rusniock, C., Glaser, P., Buchrieser,C., Goebel, W.,Chakrabort, T. 2007. Pathogenomics of *Listeria* spp. International Journal of Medical Microbiology 297, 541–557.

Harmsen, M., Lappann, M., Knøchel, S., Molin, S., 2010. Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Applied and Environmental Microbiology 76, 2271–2279.

Harvey, J., Keenan,K.P., Gilmour, M. 2007.Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiology 24, 380–392.

Health Canada. 2011. Policy on *Listeria monocytogenes* in Ready-to-Eat Foods. http://www.hc-sc.gc.ca/fn-an/legislation/pol/policy_listeria_monocytogenes_2011-eng.php#scien (Accessed September, 2015)

Hickman, J.W., Tifrea, D.F., Harwood, C.S. 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proceedings of the National Academy of Sciences of the United States of America 102, 14422–14427.

Hill, C., Cotter, P.D., Sleator, R.D., Gahan, C.G.M. 2002. Bacterial stress response in *Listeria monocytogenes*: jumping the hurdles imposed by minimal processing. International Dairy Journal 12, 273–283

Hingston, P.A., Stea, E.C., Knøchel, S., Hansen, T. 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, 46-56.

Hoffmann, S., Batz, M.B., Morris II, J.G. 2012.Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. Journal of Food Protection 75, 1292–1302.

Holah, J.T. ,Taylor, J.H. , Dawson, D.J., Hall, K.E. 2002. Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli.* Journal of Applied Microbiology Symposium 92, 111S–120S.

Holland, L.M., Conlon, B., O'Gara, J.P. 2011. Mutation of *tagO* reveals an essential role for wall teichoic acids in *Staphylococcus epidermidis* biofilm development. Microbiology 15, 408-418.

Houry, A., Briandet, R., Aymerich, S., Gohar, M. 2010. Involvement of motility and flagella in *Bacillus cereus* biofilm formation. Microbiology 156, 1009-1018.

Ibusquiza, P.S., Herrera , J.J.R., Cabo, M.L. 2011. Resistance to benzalkonium chloride, peracetic acid and nisin during formation of mature biofilms by *Listeria monocytogenes*. Food Microbiology 28, 418-425.

Jacquet, C., Doumith, M., Gordon JI, Martin PMV, Cossart P, Lecuit M. 2004. A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. Journal of Infectious Diseases 189, 2094 –2100.

Jacquet, C., Gouin, E., Jeannel, D., Cossart, P., Rocourt, J.2002. Expression of ActA, Ami, InlB, and listeriolysin O in *Listeria monocytogenes* of human and food origin. Applied and Environmental Microbiology 68, 616-22.

Jefferson, K. 2004. What drives bacteria to produce a biofilm? FEMS Microbiology Letters 236, 163-173.

Ji, G., Beavist, R.C., Novick, R.P. 1995. Cell density control of *staphylococcal* virulence mediated by an octapeptide pheromone. Proceedings of the National Academy of Sciences of the United States of America 92, 12055-12059.

Join-Lambert, O.F., Ezine, S., Le Monnier, A., Jaubert,F., Okabe, M., Berche, P., Kayal, S. 2005. *Listeria monocytogenes* -infected bone marrow myeloid cells promote bacterial invasion of the central nervous system. Cellular Microbiology 7, 167-180.

Jordan, S.J., Perni, S., Glenn, S., Fernandes, I., Barbosa, M., Sol, M., Tenreiro, R.P., Chambel, L., Barata, B., Zilhao, I., Aldsworth,T.G., Adriao, A., Faleiro,M.L., Shama, G., Andrew, P.W. 2008. *Listeria monocytogenes* biofilm-associated protein (BapL) may contribute to surface attachment of *L. monocytogenes* but is absent from many field isolates. Applied and Environmental Microbiology 74, 5451–5456.

Kaiser, T. D. L., Pereira, E.M., Netto dos Santos, K.R., Maciel, E.L.N., Schuenck, R.P., Nunes, A.P.F. 2013. Modification of the Congo red agar method to detect biofilm production by *Staphylococcus epidermidis*. Diagnostic Microbiology and Infectious Disease 75(3), 235 – 239.

Kalmokoff, M., Austin, J., Wan, X.D., Sanders, G., Banerjee, S., Farber, J. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. Journal of Applied Microbiology 91, 725-734.

Kastbjerg, V.G., Gram, L. 2009. Model systems allowing quantification of sensitivity to disinfectants and comparison of disinfectant susceptibility of persistent and presumed nonpersistent *Listeria monocytogenes*.Journal of Applied Microbiology 106, 1667–1681.

Kérouanton, A., Roche, S.M., Marault, M., Velge, P., Pourcher, A.M., Brisabois, A., Federighi Garrec, M.N. 2010. Characterization of isolates of *Listeria monocytogenes* from sludge using pulsed-field gel electrophoresis and virulence assays. Journal of Applied Microbiology 108, 1380–1388. http://dx.doi.org/10.1111/j.1365-2672.2009.04531

Kiviranta, I., Jurvelin, J., Tammi, M., Säämänen, A.M., Helminen, H.J. 1985. Microspectrophotometric quantitation of glycosaminoglycans in articular cartilage sections stained with Safranin O. Histochemisty 82, 249-55.

Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jorgensen, S. Molin, T. Tolker-Nielsen. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Molecular Microbiology 48, 1511–1524.

Knabel, S.J., Reimer, A., Verghese, B.,Lok, M., Ziegler, J., Farber, J., Pagotto, F., Graham, M., Nadon, C.A., the Canadian Public Health Laboratory Network (CPHLN), Gilmour, M.W. 2012. Sequence typing confirms that a predominant *Listeria monocytogenes* clone caused human listeriosis cases and outbreaks in Canada from 1988 to 2010. Journal of Clinical Microbiology 50, 1748-1751.

Korea, C.G., Balsamo, G., Pezzicoli, A., Merakou,C., Tavarini, S., Bagnoli, F., Serruto, D., Unnikrishnan, M. 2014. Staphylococcal Esx proteins modulate apoptosis and release of intracellular *Staphylococcus aureus* during infection in epithelial cells. Infection and Immunity 82, 4144-4153.

Köseoğlu,V.K., Heiss, C., Azadi, P.,Topchiy, E.,Güvener, Z.T., Lehmann, T.E., Miller, K.W., Gomelsky, M. 2015. *Listeria monocytogenes* exopolysaccharide: origin, structure, biosynthetic machinery and c-di-GMP-dependent regulation. Molecular Microbiology 96, 1365-2958.

Kostaki, M., Chorianopoulos, N., Braxou, E., Nychas, G., Giaourisc, E. 2012. Differential biofilm formation and chemical disinfection resistance of sessile cells of *Listeria monocytogenes* strains under monospecies and dual-species (with *Salmonella enterica*) conditions. Applied and Envioronmental Microbiology 78, 2586-2595.

Kovacevic, J., Arguedas-Villa, C., Wozniak, A., Tasara, T., Allen, K.J. 2013. Examination of food chain-derived *Listeria monocytogenes* strains of different serotypes reveals considerable diversity in *inlA* genotypes, mutability, and adaptation to cold temperatures. Applied and Environmental Microbiology 79, 1915-1922.

Kovacevic, J. 2014. Characterization of food chain-derived *Listeria monocytogenes* and the role of *Listeria* genomic island (LGI1) in virulence, survival, and tolerance to food-related stress. Ph.D. Thesis, University of British Colombia, Vancouver, BC, Canada.

Kumar, C.G. Anand, S.K. 1998.Significance of microbial biofilms in food industry: a review. International Journal of Food Microbiology 42, 9–27.

Kumar, S., Parvathi, A.,George, J.,Krohne, G., Karunasagar, I., Karunasagar, I., 2009. A study on the effects of some laboratory-derived genetic mutations on biofilm formation by *Listeria monocytogenes*. World Journal of Microbiology and Biotechnology, 25,527-531.

Lauderdale, K.J., Boles, B.R., Cheung, A.L., Horswill, A.R. 2009. Interconnections between sigma b, agr, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. Infection and Immunity 77, 1623-1635.

Lemon, K.P., Higgins, D.E., Kolter, R., 2007. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. Journal of Bacteriology 189, 4418–4424.

Lemon,K.P., Freitag,N.E., Kolter, R. 2010.The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. Journal of Bacteriology 192, 3969-3967.

Leriche, V., Carpentier, B. 1995. Viable but nonculturable *Salmonella typhimurium* in single and binary species biofilms in response to chlorine treatment. Journal of Food Protection 58(11), 1186-1191.

Liao, S., Klein, M.I., Heim, L.P., Fan, Y., Bitoun, J.P., Ahn, S.,Burne, R.A., Koo, H., Brady, L.J., Wena, Z.T. 2014. *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. Journal of Bacteriology 196, 2355–2366

Lim, B., Beyhan, S., Meir, J.,Yildiz, F.H. 2006. Cyclic-diGMP signal transduction systems in *Vibrio cholerae* : modulation of rugosity and biofilm formation. Molecular Microbiology 60, 331-348.

Linnan,M.J., Mascola, L., Lou, X.D., Goulet,V. , May, S. , Salminen,C. , Hird,D.W. ,Yanekura, M.L. , Hayes, P. , Weaver, R. , Audurier, A. , Plikayatis, B.D. , Fannin, S.L., Kleks, A., Broome, C.V..1988. Epidemic listeriosis associated with Mexican-style cheese. New England Journal of Medicine 319, 823-828.

Little, C.L., Sagoo, S.K., Gillespie, I. A., Grant, K. and McLauchlin, J. 2009. Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in selected retail ready-to-eat foods in the United Kingdom. Journal of Food Protection 72, 1869-1877

Liu, D. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. 2006. Journal of Medical Microbiology 55, 645–659.

Liu, S., Graham, J.E., Bigelow, L., Morse, P.D. 2nd, Wilkinson, B.J. 2002. Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. Applied and Environmental Microbiology 68, 1697-1705.

Loder, A.L. 2006. Effects of temperature and nutrient status on adherence of clinical and environmental *Listeria monocytogenes* strains to food grade stainless steel coupons. M.Sc. Thesis, Dalhousie University, Halifax, NS, Canada.

Lomonaco, S., Nucera, D., Filipello, V. 2015.The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. Infection, Genetics and Evolution 35, 172–183.

Longhi, C., Scoarughi, G.L., Poggiali, F., Cellini, A., Carpentieri, A., Seganti, L., Pucci, P., Amoresano, A., Cocconcelli, P.S., Artini, M., Costerton, J.W., Selan, L., 2008. Protease treatment affects both invasion ability and biofilm formation in *Listeria monocytogenes*. Microbial Pathogens 45, 45–52.

Lourenço, A., Machado, H., Brito, L., 2011. Biofilms of *Listeria monocytogenes* produced at 12°C either in pure culture or in co-culture with *Pseudomonas aeruginosa* showed reduced susceptibility to sanitizers. Journal of Food Science 76, 142–148.

Lundén, J., Autio, T., Markkula, A.,Hellström, S., Korkeala, H. 2003. Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. International Journal of Food Microbiology 82, 265-272.

Lundén, J., Miettinen, M., Autio, T., Korkeala, H., 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. Journal of Food Protection 63, 1204– 1207

Luo Q, Shang J, Feng X, Guo X, Zhang L, Zhou Q**.** 2013. PrfA led to reduced biofilm formation and contributed to altered gene expression patterns in biofilm-forming *Listeria monocytogenes*. Current Microbiology 67, 372–378. http://dx.doi.org/10.1007/s00284-013-0377-7.

Lyautey, E., Lapen, D.R., Wilkes, G., McCleary, K., Pagotto, F., Tyler, K., Topp, E. 2007. Distribution and characteristics of *Listeria monocytogenes* isolates from surface waters of the South Nation River watershed, Ontario, Canada. Applied and Environmental Microbiology 73, 5401–5410. http://dx.doi.org/10.1128/AEM.00354-07.

Machata, S., Hain, T., Rohde, M., Chakraborty, T. 2005. Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in *Listeria monocytogenes*. Journal of Bacteriology 187, 8385–8394.

Mack,D., Fischer,W., Krokotsch,A., Leopold, K., Hartmann,R., Egge, H., Laufs, R. 1996. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear B-1,6-linked glucosaminoglycan: purification and structural analysis. Journal of Bacteriology 178, 175–183.

Mafu, A.A., Roy, D., Goulet, J., Savoie. L. 1991. Characterization of physicochemical forces involved in adhesion of *Listeria monocytogenes* to surfaces. Applied and Environmental Microbiology 57, 1969-1973.

Mata, M.M., da Silva, W.P., Wilson,R., Lowe, E., Bowman, J.P. 2015.Attached and planktonic *Listeria monocytogenes* global proteomic responses and associated influence of strain genetics and temperature. Journal of Proteome Research 14 (2), 1161-1173.

Mauder,M. Williams, T., Fritsch,F., Kuhn, M., Beier, D. 2008.Response regulator DegU of *Listeria monocytogenes* controls temperature-responsive flagellar gene expression in its unphosphorylated state. Journal of Bacteriology 13, 4777-4781.

McGann, P., Ivanek, R., Wiedmann, M., Boor, K.J. 2007. Temperature-dependent expression of *Listeria monocytogenes* internalin and internalin-like genes suggests functional diversity of these proteins among the listeriae. Applied and Environmental Microbiology 73(9), 2806-2814.

McLauchlin, J. 1990. Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis. European Journal of Clinical Microbiology and Infectious Diseases 9, 210-213.

Mead, P.S.,Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro,C., Griffin, P.M., Tauxe, R.V. 2009. Food-related illness and death in the United States. Emerging Infectious Diseases 5, 607-625.

Meinersmann, R.J., Phillips, R.W., Wiedmann, M., Berrang, M.E.2004.Multilocus sequence typing of *Listeria monocytogenes* by use of hypervariable genes reveals clonal and recombination histories of three lineages. Applied and Environmental Microbiology 70,2193–2203.

Mendonca, A.F., Amoroso, T.L., Knabel, S.J. 1994.Destruction of Gram-negative food-borne pathogens by high pH involves disruption of the cytoplasmic membrane. Applied and Environmental Microbiology 60, 4009-4014.

Micallef, L., Rodgers, P. 2014. euler*APE*: Drawing area-proportional 3-Venn diagrams Using ellipses. PLoS ONE 9(7), e101717. doi:10.1371/journal.pone.0101717.

Milohanic, E., Glaser, P., Coppée, J. Y., Frangeul, L., Vega, Y., Vázquez-Boland, J. A., Kunst, F., Cossart, P., Buchrieser, C. 2003. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Molecular Microbiology 47(6), 1613-1625.

Milohanic, E., Jonquières, R., Cossart, P., Berche, P., Gaillard, J.L. 2001. The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. Molecular Microbiology 39(5),1212-1224.

Mitrakul, K., Loo, C. Y., Gyurko, C., Hughes, C. V. Ganeshkumar, N. 2005. Mutational analysis of the *adcCBA* genes in *Streptococcus gordonii* biofilm formation. Oral Microbiology and Immunology 20, 122–127.

Moltz, A.G., Martin, S.E. 2005. Formation of biofilms by *Listeria monocytogenes* under various growth conditions. Journal of Food Protection 68, 92-97.

Motulsky, H.J., Ransnas, L.A. 1987. Fitting curves to data using nonlinear regression: a practical and nonmathematical review. The FASEB Journal 1, 365-374.

Muñoz-Elías, E.J. Marcano, J., Camilli, A. 2008. Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. Infection and Immunity 76(11), 5049–5061.

Nakamura,H., Takakura, K., Sone, Y., Itano,Y., Nishikawa, Y. 2013.Biofilm formation and resistance to benzalkonium chloride in *Listeria monocytogenes* isolated from a fish processing plant. Journal of Food Protection 76, 1179–1186.

NCBI, 2015. http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=100079. Accessed September 1, 2015.

Neuhaus, K., Satorhelyi, P., Schauer, K., Scherer, S., Fuchs, T.M. 2013. Acid shock of *Listeria monocytogenes* at low environmental temperatures induces *prfA*, epithelial cell invasion, and lethality towards *Caenorhabditis elegans*. BMC Genomics 14, 285.

Ng, W.L., Bassler, B.L. 2009. Bacterial quorum-sensing network architectures. Annual Review of Genetics 43, 197-222.

Nguyen, U.T., Burrows, L.T., 2014. DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms. International Journal of Food Microbiology 187, 26-32.

Nightingale, K. K., Windham, K., Martin, K. E., Yeung, M., Wiedmann, M. 2005. Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in *inlA*, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. Applied and Environmental Microbiology 71, 8764-8772.

O'Gara, J.P. 2007. *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiology Letters 270, 179-188.

Oliver, H.F., Wiedmann M., Boor, K.J. 2007. Environmental reservoir and transmission into the mammalian host, p 111–138. In Goldfine H., Shen, H. Palmer (eds.), Pathogenesis and host response of *Listeria monocytogenes*. Springer-Verlag, New York, NY.

Orsi, R.H., den Bakker, H.C., Wiedmann, M. 2011. *Listeria* monocytogenes lineages: Genomics, evolution, ecology, and phenotypic characteristics. International Journal of Medical Microbiology 301, 79-96.

Orsi, R.H., Bowen, B.M., Wiedmann, M., 2010. Homopolymeric tracts represent a general regulatory mechanism in prokaryotes. BMC Genomics 11, 102. doi:10.1186/1471-2164-11-102

O'Toole, G.A., Kolter, R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Molecular Microbiology 28, 449-461.

Ouyang, Y., Li, J., Dong, Y., Blakely, L., Cao, M., 2012. Genome-wide screening of genes required for *Listeria monocytogenes* biofilm formation. Journal of Biotechnology Research 4, 13–25.

Pan,Y., Breidt II,F., Kathariou, S. 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Applied and Environmental Microbiology 72, 7711–7717.

Perry, R.D., Bobrov, A.G., Kirillina, O., Jones, H.A.,Pedersen, L., Abney, J., Fetherston, J.D. 2004. Temperature regulation of the hemin storage (Hms+) phenotype of *Yersinia pestis* is posttranscriptional. Journal of Bacteriology 186, 1638–1647.

Reij, M.W. Den Aantrekker. 2004. Recontamination as a source of pathogens in processed foods. International Journal of Food Microbiology 91, 1–11.

Renier, S., Chagnot, C., Deschamps, J., Caccia, N., Szlavik, J., Joyce, S.A., Popowska, M., Hill, C., Knøchel, S., Briandet, R., Hébraud, M., Desvaux, M. 2014. Inactivation of the SecA2 protein export pathway in *Listeria monocytogenes* promotes cell aggregation, impacts biofilm architecture and induces biofilm formation in environmental condition. Environmental Microbiology 16, 1462-2920.

Ribeiro, M.H., Manha, S., Brito, L. 2006. The effects of salt and pH stress on the growth rates of persistent strains of *Listeria monocytogenes* collected from specific ecological niches. Food Research International 39, 816–822

Riedel, C.U., Monk, I.R., Casey, P.G., Waidmann, M.S., Gahan, C.G.M., Hill, C. 2009. AgrD-dependent quorum sensing affects biofilm formation, invasion, virulence and global gene expression profiles in *Listeria monocytogenes*. Molecular Microbiology 71, 1177-1189.

Rieu, A., Weidmann, S., Garmyn, D., Piveteau, P., Guzzo, J. 2007. *agr* system of *Listeria monocytogenes* EGD-e: role in adherence and differential expression pattern. Applied and Environmental Microbiology 73, 6125-6133

Rieu,A., Briandet,R., Habimana,O., Garmyn, D., Guzzo,J., Piveteau, P. 2008. *Listeria monocytogenes* EGD-e biofilms: no mushrooms but a network of knitted chains. Applied and Environmental Microbiology 74, 4491-4497

Rocourt, J., Jacquet, C., Reilly, A. 2000. Epidemiology of human listeriosis and seafoods. International Journal of Food Microbiology 62, 197-209.

Ryan, S., Begley, M., Hill, C., Gahan, C.G.M. 2010. A five-gene stress survival islet (SSI-1) that contributes to the growth of *Listeria monocytogenes* in suboptimal conditions. Journal of Applied Microbiology 109, 984–995.

Sanchez-Torres, V., Maeda, T., Wood, T.K. 2010. Global regulator H-NS and lipoprotein NlpI influence production of extracellular DNA in *Escherichia coli*. Biochemical and Biophysical Research Communications 15, 197-202. doi: 10.1016/j.bbrc.2010.09.026.

Schlech III, W.F., Lavigne, P.M., Brotolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, A.H., Nicholls, E.S., Broome, C.V., 1983. Epidemic listeriosis—evidence for transmission by food. New England Journal of Medicine 308, 203–206.

Simões, M., Simões, L., Vieira, M.J. 2010. A review of current and emergent biofilm control strategies. LWT- Food Science and Technology 43, 573–583.

Smoot, L.M., Pierson, M.D. 1998a. Influence of environmental stress on the kinetics and strength of attachment of *Listeria monocytogenes* Scott A to Buna-N rubber and stainless steel. Journal of Food Protection 61,1286-1292.

Smoot, L.M.,Pierson, M.D. 1998b. Effects of environmental stress on the ability of *Listeria monocytogenes* Scott A to attach to food contact surfaces. Journal of Food Protection 61, 1293-1298.

Stanley, N.R., Lazazzera, B. A. 2004. Environmental signals and regulatory pathways that influence biofilm formation. Molecular Microbiology 52, 917–924

Stea, E.C., Purdue, L.M., Jamieson, R.C., Yost, C.K., Truelstrup Hansen, L. 2015. Comparison of the prevalences and diversities of *Listeria* species and *Listeria monocytogenes* in an urban and a rural agricultural watershed. Applied and Environmental Microbiology 81, 3812-3822.

Suo, Y., Huang, Y., Liu, Y., Shi, C., Shi, X. 2012. The expression of superoxide dismutase (SOD) and a putative ABC transporter permease is inversely correlated during biofilm formation in *Listeria monocytogenes* 4b G. PLoS ONE 7(10),e48467. doi:10.1371/journal.pone.0048467

Szlavik, J., Paiva, D.S., Mørk, N. van den Berg, F., Verran, J.,Whitehead, K., Knøchel, S., Nielsen, D.S. 2012. Initial adhesion of *Listeria monocytogenes* to solid surfaces under liquid flow. International Journal of Food Microbiology, 152, 181-188.

Takahashi, H., Kuramoto, S., Miya, S., Kimura, B. 2011. Desiccation survival of *Listeria monocytogenes* and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. Food Control 22, 633-637.

Tauxe, R.V., Doyle, M.P., Kuchenmüller, T., Schlundt, J. , Stein, C.E. 2010. Evolving public health approaches to the global challenge of foodborne infections. International Journal of Food Microbiology 139, S16-S28.

Taylor, C.M., Beresford, M., Epton, H.A., Sigee, D.C., Shama, G.,Andrew, P.W., Roberts, I.S.2002. *Listeria monocytogenes relA* and *hpt* mutants are impaired in surface-attached growth and virulence. Journal of Bacteriology 184, 621–628

To, M.S., Favrin, S., Romanova, N., Griffiths, M.W. 2002. Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of *Listeria monocytogenes*. Applied and Environmental Microbiology 68, 5258–5264.

Todd, E.C.D., Notermans, S. 2011. Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. Food Control 22, 1484-1490.

Todhanakasem, T., Young, G.M., 2008. Loss of flagellum-based motility by *Listeria monocytogenes* results in formation of hyperbiofilms. Journal of Bacteriology 190, 6030–6034.

Tongkhao, K. 2011. Defining mechanisms of foodborne pathogen survival. Ph.D. Thesis. University of California, Davis, CA, USA.

Tresse, O., Lebret, V., Garmyn, D., Dussurget, O., 2009. The impact of growth history and flagellation on the adhesion of various *Listeria monocytogenes* strains to polystyrene. Canadian Journal of Microbiology 55, 189–196.

Unnerstad, H., Bannerman, E., Bille, J., Danielsson-Tham, M. L., Waak, .E, Tham, W. 1996. Prolonged contamination of a dairy with *Listeria monocytogenes*. Netherlands Milk and Dairy Journal, 50, 493–499.

van de Mortel M, Chang W-S, Halverson L. 2004. Differential tolerance of *Pseudomonas putida* biofilm and planktonic cells to desiccation. Biofilms 1:361-368. 673 25.

van der Veen, S., Abee, T. 2010. Dependence of continuous-flow biofilm formation by *Listeria monocytogenes* EGD-e on SOSresponse factor YneA. Applied and Environmental Microbiology 76, 1992–1995.

Vergara-Irigaray, M., Maira-Litrán, T., Merino, N., Pier, G.B., Penadés, J.R., Lasa, I. 2008. Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the *Staphylococcus aureus* cell surface. Molecular Microbiology 154, 865-877.

Verghese, B., Lok, M., Wen, J., Alessandria, V., Chen, Y., Kathariou, S., Knabel, S.2011. ComK prophage junction fragments as markers for *Listeria monocytogenes* genotypes unique to individual meat and poultry processing plants and a model for rapid niche-specific adaptation, biofilm formation, and persistence. Applied and Environmental Microbiology 77, 3279 –3292.

Vilain S, Pretorius JM, Theron J, Brozel VS. 2009. DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. Applied and Environmental Microbioology 75, 2861–2868.

Vogel, B.F., Truelstrup Hansen, L., Mordhorst, H., Gram, L. 2010. The survival of *Listeria monocytogenes* during long term desiccation is facilitated by sodium chloride and organic material. International Journal of Food Microbiology 140,192-200.

Wang, J., Ray, A.R., Hammons, S.R., Oliver, H.F. 2015. Persistent and transient *Listeria monocytogenes* strains from retail deli environments vary in their ability to adhere and form biofilms and rarely have *inlA* premature stop codons. Foodborne Pathogens and Disease 12, 151-158.

Wang, X., Preston III, J.F., Romeo1, T. 2004. The pgaABCD locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. Journal of Bacteriology 186, 2724–2734.

Ward, T.J., Ducey, T.F., Usgaard, T., Dunn, K.A., Bielawski, J.P., 2008. Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. Applied and Environmental Microbiology 74, 7629–7642.

Ward, T.J., Evans, P., Wiedmann, M., Usgaard, T., Roof, S.E., Stroika, S.G., Hise, K. 2007. Molecular and phenotypic characterization of *Listeria monocytogenes* from U.S. Department of Agriculture Food Safety and Inspection Service surveillance of ready-to-eat foods and processing facilities. Journal of Food Protection 73, 861–869.

Warriner, K., Namvar, A. 2009. What is the hysteria with *Listeria*? Trends in Food Science and Technology 20, 245-254.

Weatherill, S. 2009. Report of the independent investigator into the 2008 listeriosis outbreak. Retrieved September 16, 2015 from http://publications.gc.ca/site/archivee-archived.html?url=http://publications.gc.ca/collection_2009/agr/A22-508-2009E.pdf

Weller, D., Andrus, A., Wiedmann, M., den Bakker, H.C. 2015a. *Listeria booriae* sp. nov. and *Listeria newyorkensis* sp. nov., from food processing environments in the USA. International Journal of Systematic and Evolutionary Microbiology 65, 286–292.

Weller, D., Wiedmann, M., Strawn, L. 2015b. Spatial and temporal factors associated with an increased prevalence of *Listeria monocytogenes* in spinach fields in New York state. Applied and Environmental Microbiology 81, 6059-6069.

Whittaker, C. A., Hynes, R. O. 2002. Distribution and evolution of von Willebrand/Integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Molecular Biology of the Cell 13, 3369–3387.

Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L., Batt, C.A., 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infection and Immunology 65, 2707–2716.

Williams, D., Dunn, S., Richardson, A., Frank, J.F., Smith, M.A. 2011.Time course of fetal tissue invasion by *Listeria monocytogenes* following an oral inoculation in pregnant guinea pigs. Journal of Food Protection 74, 248–253.

Xue, J., Blais, B. 2012. Improved recovery of viable *Listeria monocytogenes* from stainless steel surfaces for subsequent detection. International Journal of Food Safety 14, 1-4.

Yarwood, J.M., Bartels, D.J., Volper, E.M., Greenberg, E.P. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. Journal of Bacteriology 186, 1838-1850.

Zhang, Q., Nguyen, T., McMichael, M., Velu, S.E., Zou, J., Zhou, X., Wu, H., 2015. New small-molecule inhibitors of dihydrofolate reductase inhibit *Streptococcus mutans*. International Journal of Antimicrobial Agents 46, 174-182.

Zhu, X, Long, F., Chen, Y., Knøchel, S., She, Q., Shi, X. 2008. A putative ABC transporter is involved in negative regulation of biofilm formation by *Listeria monocytogenes*. Applied and Environmental Microbiology 74(24), 7675–7683.

Ziegler, J., 2012. The distribution, diversity and functional characterization of the *Listeria* genomic Island 1. M.Sc. Thesis. University of Manitoba, Winnipeg, MB, Canada.

Appendix A: Supplemental Information – Chapter 4

Table S1. Primers used in arbitrary PCR reaction as described by Cao *et al.* (2007).

Primers		Sequence (5' to 3')
Round I		
Arbitrary	207	GGCCACGCGTCGACTAGTACNNNNNNNNNNNGTAAT
Left	255	CAGTACAATCTGCTCTGATGCCGCATAGTT
Right	269	GCTCTGATAAATATGAACATGATGAGTGAT
Round II		
Arbitrary	208	GGCCACGCGTCGACTAGTAC
Left	256	TAGTTAAGCCAGCCCCGACACCCGCCAACA
Right	270	TGTGAAATACCGCACAGATGCGAAGGGCGA
Sequencing		
Left	257	CTTACAGACAAGCTGTGACCGTCT
Right	271	GGGAATCATTTGAAGGTTGGTACT

Table S2. Primers designed to fill in sequencing gaps and absences in a PCR reaction using the transposon sequencing primer and the gene specific primer.

Mutant	Corresponding primer used	Primer sequence (5' – 3')	Tm (°C)
lmo0057:Himar1	270	ATCTCTGCCGGTGTACTTCC	59
lmo0433:Himar1	270	CGTTGGCACGGTGATAGTCT	60
lmo0434:Himar1	256	AGTGGTTGGGTTACTCTCCC	58
lmo0683:Himar1	270	CGTCGTTTTTCCGTAATCGCA	60
lmo1224:Himar1	256	TGGCAAGTTCTGCTTCACCA	60
lmo2526:Himar1	270	TTACTTCCTGGCCACAGTGC	60
lmo2558:Himar1	270	AATTGCCGCTCCCTCTTTCC	60
lmo2563:Himar1	270	TCGCTTATTTACCCCTTTCCG	58
lmo2572:Himar1	270	TAGCGCGTGTTCCTGATCTT	59

To amplify missing sequences, PCR (total volume 25 µl) was performed with 1 mutant colony added to 0.5 µL 1.5 mM Mg2+, 12.5 µL OneTaq Hot Start 2 × master mix (New England Biolabs, Whitby, ON, CA), 0.5 µL of each 10 µM primer, and 10 µL H₂O. Temperature conditions were for 5 min 95°C denaturing step, followed by 30 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 60 s, and final extension 5 min at 72°C.

Table S3. RNA expression primers

Target gene/ primer direction	Primer sequence (5' – 3')	Tm (°C)
lmo0263 F	ACGCAAGCGGATTTAGATGGA	60
lmo0263Rev	TGCTTTGTAACCCAGCAATCG	59
lmo0433Rev	GCTAGCGGAGTTATATCTGCTATTT	59
lmo0433F	ACAGACACGGTCTCACAAACA	60
lmo0434F	GTGACAGATGCAGTGACACA	58
lmo0434Rev	AGCGAACTTAGGTCCTTAACTTTAT	58
lmo0527 F	GGCACCATTTAACACCGCAA	60
lmo0527 Rev	ACTCCTTCACAAAGTGCCCA	59
lmo0528F	GAAGGCAATATGGATGCGCT	59
lmo0528Rev	TGGTGGATTTCCGCCTATGT	59
lmo0529F	GTGAAAACGGTGGAGTCCCT	60
lmo0529Rev	CGCAAACCCGATATTGAGCG	60
lmo0530F	ACGATGTTGCGTTCTACTCTT	58
lmo0530Rev	AGCCAGTTGGCAGGTGTATC	60
lmo0531F	ACGAATTTAACGCTTGGTTTGA	57
lmo0531Rev	AGCAAAGTTTCTTGGTCAATCGT	59
lmo0532F	GTGCTCCGTTTACCATTGGC	60
lmo0532Rev	CGTTTCGCCCACTTGCTTAG	60
lmo0683F	TTTAGCCACGGACCTCGAAC	60
lmo0683Rev	AATCGGTCAGCAAATCGTGC	60
lmo0684F	GACAAACCTGGACTCGCAGA	60
lmo0684Rev	CAAATGCTTGCGAGCGGTAA	60
lmo0690F	GGCTTAGATGCAGCAAGCAAA	60
lmo0690Rev	CAAGTTCTTTGATCAAGCTACCG	58
lmo0691F	TGCCGGAAATGGATGGCTTA	60
lmo0691Rev	ACGCCTCTAAAACTCGGTCC	60
lmo0971F	ACGTTCCAAAATCTTCAGTTCGAC	60
lmo0971Rev	ACGAAAAACACGCCTGAACG	60
lmo0972F	CGCGGTCAAATTCGGAAACA	59
lmo0972Rev	TGGCTTTTCGTGAAAATGTATTAGA	58
lmo0973F	CGAGCTGCGAAAGGTTTGTT	60
lmo0973Rev	TGTTGTTCCACTAGACATCGC	58
lmo1068F	ACGAACGTGAAAAAGCAGCA	59
lmo1068Rev	TCATTATTGCCACAAGCACCT	58
lmo1069F	ACTCACTATTCGAAATCACTACTGG	58
lmo1069Rev	ATGCGAACGTGTAGATGAGC	58
lmo1080F	TGGGATTTTAGGGCAATGGCTA	60
lmo1080Rev	TTCCAGCATCGATTTGAGCAAG	60
lmo1081F	CGGAAGGATTAGCACAAGCG	59
lmo1081Rev	TCTTTCAGGATCATTCACGTGGT	60
lmo1224 F	GCGATGGTAACAAGCTAACTGA	59
lmo1224 Rev	AAAGCTTGTTTTGCTGCTAAGT	58

lmo1225 F	TGGTATCAGCGCAACAGAACT	60
lmo1225 Rev	CTCGAGCCATTCATTCACGA	58
lmo1226 F	AGACGAGCTACACGTAACGA	59
lmo1226 Rev	GTGTCAACGCCTTTTACATCCA	59
lmo2482F	ACTTGGCGCAACAACATCTG	59
lmo2482Rev	GTGGATTTGCTGATTTGGGCA	59
lmo2481F	ACCTTCAGGATCTGGTTTAGCA	59
lmo2481Rev	GGAGTTTATGAAGCGATTCGTGC	61
lmo0676F	CAAGTGCTTGTTGGACTGGC	60
lmo0676Rev	TTTTGTCAGAAGCGGCTGTG	59
16S rRNA F	AGAGTTTGATCATGGCTCAG	54
16S rRNA Rev	GTGTGACGGGCGGTGTGTAC	54

Table S4. Bands representing upstream RNA expression in mutant lmo0531:Himar1

Gene	Affected gene expression	
	mutant	Lm568
lmo00527	▬	▬
lmo0528	▬	▬
lmo0529	▬	▬
lmo0530	▬	▬
lmo0531	-	▬

Table S5. Bands representing RNA product expression in mutants lmo0433:Himar1, lmo0434:Himar1, and lmo0263:Himar1

Strain	Control genes		Affected gene expression (N terminal region)	
	16S rRNA	*fliP*	mutant	Lm568
Lmo0433:Himar1	▬	▬	▬	▬
lmo0434:Himar1	▬	▬	-	-
lmo0263:Himar1	▬	▬	-	▬

Appendix B: Supplemental Information – Chapter 5

Table S6. Primers used in the serogrouping protocol.

Primer Name	5' to 3' Sequence	reference
Lmo737F	AGGGCTTCAAGGACTTACCC	Doumith et al., 2004
Lmo737R	ACGATTTCTGCTTGCCATTC	
Lmo1118F	AGGGGTCTTAAATCCTGGAA	
Lmo1118R	CGGCTTGTTCGGCATACTTA	
Orf2819F	AGCAAAATGCCAAAACTCGT	
Orf2819R	CATCACTAAAGCCTCCCATTG	
Orf2110F	AGTGGACAATTGATTGGTGAA	
Orf2110R	CATCCATCCCTTACTTTGGAC	
LIP1 (*prfA*F)	GATACAGAAACATCGGTTGGC	Kérouanton et al., 2010
LIP2a (*prfA*R)	GTGTAATCTTGATGCCATCAGG	
2229F	TTGTTGAAGGAAGAGGTGGTC	Knabel et al., 2012
2229R	TCTTTTCGGCTCATTTTCGT	
1886F	TAACGACAACTATGCCAACG	
1886R	CGACAGTTTGTATTCCACCA	
lmo0448F	CTTGCAGCAAGCTTCATC	Ryan et al., 2010
lmo0448R	CATAAGAATCCACATAGTAG	

Appendix C: Co-authored publication "Genes Associated with Desiccation and Osmotic Stress in *Listeria monocytogenes* as Revealed by Insertional Mutagenesis"

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Author Piercey led the generation of the mutant library, performed the experiments to demonstrate random insertion of the transposon, determined plasmid retention, assisted in arbitrary PCR experiments and primer design, performed the RT-PCR experiments, and had a role in writing the paper.

Abstract

Listeria monocytogenes is a foodborne pathogen whose survival in food processing environments may be associated with its tolerance to desiccation. To probe the molecular mechanisms used by this bacterium to adapt to desiccation stress, a transposon library of 11,700 *L. monocytogenes* mutants was screened, using a microplate assay, for strains displaying increased or decreased desiccation survival (43% relative humidity, 15°C) in tryptic soy broth (TSB). The desiccation phenotypes of selected mutants were subsequently assessed on food-grade stainless steel (SS) coupons in TSB plus 1% glucose (TSB-glu). Single transposon insertions in mutants exhibiting a change in desiccation survival of >0.5 log CFU/cm^2 relative to that of the wild type were determined by sequencing arbitrary PCR products. Strain morphology, motility, and osmotic stress survival (in TSB-glu plus 20% NaCl) were also analyzed. The initial screen selected 129 desiccation-sensitive (DS) and 61 desiccation-tolerant (DT) mutants, out of which

secondary screening on SS confirmed 15 DT and 15 DS mutants. Among the DT

mutants, seven immotile and flagellum- less strains contained transposons in genes

involved in flagellum biosynthesis (*fliP*, *flhB*, *flgD*, *flgL*) and motor control (*motB*, *fliM*,

fliY), while others harbored transposons in genes involved in membrane lipid

biosynthesis, energy production, potassium uptake, and virulence. The genes that were

interrupted in the 15 DS mutants included those involved in energy production,

membrane transport, protein metabolism, lipid biosynthesis, oxidative damage control,

and putative virulence. Five DT and 14 DS mutants also demonstrated similar

significantly ($P<0.05$) different survival relative to that of the wild type when exposed to

osmotic stress, demonstrating that some genes likely have similar roles in allowing the

organism to survive the two water stresses.

The foodborne bacterial pathogen *Listeria monocytogenes* continues to be a

significant issue in the food supply chain, causing repeated recalls and outbreaks of

foodborne illness. *L. monocytogenes* is ubiquitous in nature but is transmitted to humans

predominantly through contaminated food, with the majority of listeriosis outbreaks

being associated with ready-to-eat (RTE) products, such as deli meats, soft cheeses, and

fresh produce (1, 2).Listeriosis is a very serious disease with mortality rates of 20 to 40%

(3). Populations which are more susceptible to listeriosis include the elderly, pregnant

women, newborns, and immunocompromised individuals.

Several foodborne outbreaks of listeriosis have been traced back to the processing

facilities in which the products were made (4–8). Recent literature also suggests that

contamination at the retail level may be a significant cause of listeriosis (9–11). In both

environments, *L. monocytogenes* has been isolated from food contact surfaces and non-food contact surfaces, such as floors and drains (12). Once introduced into a processing environment, *L. monocytogenes* can persist for years, despite the harsh environmental conditions that it encounters during food processing, preservation, and plant sanitation efforts (13). The persistence of *L. monocytogenes* in the food supply chain has most commonly been attributed to its ability to adapt to refrigeration temperatures and high-acid and high-salt environments (14, 15). Additionally, many strains are capable of adhering to and subsequently forming biofilms on a variety of abiotic surfaces, further enhancing the resistance of cells to sanitizers and dehydration. Recently, it was shown that *L. monocytogenes* can survive desiccation for 3 months on stainless steel (SS) surfaces in a simulated food processing environment (16), demonstrating that desiccation tolerance also plays a significant role in the bacterium's ability to persist.

Previous studies found that the survival of *L. monocytogenes* on food-grade stainless steel surfaces was enhanced by environmental factors and mechanisms, such as osmoadaptation prior to desiccation; desiccation in the presence of 5% NaCl, food residues, and lipids; and the formation of a mature biofilm (16–19). However, the genetic factors and mechanisms used by this bacterium to adapt to desiccation stress still remain largely understudied. Some hypotheses can be derived from literature describing the response of other cells (microbe, plant, or animal) to desiccation stress, as the fundamental principles of dehydration damage at the molecular level (i.e., proteins, lipids, carbohydrates, and nucleotides) are similar. Cellular dehydration causes solutes to concentrate within the cell, thereby imparting both osmotic and oxidative stresses (20). When the aqueous monolayer surrounding macromolecules is removed, fundamental

cellular machinery, such as ribosomes, enzymes, transport systems, and important

metabolic pathways, may become impaired (21). Correspondingly, transcriptome studies

on Gram-negative bacteria subjected to desiccation stress, specifically, *Salmonella* (22,

23) and *Pseudomonas* spp. (24, 25), identified the upregulation of genes involved in lipid

and amino acid biosynthesis, DNA repair, potassium ion transport, and osmolyte uptake,

among others.

The goal of the present study was to elucidate novel genetic factors contributing

to desiccation tolerance in *L. monocytogenes*. This was accomplished by constructing a

library of *L. monocytogenes* mutants with random *Himar1* transposon insertional

mutations in a serotype 1/2a food isolate. The library was then screened for mutants

displaying increased or decreased desiccation tolerance on stainless steel surfaces

compared to the desiccation tolerance of the wild type (WT). Our results demonstrate that

the desiccation tolerance of *L. monocytogenes* is a complex process involving a wide

variety of genes. Ultimately, 30 desiccation-associated loci were identified in this study,

and some of these correspond to those found previously in other bacteria and others

represent loci newly recognized to be involved in desiccation tolerance. We also report

that 19 of these genes likely have a role in adaptation to severe osmotic stress.

MATERIALS AND METHODS
Bacterial strains and media. Insertional mutagenesis was performed in *L.*

monocytogenes 568, which is a serotype 1/2a food isolate (26). All reagents and

antibiotics used in this study were purchased from Fisher Scientific (Whitby, Ontario,

Canada) and Sigma-Aldrich (Oakville, Ontario, Canada), respectively. Unless otherwise

stated, when necessary, medium was supplemented with 10 μg/ml erythromycin (Erm)

and/or kanamycin (Kan). Routine culturing was carried out on brain heart infusion (BHI; Difco) agar (15% [wt/wt] technical agar; Difco) or in tryptic soy broth (TSB; Difco). All bacterial stocks were stored at -80°C in BHI broth supplemented with 20% glycerol.

Generation of transposon library. Insertional mutagenesis was carried out using the shuttle vector pMC39 (kindly obtained from the H. Marquis Laboratory, Cornell University, Ithaca, NY, USA), which contains a temperature-sensitive origin of replication and the *Himar1* mariner transposon (27). The pMC39 plasmid was electroporated into competent *L. monocytogenes* 568 cells prepared as described by Alexander et al. (28), using 0.1-cm cuvettes in a Micropulser electroporator (Bio-Rad Laboratories, Inc., Mississauga, Ontario, Canada) set to a field strength of 1.0 kV. Following electroporation, the cells were resuspended in 1.0 ml of SOC medium (2% tryptone [Oxoid], 0.5% yeast extract [Oxoid], 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ [Mallinckrodt], 20 mM glucose) and allowed to recover for 3 h at 28°C under static conditions. Transposon mutagenesis was performed following a previously described protocol (29). *L. monocytogenes* 568 transformants were selected at 30°C on BHI agar-Erm (5 μg/ml). Individual colonies were then inoculated and grown overnight in BHI agar-Erm plus Kan at 30°C with shaking. The cultures were diluted 1/200 in BHI agar-Erm, grown for 1 h at 30°C with shaking, and then shifted to 40°C for approximately 6 h until the A_{600} was 0.3 to 0.5 (NanoPhotometer P330; Implen, Westlake Village, CA, USA). Aliquots of the culture were spread plated on BHI agar-Erm and incubated at 40°C for 2 days. Approximately 11,700 mutant colonies were inoculated into polystyrene microtiter plates (96-well plates; Costar) containing 100 μl of TSB and 100 μl of 30% (wt/wt) glycerol in distilled water (dH_2O) and grown for 2 days at room

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temperature (~22°C), before being stored at -80°C until further use. Plasmid retention was determined to be less than 2.5%, and 25 mutants were examined to confirm random insertion of the transposon in the *L. monocytogenes* 568 chromosome.

Screening the transposon library for DT and DS mutants. *L. monocytogenes* 568 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 at room temperature, and 10 μl from each well was used to inoculate 190 μl of fresh TSB distributed in microtiter plates. The plates were incubated overnight at room temperature, and final readings of the absorbance at 490nm were recorded using a BioTek* ELx808* absorbance microplate reader (Fisher Scientific) connected to a computer operating Gen5 (version 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 microtiter plates containing 190 μl of fresh TSB, and the plates were incubated at 15°C. The absorbance at 490 nm of each well was recorded every 3 h until all mutants reached stationary phase (approximately 20 h).To isolate mutants with an altered desiccation phenotype, 10 μl from the overnight culture plates was spotted on the bottom of empty microtiter plates, and the plates were placed in desiccators preconditioned to 43% relative humidity (RH; using saturated K_2CO_3) and 15°C as previously described (17). After 5 days of desiccation, the growth in each well, representing one unique transposon mutant, was rehydrated with 190 μl of TSB, and the plates were incubated at 15°C under static conditions. The plates were briefly shaken every 3 h, and the A_{490} was recorded until stationary phase was achieved by the majority

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of isolates (approximately 28 h). The resulting rehydration growth curves were used to select mutants for the second screening procedure. The premise of this assay was that desiccation-tolerant (DT) and desiccation-sensitive (DS) mutants would show faster and slower regrowth after desiccation, respectively, than the WT. The resulting curves were visually assessed, and mutants were categorized as DT if they displayed short lag phases and reached an A_{490} of 0.40 more than 2 h before the majority of mutants. Conversely, DS mutants were selected on the basis of displaying long lag phases that resulted in mutants achieving an A_{490} of 0.10 more than 2 h after the majority of isolates and the WT. DS mutants were discarded if they displayed low growth rates under regular conditions at 15°C.

Quantitative determination of desiccation survival on stainless steel coupons. In total, 190 mutants were selected from the first screening procedure and streaked out on BHI agar-Erm and BHI agar-Kan to confirm the presence of the transposon and the absence of the pMC39 plasmid, respectively. From the BHI agar-Erm plates, colonies were inoculated into 5 ml of TSB supplemented with 1% (wt/vol) glucose (TSB-glu) and incubated at 15°C for 48 h. Following incubation, the cultures were centrifuged, resuspended in fresh TSB-glu to an A_{450} of 1, and diluted to obtain a cell density of 7.5 log CFU/cm^2 when spotted onto sterile foodgrade SS (314 finish) coupons (0.5 by 0.5 cm). The inoculated coupons were desiccated (43% RH, 15°C) for 7 days. Three coupons were prepared for each mutant per sampling day (days 0, 1, 2, 4, and 7), and survivors were released from the coupons by sonication and vortexing (17), followed by enumeration via the plate count method on BHI agar. Mutants which exhibited a >0.5 log

CFU/cm^2 increase or decrease in survival relative to that of the WT during the 7-day desiccation period were selected for arbitrary PCR and DNA sequencing.

Arbitrary PCR. To determine the affected genes in the desiccation mutants, the arbitrary primer PCR method (27, 29) was used to determine the nucleotide sequences flanking the transposon insertion sites. This method uses two sequential PCRs to amplify the transposon-chromosome junctions. The first arbitrary PCR was performed using an IDTaqDNA polymerase kit (ID Labs Manufacturing Inc., London, ON, Canada). Each 24.5-µl reaction mixture contained 2.5 µl 10µ MgCl2-free buffer, 0.5 µl deoxynucleoside triphosphate mixture (2.5mMeach), 2.0 µl MgCl2, 0.25 µl ID Labs *Taq* polymerase, 18.25 µl RNA-free dH$_2$O, 0.5 µl of each primer stock (10µM; see Table S1 in the supplemental material), and one mutant colony from BHI agar-Erm plates. The following PCR cycle was conducted using a Tgradient thermocycler (Whatman Biometra, Goettingen, Germany) and consisted of a 5-min initial denaturation step at 95°C, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 34°C for 45 s, and elongation at 72°C for 1 min. This was followed by a final extension step of 10 min at 72°C. The resulting PCR products were then diluted by adding 0.5 µl to 24.5 µl of RNA-free dH$_2$O. The second PCR used 5 µl 5X buffer, 0.5 µl high-fidelity *Taq* DNA polymerase (HotStar *Taq* DNA polymerase; Qiagen, Toronto, ON, Canada), 2.5 µl of each primer stock (10 µM; see Table S1 in the supplemental material), and 14 µl RNA-free dH$_2$O for a total of 25 µl, including 0.5 µl of the diluted products from round I. The PCR cycle was as follows: a 5-min initial denaturation step at 95°C, followed by 40 cycles of 95°C for 30 s, 34°C for 30 s, and 72°C for 1 min. The final extension step was

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at 72°C for 10 min. Gel electrophoresis in an agarose gel (1.5% agarose, 1% TAE [Tris-acetate- EDTA]) was used to confirm the presence of amplicons prior to sequencing.

Identification of transposon insertion sites. Purification of the products obtained by the arbitrary PCR method was performed using a QIAquick PCR purification kit (Qiagen) as directed by the manufacturer. Purified samples were diluted in pH-adjusted dH_2O (pH 7.0 to 8.5) to concentrations of 1 to 10 ng/μl and sequenced by the Sanger method (McGill University and Génome Québec, Montréal, Québec, Canada). Sequences were aligned to the complete genome sequence of *L. monocytogenes* strain EGD-e (GenBank accession number NC_003210) (30) using BLASTn online software (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). Where the functions of some genes were not annotated in EGD-e, BLASTn searches were conducted on other *L. monocytogenes* 1/2a serotype genomes. Sequencing gaps and absences were closed in using additional primers (see Table S2 in the supplemental material).

Osmotic stress survival. Sequenced desiccation mutants were tested for survival during severe osmotic stress exposure. Colonies from BHI agar-Erm plates were precultured in 5 ml TSB-glu for 2 days at 15°C, centrifuged, resuspended in TSB-glu containing 20% (wt/wt) NaCl (water activity, 0.86) to a final concentration of 7.5 log CFU/ml, and incubated at 15°C for 49 h. Survivors were regularly enumerated in triplicate by plating on BHI agar.

Motility assay and staining of flagella. Insertion of the transposon affected genes involved in flagellum biosynthesis and motility in seven DT mutants. To elucidate any possible causal relationship between impaired motility and desiccation tolerance, the

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motility of all mutants with respect to that of the WT was assessed at 15°C. To accomplish this, colonies from BHI agar plates (grown at 20°C for 48 h) were stabbed in triplicate into petri dishes containing soft BHI agar (0.3%, wt/wt) and incubated for 72 h at 15°C. A large area of bacterial growth surrounding the initial point of inoculation was indicative of bacterial motility. The presence or absence of flagella in seven mutants immotile at 15°C was determined using a crystal violet-based method (110) with the WT serving as a control. Prior to staining, cells were grown for 2 days at 20°C on BHI agar. Stained slides were examined under x1,000 magnification with a Nikon Eclipse 80i light microscope (Nikon Canada, Mississauga, ON, Canada).

Endpoint RT-PCR to determine the expression of selected flagellum-associated genes. *L. monocytogenes* 568 and motility mutants were grown statically in TSB-glu at 15°C until they reached an A_{450} of ~0.8. One volume of each bacterial culture was added to 2 volumes of RNA stabilizer (RNAprotect bacterial reagent; catalog number 76506; Qiagen). RNA was extracted (RNeasy minikit; Qiagen) after pretreatment of the cells with 20 mg/ml lysozyme (Roche, Mississauga, ON, Canada) and 20 μg/ml proteinase K (Roche) for 15 min at 37°C. Genomic DNA (gDNA) wipeout buffer (Qiagen) was used for 2 min at 42°C to eliminate gDNA. RNA was then converted to cDNA (QuantiTect reverse transcriptase [RT] kit; Qiagen), with the reverse transcriptase reaction mixture being incubated at 42°C for 25 min, followed by quenching at 95°C for 3 min. PCR was performed on the cDNA using primers (see Table S3 in the supplemental material) designed to amplify (150 to 200 bp) the affected or adjacent downstream gene in each of the seven motility mutants. The primers were first confirmed to work on gDNA. For each gene, after gDNA removal sample RNA was used as the negative

control, while RT PCR using primers specific for 16S rRNA and *flaA* (i.e., a motility gene not impacted in any of the mutants) served as the positive control. Each PCR (25 μl) was performed using a PCR mixture consisting of 1.5 μl of 25 mM Mg2^{++}, 12.5 μl of One*Taq* Hot Start 2x master mix (NEB, Ipswich, MA, USA), 0.5 μl of each 10 μM primer, 9 μl H$_2$O, and 1 μl of a 1/10-diluted cDNA mixture or an equivalent amount of RNA following gDNA removal. The temperature conditions for the PCR were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 45 s (54°C for 16S rRNA), and 72°C for 60 s and a final extension at 72°C for 5 min.

Heat tolerance assay. Mutant DT10 contained the transposon insertion in the butyrate kinase gene (*lmo1370*, *buk*), whose interruption was previously reported to produce a heat-resistant mutant of *L. monocytogenes* 568 (31). To determine if DT10 also exhibited this phenotype, its survival during a mild heat treatment was compared to that of the WT. Cells were first cultured in TSB-glu for 2 days at 15°C and then centrifuged and resuspended in peptone saline (PS; 0.1% peptone, 0.85% NaCl) to a concentration of 7.5 log CFU/ml. Aliquots of 110 μl were distributed in PCR tubes and heated to 55°C in a thermocycler (Tgradient; Whatman Biometra) for 30 min. Treated samples (*n*=3) were serially diluted in PS, and survivors were enumerated on BHI agar.

Statistical analysis. The desiccation and osmotic stress survival of the mutants was statistically compared to that of the WTusing one-way analysisof variance (ANOVA) with a Dunnett's *post hoc* test in IBM SSPS statistics software. Differences were concluded to be significant when *P* was <0.05.

RESULTS

Discovery of DT and DS mutants. Screening of 11,700 transposon insertion mutants for desiccation survival in a microplate assay resulted in the detection of 129 desiccation-sensitive (DS) and 61 desiccation-tolerant (DT) candidates. Secondary screening of these mutants on food-grade SS coupons yielded 15 DT and 15 DS mutants, each with a ~0.5 log CFU/cm^2 increase or decrease in survival, respectively, compared to that of the WT during the 7-day desiccation trial.

After 24 h of desiccation on the SS surface, 14 DT mutants displayed significantly ($P < 0.05$) increased survival compared to that of the WT, and 9 of these maintained significantly ($P<0.05$) enhanced survival after 7 days (Table 1). Four mutants (DT02, DT06, DT10, DT11) exhibited a final mean log reduction of < 1 log CFU/cm^2, with the two most tolerant mutants (DT10 and DT11) displaying < 0.20 log CFU/cm^2 reductions in survival, whereas that for the WT was 1.72 log CFU/cm^2 (Table 1). While all other DT and DS mutants had growth rates similar to the growth rate of the WT, both DT10 and DT11 displayed reduced growth rates in TSB-glu at 15°C. The reduced growth rates of these mutants were masked in the initial microtiter plate rehydration assay due to the minimal viability loss after 5 days of desiccation. Unlike the other mutants, which reached stationary phase (A_{450}, >1.20) during preculturing (2 days, 15°C), DT10 and DT11 reached only late exponential phase (A_{450}, ~0.60) before being harvested, resuspended, and inoculated (7.5 log CFU/cm^2) onto the SS coupons. Interestingly, after 4 days of desiccation on SS coupons, DT11 colonies on BHI agar took on a crateriform donut-shaped morphology (Fig. 1).

After 1 and 7 days of desiccation, 9 and 14 of the 15 DS mutants, respectively, showed significantly ($P < 0.05$) impaired desiccation survival compared to that of the WT. For these mutants, final losses in viability ranged from 2.37 to 4.58 log CFU/cm^2, whereas a final loss in viability of 1.72 log CFU/cm^2 was observed for the WT (Table 2). All strains experienced the most inactivation within the first 24 h of desiccation. While some strains experienced little additional losses over the remaining desiccation period, the viability of others continued to steadily decrease throughout the 7 days. Five mutants (DS01, DS04, DS07, DS10, DS15) achieved a final mean reduction of >4 log CFU/cm^2. Nine DS mutants were consistently significantly ($P< 0.05$) more susceptible to desiccation than the WT throughout the experiment (Table 2). It is important to note that even the most DS mutants had survival levels of ~3.0 log CFU/cm^2 after 7 days of desiccation on SS.

Location of transposon insertion sites. DNAsequences flanking both sides (left and right) of the transposon insertion site were successfully obtained for 26 mutants using the arbitrary PCR method. For these mutants, identification of corresponding left and right sequences made it likely that only one transposon was inserted. Additional primers were designed to amplify the unsuccessfully sequenced side in four mutants (DS01, DS07, DS09, DS10). Although the location of one transposon was confirmed in these mutants, the possibility of the presence of other transposons, however unlikely, cannot be excluded. Overall, the exact insertion site of 19 transposons was identified, whereas in EGD-e the mapped locations of the left and right sequences were separated by 1 bp in seven mutants and 3, 6, 20, and 52 bp in the remaining four mutants, respectively (see Table S4 in the supplemental material). These gaps may be a result of the arbitrary PCR

method, sequence quality, or discrepancies between the sequences of the genomes of *L. monocytogenes* 568 and EGD-e. The gene identities and their known or predicted functions are listed in Tables 1 and 2 for the DT and DS mutants, respectively. The prevalence of specific gene functional groups is displayed in Fig. 2, and the distribution of insertion sites mapped to the EGD-e chromosome is shown in Fig. 3.

The interrupted genes in seven DT mutants were associated with motility, including flagellar biosynthesis (*fliP*, *flhB*, *flgD*, *flgL*) and motor control (*motB*, *fliM*, *fliY*). Other affected genes in DT mutants were classified as contributing to membrane lipid biosynthesis (*buk*, *lpd*, *yycH*), potassium uptake (*lmo0771*), energy production (*adeC*), and virulence (*inlC*). The extremely desiccation-tolerant mutants DT10 and DT11 harbored transposons in neighboring genes (*lmo1370* and *lmo1371*, respectively) encoding butyrate kinase (*buk*) and dihydrolipoamide dehydrogenase (*lpd*), respectively, which are involved in fatty acid (FA) metabolism. Mutants DT13 and DT14 also exhibited insertions in nearby genes (*lmo1742* and *lmo1744*, respectively) encoding adenine deaminase and an NAD-dependent epimerase/dehydratase, respectively. The transposon location in the remaining four DT mutants was mapped to dispersed locations in the EGD-e chromosome. In mutant DT01, the affected gene (*lmo0289*, *yycH*) encodes a regulatory protein of the sensory box histidine kinase two-component system (TCS) YycFG which is involved in cell wall homeostasis, membrane integrity, and cell division. Mutant DT09 had an affected ATP-sensitive inward rectifier potassium channel (*lmo0771*), and mutants DT12 and DT15 had an interrupted adenine- specific DNA methylase (*lmo1582*) and internalin C (*inlC*), respectively.

In the DS mutants, most transposon insertions were mapped to one of three areas of the EGD-e chromosome. The first cluster encompassed mutants DS05 to DS07 with insertions in *lmo1174*, *lmo1194*, and either the intergenic region of *lmo1219-lmo1220* or *lmo1220*, respectively. These genes encode an ethanolamine utilization protein (EutA), a cobalt-precorrin-6A synthase (CbiD), and an intergenic region or an HxIR DNA binding protein (*lmo1220*), respectively. The second area included mutants DS09 to DS12 and involved the genes *lmo2443*, *lmo2470*, *lmo2490*, and *lmo2503*, respectively. The first gene, *lmo2443*, encodes glutamate tRNA ligase, while *lmo2470* is a *L. monocytogenes*-specific secreted internalin. The gene *lmo2490* encodes CsbA, a σ^B-regulated transmembrane general stress response protein, and *lmo2503* is a cardiolipin synthase.

The last cluster of DS-associated transposon insertion sites were found in mutants DS13 and DS14, both of which contained the transposon in *lmo2768*, an uncharacterized membrane transport protein, and mutant DS15, which contained a transposon in *lmo2778*, encoding an uncharacterized protein consisting of 170 amino acids.

Among the remaining five unclustered DS mutants were DS01 to DS04 and DS08. DS01 contained a transposon in the intergenic region between the genes *lmo0371* and *lmo0372*, which encode a GntR family transcriptional regulator and beta-glucosidase, respectively, with *lmo0371* being individually transcribed and encoded on the complement strand. Mutant DS02 contained the transposon in *lmo0565*, corresponding to the HisH subunit of imidazole glycerol phosphate synthase. The affected gene (*lmo0616*) in mutant DS03 was that for glycerophosphoryl diester phosphodiesterase, which acts on membrane glycerophospholipids. Lastly, mutants DS04 and DS08 contained transposons

in genes *lmo0983* and *lmo1728*, respectively, encoding glutathione peroxidase and cellobiose-phosphorylase, respectively.

Osmotic stress survival. Five DT mutants also demonstratedsignificantly ($P<0.05$) enhanced osmotic stress survival after 49 h of exposure to 20% NaCl (Table 1). For these mutants, mean final losses ranged from 0.10 to 0.41 log CFU/ml, whereas the loss for the WT was 0.74 log CFU/ml (Table 1). Three of the most osmotolerant mutants harbored transposons in flagellar biosynthesis and structure-related genes (*fliP*, *flhB*, *flgL*), whereas two DT flagellar mutants with interrupted motor control genes (*fliM*, *motB*) exhibited significantly ($P< 0.05$) reduced osmotolerance. Seven other DT mutants showed initial osmotolerance relative to the tolerance of the WT; however, the overall losses after 49 h were not significantly ($P>0.05$) different. The colony morphology of mutant DT11, which took on a donut shape when cultured after desiccation on SS coupons, displayed irregularly shaped colonies after exposure to high salt concentrations (Fig. 1).

Of the 15 DS mutants, 14 displayed significantly ($P < 0.05$) impaired osmotolerance after 33 h (data not shown). This impairment remained significant for seven mutants after 49 h of exposure, with mean final losses ranging from 0.92 to 1.25 log CFU/ml (Table 2). The most osmosensitive (OS) mutants (DS05, DS07, and DS08) displayed >1.15 log CFU/ml mean final reductions in survival, and all contained transposons in genes associated with energy production.
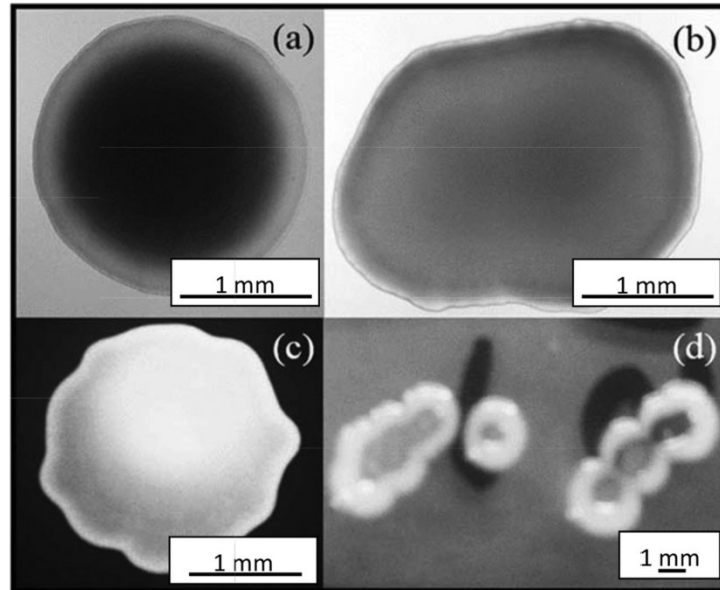
FIG 1 Colony morphology of the *Listeria monocytogenes* 568 wild type and its DT transposon mutants on BHI agar after 2 days of incubation at 30°C. (a) *L. monocytogenes* 568; (b) DT01 (*lmo0289*, *yycH*); (c and d) DT11 (*lmo1371*, *lpd*) plated on BHI after 49 h of osmotic stress (TSB-glu plus 20% NaCl, 15°C) (c) and 4 days of desiccation (43% RH, 15°C) (d).

When directly comparing the effect of osmotic and desiccation stresses after 49 and 48 h, respectively, four DT mutants (DT04, DT10, DT11, and DT15) displayed significantly ($P < 0.05$) higher survival levels after desiccation stress than after osmotic stress, while four mutants (DT01, DT03, DT05, and 09) displayed significantly ($P < 0.05$) lower survival levels after osmotic stress (Table 1). In contrast, all DS mutants had significantly ($P < 0.05$) lower survival levels following desiccation stress than following osmotic stress (Table 2). With the exception of the extremely desiccation-tolerant mutants DT10 and DT11, all mutants exhibited lower viability losses after they were exposed to 20% NaCl for 49 h than after they were desiccated at 43% RH for 7 days.

TABLE 1 Transposon insertion sites identified in *Listeria monocytogenes* DT mutants mapped to the complete EGD-e sequenced genome and the corresponding losses in viability observed during desiccation and exposure to severe osmotic stress[a]

Lm strain	Gene	Gene locus tag	Function	Survival reduction (mean±SD Δlog CFU/cm^2 [desiccation] or Δlog CFU/ml [osmotic stress])				
				Desiccation				Osmotic Stress at
				Day 1	Day 2	Day 4	Day 7	49 h
Lm 568				-1.48 ± 0.14	-1.52 ± 0.12	-1.74 ± 0.03	-1.72 ± 0.15	-0.74 ± 0.17[b]
DT01	*lmo_0289*	*yycH*	Sensory box histidine kinase two-component system regulatory protein [a]	-0.67 ± 0.09 [d]	-0.87 ± 0.05 [d]	-1.20 ± 0.25 [e]	-1.33 ± 0.28	-0.53 ± 0.14[b]
DT02	*lmo_0676*	*fliP*	Flagellar biosynthesis protein	-0.49 ± 0.26 [f]	-0.54 ± 0.11[f]	-0.71 ± 0.19 [f]	-0.91 ± 0.23 [d]	-0.22 ± 0.16 [f]
DT03	*lmo_0679*	*flhB*	Flagellar biosynthesis protein	-0.75 ± 0.23 [d]	-0.80 ± 0.20 [f]	-1.03 ± 0.45 [e]	-1.51 ± 0.53	-0.10 ± 0.09 [bf]
DT04	*lmo_0689*	*motB*	Chemotaxis flagella motor rotation protein [a]	-0.97 ± 0.03 [e]	-0.79 ± 0.17 [f]	-1.47 ± 0.36	-1.20 ± 0.04	-1.13 ± 0.10 [dg]
DT05	*lmo_0696*	*flgD*	Flagellar basal body rod modification protein	-1.12 ± 0.14	-1.40 ± 0.09	-1.28 ± 0.30	-1.16 ± 0.24 [d]	-1.00 ± 0.10[b]

DT06	*lmo_0699*	*fliM*	Flagellar motor switch protein	-0.89 ± 0.16 [e]	-1.06 ± 0.15 [e]	-0.97 ± 0.18 [d]	-0.91 ± 0.14 [d]	-1.07 ± 0.14 [d]
DT07	*lmo_0700*	*fliY*	Flagellar motor switch protein	-0.87 ± 0.27 [e]	-0.96 ± 0.05 [d]	-1.20 ± 0.15 [e]	-1.44 ± 0.19	-0.72 ± 0.05
DT08	*lmo_0706*	*flgL*	Flagellar hook-associated protein	-0.99 ± 0.40 [e]	-0.83 ± 0.36 [f]	-1.30 ± 0.55	-1.26 ± 0.31	-0.14 ± 0.02 [f]
DT09	*lmo_0771*		ATP-sensitive inward rectifier potassium channel 15 [a]	-0.74 ± 0.26 [d]	-1.07 ± 0.27 [e]	-1.05 ± 0.14 [d]	-1.18 ± 0.02 [e]	-0.47 ± 0.10 [b]
DT10	*lmo_1370*	*buk*	Butyrate kinase	-0.01 ± 0.09 [f]	-0.05 ± 0.11 [f]	-0.10 ± 0.05 [f]	-0.10 ± 0.19 [f]	-0.41 ± 0.15 [rg]
DT11	*lmo_1371*	*lpd*	Branched-chain alpha-keto acid dehydrogenase E3 subunit	-0.05 ± 0.07 [f]	-0.14 ± 0.20 [f]	-0.09 ± 0.04 [f]	-0.19 ± 0.13 [f]	-0.51 ± 0.04 [g]
DT12	*lmo_1582*		Acetate kinase [a]	-0.83 ± 0.29 [d]	-0.91 ± 0.23 [d]	-1.20 ± 0.07 [e]	-1.26 ± 0.26	-0.70 ± 0.09
DT13	*lmo_1742*	*adeC*	Adenine deaminase	-0.70 ± 0.12 [d]	-0.73 ± 0.21 [f]	-0.95 ± 0.08 [d]	-1.10 ± 0.17 [e]	-0.66 ± 0.12
DT14	*lmo_1744*		Uncharacterized protein	-0.66 ± 0.26 [f]	-0.29 ± 0.04 [f]	-0.72 ± 0.16 [f]	-1.11 ± 0.10 [e]	-0.39 ± 0.07 [e]
DT15	*lmo_1786*	*inlC*	Internalin C	-0.55 ± 0.32 [f]	-0.41 ± 0.08 [f]	-0.64 ± 0.12 [f]	-1.12 ± 0.33 [e]	-0.64 ± 0.12 [g]

a Cells were desiccated for 7 days at 43% RH and 15°C in TSB plus 1% glucose on SS coupons at an initial concentration of 7.5 log CFU/cm^2. Osmotic stress survival was determined by resuspending TSB-glu-grown cultures to 7.5 log CFU/ml in TSB-glu plus 20% NaCl for 49 h at 15°C ($n = 3$). The complete *L. monocytogenes* EGD-e sequenced genome can be found in GenBank under accession number NC_003210.

b Significantly ($P < 0.05$) higher survival during osmotic (49 h) stress than during desiccation (48 h) stress.

c Annotation information was derived from *L. monocytogenes* N53-1 (GenBank accession no. CCQ22741.1).

d $P < 0.01$ for the difference in survival compared to that of the WT.

e P < 0.05 for the difference in survival compared to that of the WT.

f P < 0.001 for the difference in survival compared to that of the WT.

g Significantly (*P* < 0.05) lower survival during osmotic (49 h) stress than during desiccation (48 h) stress.

h Annotation information was derived from *L. monocytogenes* N53-1 (GenBank accession no. CCQ22741.1)

TABLE 2 Transposon insertion sites identified in *Listeria monocytogenes* DS mutants mapped to the complete EGD-e sequenced genome and the corresponding losses in viability observed during desiccation and exposure to severe osmotic stress[a]

Lm strain	Gene	Gene locus tag	Function	Survival reduction (mean±SD Δlog CFU/cm^2 [desiccation] or Δlog CFU/ml [osmotic stress])				
				Desiccation				Osmotic Stress at
				Day 1	Day 2	Day 4	Day 7	49 h
Lm 568				-1.48 ± 0.14	-1.52 ± 0.12	-1.74 ± 0.03	-1.72 ± 0.15	-0.74 ± 0.17
DS01	*lmo_0371-0372*		Intergenic space	-2.71 ± 0.58 [b]	-2.42 ± 0.34 [c]	-2.66 ± 0.26 [d]	-4.58 ± 0.99 [b]	-0.76 ± 0.15 [e]
DS02	*lmo_0368*	*hisH*	Imidazole glycerol phosphate synthase subunit	-2.02 ± 0.15	-3.71 ± 0.82 [b]	-3.00 ± 0.56 [c]	-3.54 ± 0.19 [b]	-0.61 ± 0.21 [e]
DS03	*lmo_0616*		Glycerophosphoryl diester phosphodiesterase	-2.15 ± 0.15	-2.66 ± 0.16 [b]	-3.23 ± 0.67 [b]	-3.39 ± 0.05 [b]	-0.66 ± 0.07 [e]
DS04	*lmo_0983*		Hypothetical protein similar to glutathione peroxidase	-2.32 ± 0.12 [d]	-2.39 ± 0.12 [c]	-2.73 ± 0.14 [d]	-4.09 ± 0.02 [b]	-1.04 ± 0.00 [c]
DS05	*lmo_1174*	*eutA*	Reactivating factor for ethanolamine ammonia lyase	-2.13 ± 0.18	-2.33 ± 0.13 [d]	-2.27 ± 0.20	-2.76 ± 0.04 [d]	-1.19 ± 0.05 [b]

DS06	*lmo_1194*	*cbiD*	Cobalt-precorrin-6A synthase	-3.45 ± 0.50 [b]	-3.43 ± 0.07 [b]	-3.71 ± 0.36 [b]	-3.97 ± 0.19 [b]	-0.92 ± 0.06 [e]
DS07	*lmo_1219-1220 or lmo_1220*		Intergenic space or hypothetical protein	-2.29 ± 0.18 [d]	-2.56 ± 0.14 [c]	-2.64 ± 0.06 [d]	-4.07 ± 0.45 [b]	-1.16 ±0.05 [b]
DS08	*lmo_1728*		Hypothetical protein similar to cellobiose phosphorylase [a]	-2.80 ± 0.09 [b]	-3.03 ± 0.29 [b]	-3.05 ± 0.20 *	-3.27 ± 0.96 [b]	-1.25 ± 0.06 [b]
DS09	*lmo_2443*		Glutamate tRNA ligase [a]	-3.11 ± 0.76 [b]	-2.96 ± 0.04 [b]	-3.09 ± 0.89 [c]	-3.71 ± 0.27 [b]	-0.62 ± 0.15 [e]
DS10	*lmo_2470*		Leucine-rich repeat domain protein [a]	-2.78 ± 0.21 [b]	-2.85 ± 0.18 [b]	-3.05 ± 0.32 [c]	-4.06 ± 0.33 [b]	-0.84 ± 0.17 [e]
DS11	*lmo_2490*	*csbA*	CsbA putative membrane protein [a]	-1.54 ± 0.37	-2.05 ± 0.32	-1.97 ± 0.31	-2.48 ± 0.10	-0.48 ± 0.13
DS12	*lmo_2503*		Cardiolipin synthase [a]	-1.96 ± 0.18	-2.42 ± 0.32 [c]	-2.59 ± 0.28	-3.78 ± 0.24 [b]	-1.06 ± 0.06 [c]
DS13	*lmo_2768*		Hypothetical membrane protein	-1.54 ± 0.37	-1.90 ± 0.48	-2.55 ± 0.69	-2.81 ± 0.20 [+]	-0.88 ± 0.17 [b]
DS14	*lmo_2768*		Hypothetical membrane protein	-2.44 ± 0.00 [c]	-2.78 ± 0.17 [b]	-3.06 ± 0.03 [c]	-2.99 ± 0.16 [c]	-0.73 ± 0.03 [e]
DS15	*lmo_2778*		Uncharacterized protein [a]	-2.48 ± 0.24 [c]	-2.88 ± 0.09 [b]	-3.20 ± 0.12 [c]	-4.10 ± 0.21 [b]	-1.06 ± 0.07 [c]

a Cells were desiccated for 7 days at 43% RH and 15°C in TSB plus 1% glucose on SS coupons at an initial concentration of 7.5 log CFU/cm^2. Osmotic stress survival was determined by resuspending TSB-glu-grown cultures to 7.5 log CFU/ml in TSB-glu plus 20% NaCl for 49 h at 15°C (*n* = 3).

The complete *L. monocytogenes* EGD-e sequenced genome can be found in GenBank under accession number NC_003210.

b $P < 0.001$ for the difference in survival compared to that of the WT.
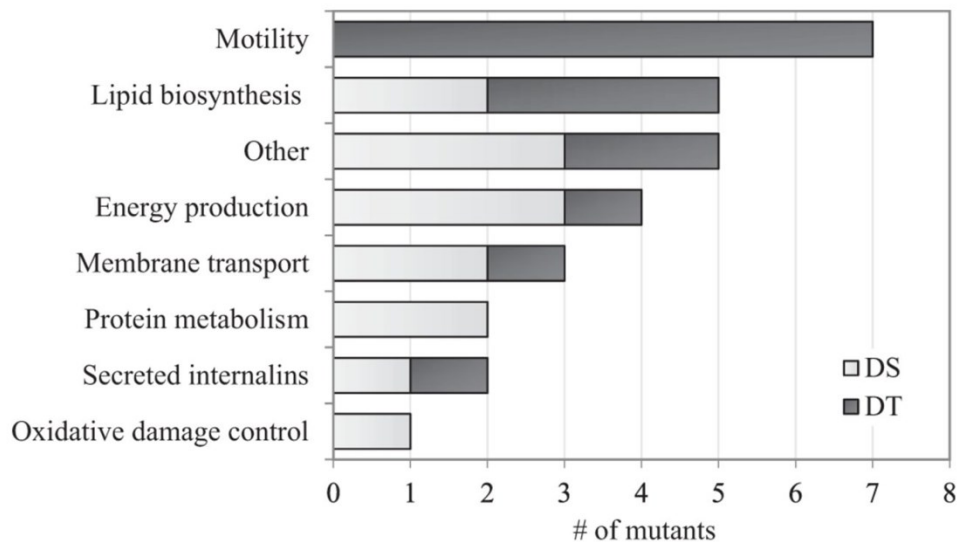
c $P < 0.01$ for the difference in survival compared to that of the WT.

d $P < 0.05$ for the difference in survival compared to that of the WT.

e The mutants displayed significantly ($P < 0.05$) decreased survival after 33 h of osmotic stress compared to the wild type.

f Annotation information was derived from *L. monocytogenes* N53-1 (GenBank accession no. CCQ25113.1).

FIG 2 Putative functions of the interrupted genes in 15 desiccation-tolerant (DT) and 15 desiccation-sensitive (DS) *Listeria monocytogenes* 568 *Himar1* transposon insertion mutants. Note that two DS mutants contained transposons in the same gene, making the total number of affected genes 14.

Motility assay and flagellum staining. Only mutants that contained transposon insertions in flagellum-associated genes exhibited a lack of motility at 15°C. Light microscopy further demonstrated that these seven mutants were devoid of flagella (data not shown). Additionally, the motility of the *lmo1582* mutant was reduced in comparison to that of the WT.

Heat tolerance assay. After 30 min at 55°C, mutant DT10 (*buk*) showed only a 0.84 log CFU/ml reduction in survival, which was significantly ($P < 0.05$) less than the 2.15 log CFU/ml reduction demonstrated by the WT.

Expression of flagellum-associated genes in desiccation-tolerant immotile mutants. To elucidate whether the transposon insertion caused any downstream polar effects in the mutants with mutations in flagellum-associated genes, endpoint RT-PCR was performed to detect the expression of the affected gene and the gene directly downstream in each of the seven mutants (see Fig. S1 in the supplemental material). Expression of 16S rRNA and *flaA* in all mutants and the WT confirmed successful cDNA conversions. While stationary-phase cells of the WT strain grown at 15°C expressed all of the tested flagellum-associated genes, none of the affected genes were transcribed in the mutants with mutations in flagellum-associated genes (see Table S5 in the supplemental material). While the immediately adjacent downstream genes were expressed in the *fliP*, *flhB*, *flgD*, and *motB* insertional mutants, they were not expressed in the *fliM*, *fliY*, and *flgL* mutants, demonstrating possible polar effects of the transposon insertion in some mutants.
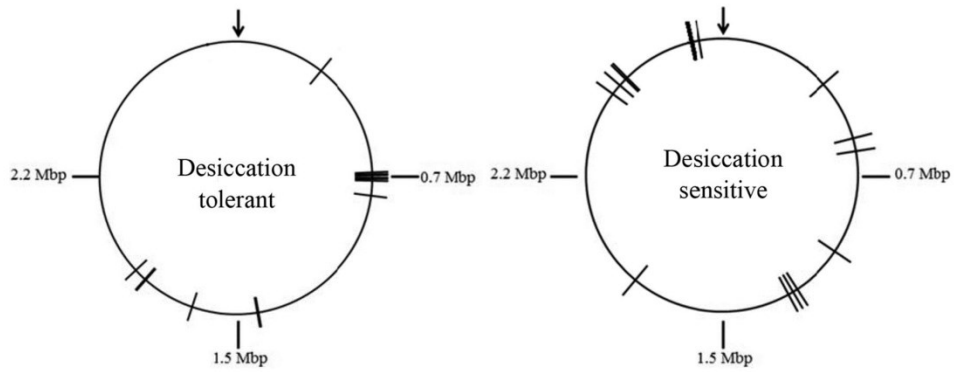
FIG 3 Locations of the sequenced *Himar1* transposon insertion sites in desiccation-tolerant and -sensitive *Listeria monocytogenes* 568 mutants mapped to the EGD-e chromosome. Each quarter of the chromosome is identified with the approximate base pair, except for bp 1, which is identified by an arrow.

DISCUSSION

In this study, insertional mutagenesis in *L. monocytogenes* 568 led to the identification of 30 loci with putative roles in desiccation stress survival, and 19 of these similarly influenced osmotic stress survival.

Impaired motility is associated with enhanced desiccation tolerance. Desiccation tolerance and impaired motility in seven mutants were attributed to interruptions in flagellum-associated genes. Additionally, three of these mutants displayed enhanced osmotolerance, suggesting that the downregulation of flagellumassociated gene expression may be a valuable survival mechanism for cells experiencing water stress. A study which compared the transcriptome profiles of a DT strain and a DS strain of *Salmonella* after 2 h of drying saw inhibition of flagellum-associated gene expression in the DT strain relative to the level of flagellum-associated gene expression in the DS strain (23). The downregulation of flagellum-associated genes has also been observed in *Pseudomonas putida* cells exposed to matric and, to a lesser extent, solute stresses (32) and *Bacillus subtilis* exposed to osmotic stress (33). Additional studies report conflicting results on the importance of flagella in desiccation survival (25, 34). In the present study, three mutants with mutations in flagellum-associated structural gene (*fliP*, *flhB*, *flgL*) displayed the greatest osmotolerance observed among all the mutants, whereas two mutants with mutations in flagellum motor control (*motB*, *fliM*) showed significantly decreased osmotolerance. This demonstrates that different osmotolerance phenotypes can exist within motility-impaired DT strains of *L. monocytogenes*.

Flagellum biosynthesis is an energy-consuming endeavor, and so it would likely be beneficial for bacterial cells experiencing stress to downregulate flagellum-associated gene expression and redirect their energy into more critical metabolic processes. Recently, it was shown that immotile *L. monocytogenes* mutants were capable of forming hyperbiofilms under dynamic but not static conditions (35). Similar results have also been reported in *Bacillus cereus* (36). Together with the possibility of increased desiccation tolerance and osmotolerance, immotile strains of *L. monocytogenes* may pose a greater threat to the food industry. To the best of our knowledge, this study is the first to report an association between flagellum-associated gene expression and desiccation survival in *L. monocytogenes*. The insertion sites in these mutants were mapped to four transcriptional units (see Fig. S1 in the supplemental material) that encompass 33 of the 41 flagellum-associated genes in *L. monocytogenes*.

The affected gene was not expressed in any of the seven mutants (see Table S5 in the supplemental material), making this likely true for all the transposon mutants. In four mutants (with mutations in *fliP*, *flhB*, *motB*, and *flgD*) the downstream gene continued to be expressed, whereas in the other three (with mutations in *fliM*, *fliY*, and *flgL*), the transposon insertion caused a polar effect and abolished the expression of the downstream gene. The successful amplification of *flaA*, which is an individually transcribed gene upstream of the transposon in these three mutants, demonstrates that the polar effect remained local. With the exception of *motB*, previous literature confirms that when these genes are deleted in *L. monocytogenes* and other motile bacteria, nonflagellated mutants result (37–41).

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Unlike the other proteins, MotB is not part of the core flagellum structure but is an associated transmembrane protein required for the rotation of the flagellum motor. Therefore, clean *motB* mutants are flagellated but immotile (42, 43). It remains unknown why flagella were not observed on the *motB* mutant in this study, since the mutation did not appear to impact the expression of downstream genes. However, regardless of whether an immotile strain is flagellated or not, strains of both phenotypes would largely benefit from the reduced energy commitment of powering flagellar rotation (reviewed in reference 44).

Fatty acid metabolism and a putatively less fluid membrane are involved in desiccation tolerance. Many of the desiccation mutants, both tolerant and sensitive, contained an affected gene which is associated with FA biosynthesis and, putatively, the cell membrane composition. This is not surprising, given the importance of membrane viscosity and integrity in the survival of bacteriaexposed to other environmental stresses, including pH and temperature extremes, high osmolarity, hydrostatic pressure, and transition to stationary phase (45–49). In *Listeria* and related bacteria, such as *Staphylococcus aureus* and *B. subtilis*, >90% of cellular membrane FAs are branched-chain FAs (BCFAs) and therefore the major determinants of membrane fluidity (50). Critical for the synthesis of BCFAs is a multisubunit branched-chain -keto acid dehydrogenase (BKD) complex. The first two genes of the *bkd* locus encode dihydrolipoamide dehydrogenase (*lpd*) and butyrate kinase (*buk*), the affected genes in two mutants (DT10 and DT11) which exhibited extreme desiccation tolerance (<0.2 log CFU/ cm^2 reduction after 7 days). In agreement with an earlier report (31), the *buk* mutant also exhibited increased heat

resistance, and both mutants were significantly ($P < 0.05$) more osmotolerant than the WT. In *B. subtilis*, *L. monocytogenes*, and *S. aureus*, it has been demonstrated that BKD-deficient mutants have less fluid membranes due to reduced levels of BCFAs and increased levels of straight-chain FAs (50–52). Severe growth defects, longer generation times, and reduced maximal growth in rich media (52) have also been reported, in agreement with our observations of low growth rates for the *lpd* and *buk* mutants and the unusual colony morphologies exhibited by the *lpd* mutant under desiccation and osmotic stress conditions (Fig. 1). These results suggest that inactivation of the listerial BKD complex may be associated with enhanced desiccation tolerance and, in some cases, heat tolerance and osmotolerance.

Exponential-phase bacterial cells are normally less resistant to various stresses than stationary-phase cells (53–56). However, this was not observed with the *lpd* and *buk* mutants, where late-exponential- phase cells had increased resistance relative to WT stationary- phase cells. The interrupted gene in another DT mutant encodes the regulatory protein YycH of the YycFG (recently renamed WalKR) two-component system essential for cell viability in Gram-positive bacteria, including *S. aureus*, *B. subtilis*, *L. monocytogenes*, and *Enterococcus faecalis* (57). YycH negatively controls WalK activity, and without this control, the overexpression of WalKR results in increased synthesis of longer-chain FAs, producing a less fluid membrane (58). *yycH* deletion mutants demonstrated growth impairments and cell wall defects (59, 60), which may partly explain the irregularly shaped colonies observed for the *yycH* transposon mutant in our study (Fig. 1).

Numerous studies have reported the upregulation of FA biosynthesis genes in bacteria subjected to dehydration or matric stresses (22, 23, 25, 32, 34). In both *Salmonella* (23) and *Pseudomonas putida* (32), exposure to matric stress resulted in the upregulation of enzymes that degrade long-chain FAs and damaged phospholipids. This may increase the energy yield under starvation conditions, as FA oxidation generates significantly more ATPs per carbon atom than glucose (61). Additionally, these studies also saw the upregulation of enzymes involved in altering the membrane composition to produce a more rigid, less fluid membrane. Such changes, including increased levels of saturated FAs and cardiolipin, have also been reported in *B. subtilis* (62, 63), *Escherichia coli* (64), and *S. aureus* (65) subjected to salt stress, which produced thicker cell walls, possibly preventing NaCl entrance and the escape of compatible solutes (62). Increased levels of saturated FAs also increase the melting point of the membrane, making cells more resistant to higher temperatures (66). *Salmonella* cells preexposed to alkaline and acidic conditions developed membranes which contained higher levels of saturated FAs and lower levels of unsaturated FAs that subsequently induced thermotolerance (67, 68). An increased percentage of saturated FAs may also limit the effects of lipid oxidation imposed by high temperatures, dehydration, and osmotic stress. Overlaps among stress adaptation mechanisms are logical due to redundancies in regulatory networks contributing to general and specific stress responses (69).

As mentioned previously, cardiolipin levels positively correlate with increased osmotolerance, cold tolerance, and acid tolerance in both *L. monocytogenes* and other bacteria alike (62, 65, 70). This diphosphatidylglycerol lipid is a multifunctional

molecule that participates in cell division, energy metabolism, and membrane transport (71). In this study, interruption of cardiolipin synthase produced a desiccation- and osmosensitive mutant, further underscoring its role in adapting to environments with low water levels. Similarly, a mutant containing an interrupted glycerophosphoryl diester phosphodiesterase, an enzyme involved in the production of glycerophospholipids and cardiolipin, also demonstrated desiccation sensitivity and osmosensitivity.

Energy sources related to desiccation survival. Several desiccation mutants harbored transposons in genes that may provide alternative energy sources during desiccation stress. Two DS mutants contained nearby affected genes involved in ethanolamine utilization (*eutA*) and vitamin B12 synthesis (*cbiD*). EutA is a putative chaperonin that prevents the inhibition of ethanolamine ammonia lyase (EutBC), an enzyme believed to be necessary for *L. monocytogenes* to utilize ethanolamine as a carbon and nitrogen source under intracellular nutrient-limited conditions (72–74). The means of regulation of the *eut* operon in *Listeria* remains unknown, but in *Salmonella enterica* serovar Typhimurium it is activated by both ethanolamine and vitamin B12 under anaerobic conditions (75, 76). In *L. monocytogenes*, transcription of vitamin B12 biosynthesis genes is induced during intracellular growth (73), suggesting that its *eut* operon is similarly regulated. This points to the putative importance of mobilizing ethanolamine at the expense of membrane phosphatidylethanolamine as an energy source to survive the starvation conditions induced under desiccation stress conditions. In support of this suggestion is the finding that enzymes involved in the degradation of phospholipids are upregulated

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during exposure to dehydration stress (32). These mutants also displayed significant osmosensitivity. Interruption of adenine deaminase (*adeC*) was found to produce a desiccation- and osmotolerant mutant. As adenine is taken up by cells, it is either phosphoribosylated to AMP or deaminated to produce nucleotide precursors (77). Under starvation conditions, adenine can act as a sole C and N source for bacteria via the AMP pathway. Since desiccated cells also experience starvation stress, it may be hypothesized that inactivation of *adeC* allows cells to utilize adenine solely as an energy source, which could be more advantageous for survival.

Lastly, the interrupted gene in a desiccation- and osmosensitive mutant was identified to be cellobiose phosphorylase (*lmo1728*). Cellobiose phosphorylase is responsible for the intracellular breakdown of cellobiose to glucose monomers (78). A mutant defective in cellobiose metabolism would be unable to use the cellobiose in the TSB used in both the desiccation tolerance and osmotolerance assays, which would possibly cause starvation and decreased survival compared to that of the WT.

Amino acid metabolism and desiccation survival. Two DS mutants contained inserts in genes involved in protein metabolism, specifically, histidine biosynthesis and glutamate utilization. The affected gene, *hisH*, is cotranscribed with *hisB*, *hisD*, *hisG*, and *hisZ* to produce enzymes required for histidine biosynthesis. The other identified gene encodes glutamate-tRNA ligase, 1 of 20 aminoacyl- tRNA synthetases essential for protein synthesis. The upregulation of glutamate metabolism genes, including Glu-tRNA ligase (79), and the intracellular accumulation of glutamate have been shown to enhance survival during osmotic, acid, and bile stress in *L. monocytogenes* (80, 81) and other bacteria (82). Interruption of *lmo2443* may

cause glutamate to accumulate in the cell, which could be an explanation for the increased osmotolerance observed in this mutant. The *hisH* mutant also displayed significantly enhanced osmotolerance; however, the reasons for this remain speculative. In a transcriptomic analysis of desiccated *Salmonella*, nearly half of the upregulated genes belonged to 10 operons, 3 of which were involved in the metabolism of arginine, histidine, and glutamate (22), suggesting that these amino acids may play a specific role in desiccation survival.

Oxidation damage control and desiccation survival. Biomolecules in low-water-activity environments are more susceptible to oxidation due to increased cytoplasm ion concentrations and the formation of reactive oxygen species (83). These reactive species can damage proteins by modifying amino acid side chains, forming cross-links between proteins, and causing DNA backbone chain breaks (84, 85). Additionally, they can cause lipid peroxidation in cell membranes (86). Therefore, it is not surprising that we identified a desiccation- and osmosensitive mutant with an affected glutathione peroxidase gene (*lmo0983*), known to prevent oxidative damage.

Internalins with putative impacts on desiccation survival. Internalin C (*inlC*) and *lmo2470*, the affected genes in a DT mutant and a DS mutant, respectively, are among 25 known *Listeria* specific internalin proteins (87, 88). Aside from their most recognized roles in intracellular invasion (*inlA*, *inlB*), internalins contain a leucine-rich repeat domain that allows them to bind structurally unrelated protein ligands, thereby implicating them in a wide range of functions. Most internalins are cell wall-anchored proteins, but both *inlC* and *lmo2470* fall into a

small class of four secreted internalins. Studies have shown that *inlC* is upregulated during late stages of intracellular infection (89); however, the role of *lmo2470* remains to be elucidated. Once they are *in vivo*, intracellular pathogens are faced with a number of harsh conditions, including nutrient and oxygen starvation and osmotic and acid stress. One of the main stress response regulators in *L. monocytogenes* is σ^B, which also regulates genes expressed during infection (90), including *inlC* (91). In desiccated *Salmonella*, four of seven downregulated genes were found to be located on the virulence plasmid (22). This suggests that the inactivation of virulence genes with little or no role in stress survival may be an advantageous energy conservation mechanism for surviving desiccation.

Contrary to the results of inhibition of *inlC*, inhibition of *lmo2470* produced desiccation- and osmosensitive mutant. Although the function of this internalin remains speculative, it is one of eight internalins found among *Listeria* spp. that is unique to *L. monocytogenes* (92). Therefore, any role that it may have in the stress response of the pathogen could potentially allow *L. monocytogenes* to outcompete nonpathogenic *Listeria* strains in food processing environments.

Membrane transport proteins associated with desiccation survival. Two DS mutants that also displayed osmosensitivity harbored their transposon in the same gene, *lmo2768*, encoding a membrane transport protein. A third DS mutant also contained an interrupted membrane protein (*csbA*) that is regulated by σ^B and involved in the general stress response (93). Membrane transport proteins are important for adaptation to low-water-activity environments to facilitate uptake of the different nutrients and/or osmolytes required by dehydrated cells (32).

When cells encounter osmotic stress, their first response mechanism is the upregulation of membrane transporters that scavenge K^+ from the environment to help maintain cell turgor pressure (94, 95). Surprisingly, in this study we identified a desiccation and osmotolerant mutant with an interrupted ATP-sensitive inward potassium channel (*lmo0771*). 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 upregulated in *Salmonella* (22) and cyanobacteria (96) in response to desiccation. The importance of this system in *L. monocytogenes* and *E. coli* survival under conditions of osmotic and low-temperature stress has also been documented (95, 97), indicating a possible role for this operon during desiccation stress.

Other affected genes. A DT mutant contained a mutated adenine- specific DNA methylase (*dam*, *lmo1582*) which has been elucidated in *E. coli* and *S. enterica* to methylate adenosine residues in 5'-GATC-3' sites postreplication, therefore altering the DNA structure and subsequent protein binding (98, 99). In these bacteria Dam -mutants exhibited attenuated virulence related to decreased expression of virulence-related fimbriae and invasion proteins (100, 101). Motility-related genes were also found to be downregulated relative to their levels of regulation in the WT, whereas genes belonging to the SOS response regulon were upregulated (102, 103). Possible reasons why decreased motility, which was also observed in the *L. monocytogenes* DT mutant, and virulence gene expression might increase desiccation survival have been previously discussed; however, given that the SOS response in *L.*

monocytogenes has been associated with stress resistance (104) and heat shock (105), it may be that this regulon also has a role in desiccation survival.

Another DT mutant also displaying osmotolerance contained an interrupted NAD-dependent epimerase/dehydratase. This class of enzymes possesses oxidoreductase activity that is central to both carbohydrate and lipid metabolic processes. More research is needed to determine the exact role of this enzyme in desiccation and osmotic stress.

In the desiccation and osmosensitive mutant DS07, a 20-bp gap in the sequencing results made it unknown whether the transposon was inserted in *lmo1220* or the *lmo1219-lmo1220* intergenic space. Regardless, the close proximity of any insertion in the intergenic region may alter the expression of *lmo1220*, which encodes the DNA binding protein HxIR. This protein positively regulates the *hxlAB* operon, encoding two key enzymes in the ribulose monophosphate pathway responsible for the detoxification of formaldehyde in *Bacillus subtilis* (106–108). Formaldehyde is produced in many microorganisms through the degradation of organic compounds that have methyl or methoxy groups, e.g., lignin and pectin. Interruption of HxIR expression in this mutant may be hypothesized to result in a toxic intracellular buildup of formaldehyde, which would decrease its survival under both stresses. A noteworthy finding is that all DS mutants still retained cell viabilities of >3.0 log CFU/cm^2 after 7 days of desiccation. Recently,we compared the desiccation survival of three Gram-negative spoilage bacteria and *L. monocytogenes* on stainless steel and found that the levels of *Shewanella baltica* and *Pseudomonas fluorescens* dropped

below the detection limit after 7 days of desiccation (43% RH, 15°C), while *Serratia proteamaculans* displayed survival similar to that of *L. monocytogenes* (109).

Similarly, *L. monocytogenes* has been shown to have desiccation tolerance superior to that of *S. aureus* and *Salmonella* Typhimurium (18). This implies that even desiccation-sensitive strains of *L. monocytogenes* may still outcompete less-desiccation-tolerant microorganisms found in food processing environments.

This study is the first to report on the multiple genes influencing the desiccation survival of *L. monocytogenes*, with many being found to be involved in motility, membrane lipid biosynthesis, energy production, membrane transport, protein metabolism, and putative virulence. Gene interruptions in 19 mutants led to survival trends similar to those for theWTunder both desiccation and osmotic stress conditions, whereas the affected genes in the remaining 11 mutants solely affected desiccation survival. This demonstrates that both similar and different mechanisms are used by *L. monocytogenes* to survive the dehydration imposed by solute and matric stresses. Future characterization of the affected regulons will help identify desiccation resistance mechanisms in *L. monocytogenes* and ultimately lead to improved control of *Listeria* in food processing environments.

REFERENCES

1. **Cartwright EJ, Jackson KA, Johnson SD, Graves LM, Silk BJ, Mahon BE.** 2013. Listeriosis outbreaks and associated food vehicles, United States, 1998–2008. Emerg Infect Dis **19:**1–9. http://dx.doi.org/10.3201 /eid1901.120393.

2. **Todd E, Notermans S.** 2011. Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. Food Control **22:**1484–1490. http://dx .doi.org/10.1016/j.foodcont.2010.07.021.

3. **Yildiz O, Aygen B, Esel D, Kayabas U, Alp E, Sumerkan B, Doganay M.** 2007. Sepsis and meningitis due to *Listeria monocytogenes*. Yonsei Med J **48:**433–439. http://dx.doi.org/10.3349/ymj.2007.48.3.433.

4. **Olsen SJ, Patrick M, Hunter SB, Reddy V, Kornstein L, MacKenzie WR, Lane K, Bidol S, Stoltman GA, Frye DM.** 2005. Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. Clin Infect Dis **40:**962–967. http://dx.doi.org/10.1086/428575.

5. **Centers for Disease Control and Prevention.** 2011. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe—United States, August-September 2011. MMWR Morb Mortal Wkly Rep **60:**1357.

6. **Mead P, Dunne E, Graves L, Wiedmann M, Patrick M, Hunter S, Salehi E, Mostashari F, Craig A, Mshar P.** 2006. Nationwide outbreak of listeriosis due to contaminated meat. Epidemiol Infect **134:**744–751. http://dx.doi.org/10.1017/S0950268805005376.

7. **Centers for Disease Control and Prevention.** 2008. Outbreak of *Listeria monocytogenes* infections associated with pasteurized milk from a local dairy—Massachusetts, 2007. MMWR Morb Mortal Wkly Rep **57:**1097.

8. **Jackson K, Biggerstaff M, Tobin-D'Angelo M, Sweat D, Klos R, Nosari J, Garrison O, Boothe E, Saathoff-Huber L, Hainstock L.** 2011. Multistate outbreak of *Listeria monocytogenes* associated with Mexican-style cheese made from pasteurized milk among pregnant, Hispanic women. J Food Prot **74:**949–953. http://dx.doi.org/10.4315/0362-028X.JFP-10-536.

9. **Simmons C, Stasiewicz MJ, Wright E, Warchocki S, Roof S, Kause JR, Bauer N, Ibrahim S, Wiedmann M, Oliver HF.** 2014. *Listeria monocytogenes* and *Listeria* spp. contamination patterns in retail delicatessen establishments in three U.S. states. J Food Prot **77:**1929–1939. http://dx .doi.org/10.4315/0362-028X.JFP-14-183.

10. **Varma JK, Samuel MC, Marcus R, Hoekstra RM, Medus C, Segler S, Anderson BJ, Jones TF, Shiferaw B, Haubert N.** 2007. *Listeria monocytogenes* infection from foods prepared in a commercial establishment: a case-control study of potential sources of sporadic illness in the United States. Clin Infect Dis **44:**521–528. http://dx.doi.org/10.1086/509920.

11. **Hoelzer K, Oliver HF, Kohl LR, Hollingsworth J, Wells MT, Wiedmann M.** 2012. Structured expert elicitation about *Listeria monocytogenes* cross-contamination in the environment of retail deli operations in the United States. Risk Anal **32:**1139 1156. http://dx.doi.org/10.1111/j.1539-6924.2011.01729.x.

12. **Destro M, Leitao M, Farber J.** 1996. Use of molecular typing methods to trace the dissemination of *Listeria monocytogenes* in a shrimp processing plant. Appl Environ Microbiol **62:**705–711.

13. **Lundén JM, Miettinen MK, Autio TJ, Korkeala HJ.** 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. J Food Prot **63:**1204–1207.

14. **Walker S, Archer P, Banks JG.** 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. J Appl Bacteriol **68:**157–162. http://dx.doi .org/10.1111/j.1365-2672.1990.tb02561.x.

15. **Liu D, Lawrence ML, Ainsworth AJ, Austin FW.** 2005. Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. FEMS Microbiol Lett **243:**373–378. http: //dx.doi.org/10.1016/j.femsle.2004.12.025.

16. **Vogel BF, Hansen LT, Mordhorst H, Gram L.** 2010. The survival of *Listeria monocytogenes* during long term desiccation is facilitated by sodium chloride and organic material. Int J Food Microbiol **140:**192–200. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.03.035.

17. **Hingston PA, Stea EC, Knøchel S, Hansen T.** 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 Microbiol **36:**46–56. http://dx.doi.org/10.1016/j.fm.2013.04.011.

18. **Takahashi H, Kuramoto S, Miya S, Kimura B.** 2011. Desiccation survival of *Listeria monocytogenes* and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. Food Control **22:**633–637. http://dx.doi.org/10.1016/j.foodcont.2010.09.003.

19. **Truelstrup Hansen L, Vogel BF.** 2011. Desiccation of adhering and biofilm *Listeria monocytogenes* on stainless steel: survival and transfer to Genes Involved in salmon products. Int J Food Microbiol **146:**88–93. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.01.032.

20. **França M, Panek A, Eleutherio E.** 2007. Oxidative stress and its effects during dehydration. Comp Biochem Phys A **146:**621–631. http://dx.doi.org/10.1016/j.cbpa.2006.02.030.

21. **Billi D, Potts M.** 2002. Life and death of dried prokaryotes. Res Microbiol **153:**7–12. http://dx.doi.org/10.1016/S0923-2508(01)01279-7.

22. **Gruzdev N, McClelland M, Porwollik S, Ofaim S, Pinto R, Saldinger-Sela S.** 2012. Global transcriptional analysis of dehydrated *Salmonella enterica* serovar Typhimurium. Appl Environ Microbiol **78:**7866–7875. http://dx.doi.org/10.1128/AEM.01822-12.

23. **Li H, Bhaskara A, Megalis C, Tortorello ML.** 2012. Transcriptomic analysis of *Salmonella* desiccation resistance. Foodborne Pathog Dis**9:**1143–1151. http://dx.doi.org/10.1089/fpd.2012.1254.

24. **van de Mortel M, Chang W-S, Halverson L.** 2004. Differential tolerance of *Pseudomonas putida* biofilm and planktonic cells to desiccation. Biofilms **1:**361–368. http://dx.doi.org/10.1017/S1479050504001528.

25. **Gülez G, Dechesne A, Workman CT, Smets BF.** 2012. Transcriptome dynamics of *Pseudomonas putida* KT2440 under water stress. Appl Environ Microbiol **78:**676–683. http://dx.doi.org/10.1128/AEM.06150-11.

26. **Kalmokoff M, Austin J, Wan XD, Sanders G, Banerjee S, Farber J.** 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. J Appl Microbiol **91:**725– 734. http://dx.doi.org/10.1046/j.1365-2672.2001.01419.x.

27. **Cao M, Bitar AP, Marquis H.** 2007. A *mariner*-based transposition system for *Listeria monocytogenes*. Appl Environ Microbiol **73:**2758– 2761. http://dx.doi.org/10.1128/AEM.02844-06.

28. **Alexander JE, Andrew P, Jones D, Roberts I.** 1990. Development of an optimized system for electroporation of *Listeria* species. Lett Appl Microbiol **10:**179–181. http://dx.doi.org/10.1111/j.1472- 765X.1990.tb00109.x.

29. **Garsin DA, Urbach J, Huguet-Tapia JC, Peters JE, Ausubel FM.** 2004. Construction of an *Enterococcus faecalis* Tn*917*-mediated-genedisruption library offers insight into Tn*917* insertion patterns. J Bacteriol **186:**7280–7289. http://dx.doi.org/10.1128/JB.186.21.7280-7289.2004.

30. **Glaser P, Frangeul L, Buchrieser C, Rusniok C, Amend A, Baquero F, Berche P, Bloecker H, Brandt P, Chakraborty T, Charbit A, Chetouani F, Couvé E, de Daruvar A, Dehoux P, Domann E, Domínguez-Bernal G, Duchaud E, Durant L, Dussurget O, Entian K-D, Fsihi H, Garcia- Del Portillo F, Garrido P, Gautier L, Goebel W, Gómez-López N, Hain T, Hauf J, Jackson D, Jones L-M, Kaerst U, Kreft J, Kuhn M, Kunst F, Kurapkat G, Madueño E, Maitournam A, Mata Vicente J, Ng E, Nedjari H, Nordsiek G, Novella S, de Pablos B, Pérez-Diaz J-C, Purcell R, Remmel B, Rose M, Schlueter T, Simoes N, et al.** 2001. Comparative genomics of *Listeria* species. Science **294:**849–852. http://dx.doi.org/10.1126/science.1063447.

31. **Ells TC, Speers RA, Truelstrup Hansen L.** 2009. Insertional mutagenesis of *Listeria monocytogenes* 568 reveals genes that contribute to enhanced thermotolerance. Int J Food Microbiol **136:**1–9. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.09.020.

32. **Van De Mortel M, Halverson LJ.** 2004. Cell envelope components contributing to biofilm growth and survival of *Pseudomonas putida* in low-water-content habitats. Mol Microbiol **52:**735–750. http://dx.doi.org/10.1111/j.1365-2958.2004.04008.x.

33. **Hahne H, Mäder U, Otto A, Bonn F, Steil L, Bremer E, Hecker M, Becher D.** 2010. A comprehensive proteomics and transcriptomics analysis of *Bacillus subtilis* salt stress adaptation. J Bacteriol **192:**870–882. http://dx.doi.org/10.1128/JB.01106-09.

34. **Cytryn EJ, Sangurdekar DP, Streeter JG, Franck WL, Chang W-S, Stacey G, Emerich DW, Joshi T, Xu D, Sadowsky MJ.** 2007. Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation- induced stress. J Bacteriol **189:**6751–6762. http://dx.doi.org/10.1128/JB.00533-07.

35. **Todhanakasem T, Young GM.** 2008. Loss of flagellum-based motility by *Listeria monocytogenes* results in formation of hyperbiofilms. J Bacteriol **190:**6030–6034. http://dx.doi.org/10.1128/JB.00155-08.

36. **Houry A, Briandet R, Aymerich S, Gohar M.** 2010. Involvement of motility and flagella in *Bacillus cereus* biofilm formation. Microbiology **156:**1009–1018. http://dx.doi.org/10.1099/mic.0.034827-0.

37. **Garbe J, Bunk B, Rohde M, Schobert M.** 2011. Sequencing and characterization of *Pseudomonas aeruginosa* phage JG004. BMC Microbiol **11:**102. http://dx.doi.org/10.1186/1471-2180-11-102.

38. **Ohnishi K, Ohto Y, Aizawa S, Macnab RM, Iino T.** 1994. FlgD is a scaffolding protein needed for flagellar hook assembly in *Salmonella typhimurium*. J Bacteriol **176:**2272–2281.

39. **Foynes S, Dorrell N, Ward SJ, Zhang ZW, McColm AA, Farthing MJ, Wren BW.** 1999. Functional analysis of the roles of FliQ and FlhB in flagellar expression in *Helicobacter pylori*. FEMS Microbiol Lett **174:**33–39. http://dx.doi.org/10.1111/j.1574-6968.1999.tb13546.x.

40. **Bischoff D, Weinreich M, Ordal G.** 1992. Nucleotide sequences of *Bacillus subtilis* flagellar biosynthetic genes *fliP* and *fliQ* and identification of a novel flagellar gene, *fliZ*. J Bacteriol **174:**4017–4025.

41. **Bischoff DS, Ordal GW.** 1992. Identification and characterization of FliY, a novel component of the *Bacillus subtilis* flagellar switch complex. Mol Microbiol **6:**2715–2723. http://dx.doi.org/10.1111/j.1365-2958.1992.tb01448.x.

42. **Gorski L, Duhé JM, Flaherty D.** 2009. The use of flagella and motility for plant colonization and fitness by different strains of the foodborne pathogen *Listeria monocytogenes*. PLoS One **4:**e5142. http://dx.doi.org /10.1371/journal.pone.0005142.

43. **Doyscher D, Fieseler L, Dons L, Loessner MJ, Schuppler M.** 2013. *Acanthamoeba* feature a unique backpacking strategy to trap and feed on *Listeria monocytogenes* and other motile bacteria. Environ Microbiol **15:** 433–446. http://dx.doi.org/10.1111/j.1462-2920.2012.02858.x.

44. **Berg HC.** 2003. The rotary motor of bacterial flagella. Biochemistry **72:**19. http://dx.doi.org/10.1146/annurev.biochem.72.121801.161737.

45. **Juneja VK, Foglia TA, Marmer BS.** 1998. Heat resistance and fatty acid composition of *Listeria monocytogenes*: effect of pH, acidulant, and growth temperature. J Food Prot **61:**683–687.

46. **Annous BA, Kozempel MF, Kurantz MJ.** 1999. Changes in membrane fatty acid composition of *Pediococcus* sp. strain NRRL B-2354 in response to growth conditions and its effect on thermal resistance. Appl Environ Microbiol **65:**2857–2862.

47. **Casadei M, Manas P, Niven G, Needs E, Mackey B.** 2002. Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. Appl Environ Microbiol **68:**5965–5972. http://dx.doi.org/10.1128/AEM.68.12.5965-5972.2002.

48. **Russell N, Evans R, Ter Steeg P, Hellemons J, Verheul A, Abee T.** 1995. Membranes as a target for stress adaptation. Int J Food Microbiol **28:**255–261. http://dx.doi.org/10.1016/0168-1605(95)00061-5.

49. **Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (ed).** 1996. *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed, vol 2. ASM Press, Washington, DC.

50. **Singh VK, Hattangady DS, Giotis ES, Singh AK, Chamberlain NR, Stuart MK, Wilkinson BJ.** 2008. Insertional inactivation of branchedchain -keto acid dehydrogenase in *Staphylococcus aureus* leads to decreased branched-chain membrane fatty acid content and increased susceptibility to certain stresses. Appl Environ Microbiol **74:**5882–5890. http://dx.doi.org/10.1128/AEM.00882-08.

51. **Willecke K, Pardee AB.** 1971. Fatty acid-requiring mutant of *Bacillus subtilis* defective in branched chain -keto acid dehydrogenase. J Biol Chem **246:**5264–5272.

52. **Zhu K, Ding X, Julotok M, Wilkinson BJ.** 2005. Exogenous isoleucine and fatty acid shortening ensure the high content of anteiso-C15:0 fatty acid required for low-temperature growth of *Listeria monocytogenes*. Appl Environ Microbiol **71:**8002–8007. http://dx.doi.org/10.1128/AEM.71.12.8002-8007.2005.

53. **Aryani D, den Besten H, Hazeleger W, Zwietering M.** 2015. Quantifying variability on thermal resistance of *Listeria monocytogenes*. Int J Food Microbiol **193:**130–138. http://dx.doi.org/10.1016/j.ijfoodmicro 2014.10.021.

54. **Nair S, Finkel SE.** 2004. Dps protects cells against multiple stresses during stationary phase. J Bacteriol **186:**4192–4198. http://dx.doi.org/10.1128/JB.186.13.4192-4198.2004.

55. **Mackey B, Forestiere K, Isaacs N.** 1995. Factors affecting the resistance of *Listeria monocytogenes* to high hydrostatic pressure. Food Biotechnol **9:**1–11. http://dx.doi.org/10.1080/08905439509549881.

56. **Luppens S, Abee T, Oosterom J.** 2001. Effect of benzalkonium chloride on viability and energy metabolism in exponential- and stationarygrowth-phase cells of *Listeria monocytogenes*. J Food Prot **64:**476–482.

57. **Dubrac S, Msadek T.** 2008. Tearing down the wall: peptidoglycan metabolism and the WalK/WalR (YycG/YycF) essential two-component system, p 214–228. *In* Bacterial signal transduction: networks and drug targets. Springer, New York, NY.

58. **Mohedano ML, Overweg K, de la Fuente A, Reuter M, Altabe S, Mulholland F, de Mendoza D, López P, Wells JM.** 2005. Evidence that the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of fatty acid biosynthesis genes and alters membrane composition. J Bacteriol **187:**2357–2367. http://dx.doi.org/10.1128/JB.187.7.2357-2367.2005.

59. **Szurmant H, Nelson K, Kim E-J, Perego M, Hoch JA.** 2005. YycH regulates the activity of the essential YycFG two-component system in *Bacillus subtilis*. J Bacteriol **187:**5419–5426. http://dx.doi.org/10.1128/JB.187.15.5419-5426.2005.

60. **Szurmant H, Mohan MA, Imus PM, Hoch JA.** 2007. YycH and YycI interact to regulate the essential YycFG two-component system in *Bacillus subtilis*. J Bacteriol **189:**3280–3289. http://dx.doi.org/10.1128/JB.01936-06.

61. **James BW, Mauchline WS, Dennis PJ, Keevil CW, Wait R.** 1999. Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. Appl Environ Microbiol **65:**822–827.

62. **López C, Heras H, Garda H, Ruzal S, Sánchez-Rivas C, Rivas E.** 2000. Biochemical and biophysical studies of *Bacillus subtilis* envelopes under hyperosmotic stress. Int J Food Microbiol **55:**137–142. http://dx.doi.org /10.1016/S0168-1605(00)00171-9.

63. **López CS, Heras H, Ruzal SM, Sánchez-Rivas C, Rivas EA.** 1998. Variations of the envelope composition of *Bacillus subtilis* during growth in hyperosmotic medium. Curr Microbiol **36:**55–61. http://dx.doi.org/10.1007/s002849900279.

64. **Lusk JE, Kennedy EP.** 1972. Altered phospholipid metabolism in a sodium-sensitive mutant of *Escherichia coli*. J Bacteriol **109:**1034–1046.

65. **Ohniwa RL, Kitabayashi K, Morikawa K.** 2013. Alternative cardiolipin synthase Cls1 compensates for stalled Cls2 function in *Staphylococcus aureus* under conditions of acute acid stress. FEMS Microbiol Lett **338:** 141–146. http://dx.doi.org/10.1111/1574-6968.12037.

66. **Annous BA, Becker LA, Bayles DO, Labeda DP, Wilkinson BJ.** 1997. Critical role of anteiso-C15:0 fatty acid in the growth of *Listeria monocytogenes* at low temperatures. Appl Environ Microbiol **63:**3887–3894.

67. **Sampathkumar B, Khachatourians GG, Korber DR.** 2004. Treatment of *Salmonella enterica* serovar Enteritidis with a sublethal concentration of trisodium phosphate or alkaline pH induces thermotolerance. Appl Environ Microbiol **70:**4613–4620. http://dx.doi.org/10.1128/AEM.70.8.4613-4620.2004.

68. **Álvarez-Ordóñez A, Fernández A, López M, Arenas R, Bernardo A.** 2008. Modifications in membrane fatty acid composition of *Salmonella typhimurium* in response to growth conditions and their effect on heat resistance. Int J Food Microbiol **123:**212–219. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.01.015.

69. **Martinez-Bueno MA, Tobes R, Rey M, Ramos JL.** 2002. Detection of multiple extracytoplasmic function (ECF) sigma factors in the genome of *Pseudomonas putida* KT2440 and their counterparts in *Pseudomonas aeruginosa* PA01. Environ Microbiol **4:**842–855. http://dx.doi.org/10.1046/j.1462-2920.2002.00371.x.

70. **Mastronicolis SK, Berberi A, Diakogiannis I, Petrova E, Kiaki I, Baltzi T, Xenikakis P.** 2010. Alteration of the phospho- or neutral lipid content and fatty acid composition in *Listeria monocytogenes* due to acid adaptation mechanisms for hydrochloric, acetic and lactic acids at pH 5.5 or benzoic acid at neutral pH. Antonie Van Leeuwenhoek **98:**307–316. http://dx.doi.org/10.1007/s10482-010-9439-z.

71. **Romantsov T, Guan Z, Wood JM.** 2009. Cardiolipin and the osmotic stress responses of bacteria. Biochim Biophys Acta **1788:**2092–2100. http://dx.doi.org/10.1016/j.bbamem.2009.06.010.

72. **Joseph B, Goebel W.** 2007. Life of *Listeria monocytogenes* in the host cells' cytosol. Microbes Infect **9:**1188–1195. http://dx.doi.org/10.1016/j .micinf.2007.05.006.

73. **Joseph B, Przybilla K, Stühler C, Schauer K, Slaghuis J, Fuchs TM, Goebel W.** 2006. Identification of *Listeria monocytogenes* genes contributing to intracellular replication by expression profiling and mutant screening. J Bacteriol **188:**556–568. http://dx.doi.org/10.1128/JB.188.2.556-568.2006.

74. **Tsoy O, Ravcheev D, Mushegian A.** 2009. Comparative genomics of ethanolamine utilization. J Bacteriol **191:**7157–7164. http://dx.doi.org /10.1128/JB.00838-09.

75. **Roof DM, Roth JR.** 1988. Ethanolamine utilization in *Salmonella typhimurium*.J Bacteriol **170:**3855–3863.

76. **Roof DM, Roth JR.** 1989. Functions required for vitamin B12-dependent ethanolamine utilization in *Salmonella typhimurium*. J Bacteriol **171:**3316–3323.

77. **Nygaard P, Duckert P, Saxild HH.** 1996. Role of adenine deaminase in purine salvage and nitrogen metabolism and characterization of the *ade* gene in *Bacillus subtilis*. J Bacteriol **178:**846–853.

78. **Hidaka M, Kitaoka M, Hayashi K, Wakagi T, Shoun H, Fushinobu S.** 2006. Structural dissection of the reaction mechanism of cellobiose phosphorylase. Biochem J **398:**37– 43. http://dx.doi.org/10.1042/BJ20060274.

79. **Bron PA, Molenaar D, de Vos WM, Kleerebezem M.** 2006. DNA micro-array-based identification of bile-responsive genes in *Lactobacillus plantarum*. J Appl Microbiol **100:**728–738. http://dx.doi.org/10.1111 /j.1365-2672.2006.02891.x.

80. **Cotter PD, Gahan CG, Hill C.** 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol Microbiol **40:**465– 475. http://dx.doi.org/10.1046/j.1365-2958.2001.02398.x.

81. **De Biase D, Tramonti A, Bossa F, Visca P.** 1999. The response to stationary-phase stress conditions in *Escherichia coli*: role and regulation of the glutamic acid decarboxylase system. Mol Microbiol **32:**1198–1211. http://dx.doi.org/10.1046/j.1365-2958.1999.01430.x.

82. **Kempf B, Bremer E.** 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch Microbiol **170:**319–330. http://dx.doi.org/10.1007/s002030050649.
83. **Potts M.** 1994. Desiccation tolerance of prokaryotes. Microbiol Rev **58:** 755–805.

84. **Berlett BS, Stadtman ER.** 1997. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem **272:**20313–20316. http://dx.doi.org/10.1074/jbc.272.33.20313.

85. **Storz G, Christman MF, Sies H, Ames BN.** 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. Proc Natl Acad Sci U S A **84:**8917–8921. http://dx.doi.org/10.1073/pnas 84.24.8917.

86. **Wolff SP, Garner A, Dean RT.** 1986. Free radicals, lipids and protein degradation. Trends Biochem Sci **11:**27–31. http://dx.doi.org/10.1016 /0968-0004(86)90228-8.

87. **Kim H, Boor KJ, Marquis H.** 2004. *Listeria monocytogenes* σ^B contributes to invasion of human intestinal epithelial cells. Infect Immun **72:** 7374–7378. http://dx.doi.org/10.1128/IAI.72.12.7374-7378.2004.

88. **Kim H, Marquis H, Boor KJ.** 2005. σ^B contributes to *Listeria monocytogenes* invasion by controlling expression of *inlA* and *inlB*. Microbiology **151:**3215–3222. http://dx.doi.org/10.1099/mic.0.28070-0.

89. **Gouin E, Adib-Conquy M, Balestrino D, Nahori M-A, Villiers V, Colland F, Dramsi S, Dussurget O, Cossart P.** 2010. The *Listeria monocytogenes* InlC protein interferes with innate immune responses by targeting the IKB kinase subunit IKK. Proc Natl Acad Sci U S A **107:**17333–17338. http://dx.doi.org/10.1073/pnas.1007765107.

90. **Kazmierczak MJ, Mithoe SC, Boor KJ, Wiedmann M.** 2003. *Listeria monocytogenes* σ^B regulates stress response and virulence functions. J Bacteriol **185:**5722–5734. http://dx.doi.org/10.1128/JB.185.19.5722-573 4.2003.

91. **McGann P, Wiedmann M, Boor KJ.** 2007. The alternative sigma factorB and the virulence gene regulator PrfA both regulate transcription of *Listeria monocytogenes* internalins. Appl Environ Microbiol **73:**2919– 2930. http://dx.doi.org/10.1128/AEM.02664-06.

92. **Bierne H, Sabet C, Personnic N, Cossart P.** 2007. Internalins: a complex family of leucine-rich repeat-containing proteins in *Listeria monocytogenes*. Microbes Infect **9:**1156–1166. http://dx.doi.org/10.1016/j.micinf.2007.05.003.

93. **Boylan S, Thomas M, Price C.** 1991. Genetic method to identify regulons controlled by nonessential elements: isolation of a gene dependent on alternate transcription factor sigma B of *Bacillus subtilis*. J Bacteriol **173:**7856–7866.

94. **Epstein W.** 1986. Osmoregulation by potassium transport in *Escherichia coli*. FEMS Microbiol Lett **39:**73–78. http://dx.doi.org/10.1111/j.1574-6968.1986.tb01845.x.

95. **Schniederberend M.** 2010. Influence of K^+-dependent membrane lipid composition on the expression of the *kdpFABC* operon in *Escherichia coli*. Biochim Biophys Acta **1798:**32–39. http://dx.doi.org/10.1016/j.bbamem.2009.10.002.

96. **Katoh H, Asthana R, Ohmori M.** 2004. Gene expression in the cyanobacterium *Anabaena* sp. PCC7120 under desiccation. Microb Ecol **47:** 164–174.

97. **Brøndsted L, Kallipolitis BH, Ingmer H, Knöchel S.** 2003. KdpE and a putative RsbQ homologue contribute to growth of *Listeria monocytogenes* at high osmolarity and low temperature. FEMS Microbiol Lett **219:** 233–239. http://dx.doi.org/10.1016/S0378-1097(03)00052-1.

98. **Low DA, Weyand NJ, Mahan MJ.** 2001. Roles ofDNAadenine methylation in regulating bacterial gene expression and virulence. Infect Immun **69:** 7197–7204. http://dx.doi.org/10.1128/IAI.69.12.7197-7204.2001.

99. **Polaczek P, Kwan K, Campbell J.** 1998. GATC motifs may alter the conformation of DNA depending on sequence context and N6- adenine methylation status: possible implications for DNA-proteinrecognition. Mol Gen Genet **258:**488 – 493. http://dx.doi.org/10.1007/s004380050759.

100. **García-Del Portillo F, Pucciarelli MG, Casadesús J.** 1999. DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. Proc Natl Acad Sci U S A **96:**11578 –11583. http://dx.doi.org/10.1073/pnas.96.20.11578.

101. **Pucciarelli MG, Prieto AI, Casadesús J, García-del Portillo F.** 2002. Envelope instability in DNA adenine methylase mutants of *Salmonella enterica*. Microbiology **148:**1171–1182.

102. **Balbontín R, Rowley G, Pucciarelli MG, López-Garrido J, Wormstone Y, Lucchini S, García-del Portillo F, Hinton JC, Casadesús J.** 2006. DNA adenine methylation regulates virulence gene expression in *Salmonella enterica* serovar Typhimurium. J Bacteriol **188:**8160–8168. http://dx.doi.org/10.1128/JB.00847-06.

103. **Løbner-Olesen A, Marinus MG, Hansen FG.** 2003. Role of SeqA and Dam in *Escherichia coli* gene expression: a global/microarray analysis. Proc Natl Acad SciUSA**100:**4672–4677. http://dx.doi.org/10.1073/pnas.0538053100.

104. **van der Veen S, van Schalkwijk S, Molenaar D, de Vos WM, Abee T, Wells-Bennik MH.** 2010. The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis. Microbiology **156:**374–384. http://dx.doi.org/10.1099/mic.0.035196-0.

105. **van der Veen S, Hain T, Wouters JA, Hossain H, de Vos WM, Abee T, Chakraborty T, Wells-Bennik MH.** 2007. The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. Microbiology **153:**3593– 3607. http://dx.doi.org/10.1099/mic.0.2007/006361-0.

106. **Yurimoto H, Hirai R, Matsuno N, Yasueda H, Kato N, Sakai Y.** 2005. HxlR, a member of the DUF24 protein family, is a DNA-binding protein that acts as a positive regulator of the formaldehyde-inducible *hxlAB* operon in *Bacillus subtilis*. Mol Microbiol **57:**511–519. http://dx.doi.org/10.1111/j.1365-2958.2005.04702.x.

107. **Kato N, Yurimoto H, Thauer RK.** 2006. The physiological role of the ribulose monophosphate pathway in bacteria and archaea. Biosci Biotechnol Biochem **70:**10–21. http://dx.doi.org/10.1271/bbb.70.10.

108. **Yasueda H, Kawahara Y, Sugimoto S-I.** 1999. *Bacillus subtilis yckG* and *yckF* encode two key enzymes of the ribulose monophosphate pathway used by methylotrophs, and *yckH* is required for their expression. J Bacteriol **181:**7154–7160.

109. **Daneshvar Alavi HE, Truelstrup Hansen L.** 2013. Kinetics of biofilm formation and desiccation survival of *Listeria monocytogenes* in single and dual species biofilms with *Pseudomonas fluorescens*, *Serratia proteamaculan*s or *Shewanella baltica* on food-grade stainless steel surfaces. Biofouling **29:**1253–1268. http://dx.doi.org/10.1080/08927014.2013.835805.

110. **Heimbrook ME, Wang WL, Campbell G.** 1989. Staining bacterial flagella easily. J Clin Microbiol **27:**2612–2615.