Characterization of *Listeria monocytogenes* Plasmids that were Newly Identified in Whole-genome Sequences of Listeriosis Outbreak Isolates

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Abstract

Listeria monocytogenes is a Gram-positive bacterium that is found ubiquitously throughout nature and is the etiologic agent of listeriosis. The majority of human listeriosis is foodborne, resulting from the consumption of unpasteurized and ready-to-eat foods that are contaminated during food processing. During the 2008 nationwide outbreak, the Gilmour laboratory performed the first real-time application of high-throughput whole-genome sequencing (WGS) of outbreak strains. Within this genomic data, the 77 kb plasmid, pLM5578, was newly identified in a clinical isolate, and additional *Listeria* plasmids (the 80 kb pLM5026 and the 60 kb pLM0813) were subsequently identified after WGS was completed on an expanded panel of outbreak isolates. Little was known regarding how plasmids contribute to persistence and virulence of *L. monocytogenes*, and to investigate these potential relationships, a panel of 147 *L. monocytogenes* food, environmental, and clinical isolates from Canadian public health events from the last three decades was selected for further study of the plasmids they might contain. Strain carriage of plasmids was determined using conventional PCR targeting known plasmid gene targets. Bioinformatic analyses were then used to predict the functions of individual genes encoded by each sequenced plasmid. These analyses were then used to direct experiments investigating the functions and associated phenotypes conferred by plasmid carriage. Phenotypic analyses included antimicrobial susceptibility testing, heavy metal resistance, and biofilm formation assays. Finally, WGS analyses was performed on isolates with plasmid screening patterns that indicated carriage of potential novel plasmids. Screening revealed that 75 of 147 isolates

were positive for the presence of a plasmid, for which WGS analysis identified 24 unique newly identified *L. monocytogenes* plasmids. Phenotypically, 15 of these plasmids were found to contribute to a decreased susceptibility to the heavy metal cadmium, whereas 4 conferred resistance to the sanitizer benzalkonium chloride. Plasmid carriage was also found to affect biofilm formation. Nine plasmids correlated with stronger biofilm formation phenotypes; whereas 5 plasmids were correlated with weaker biofilm formation phenotypes. No known virulence factors or antibiotic resistance determinants were present in the DNA sequences of these 24 newly identified plasmids. Numerous coding sequences predicted to assist with survival under environmental stress were identified, and it is hypothesized that these plasmids likely contributed to persistence of *L. monocytogenes* within food processing environments.

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List of Abbreviations

σ^{β}	Alternative sigma factor β
ActA	Actin assembly-inducing protein
BHI	Brain heart infusion
BKC	Benzalkonium chloride
CBAB	Columbian blood agar base
CDS	Coding DNA sequence
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
DDAC	Didecyldimethylammonium chloride
DDH_2O	Double distilled water
HGT	Horizontal gene transfer
Hpt	Hexose-6-phosphate transporter
InlA	Internalin A
InlB	Internalin B
Kbp	Kilobase pairs
LGI1	*Listeria* genomic island 1
LLO	Listeriolysin O
MDR	Multi-drug resistant
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing

MLVA	Multi-locus variable-number tandem repeat (analysis)
MPF	Mating-pair formation complex
MWB	Modified Welshimer’s broth
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NML	National Microbiology Laboratory
PCR	Polymerase Chain Reaction
PC-PLC	Phosphatidylcholine-specific phospholipase C
PI-PLC	Phosphatidylinositol-specific phospholipase C
PFGE	Pulsed-field gel electrophoresis
PrfA	Positive regulatory factor A
PVC	Polyvinyl chloride
QAC	Quaternary ammonium compound
RTE	Ready-to-eat
SDS	Sodium dodecyl sulfate
T2SS	Type II secretion system
T4SS	Type IV secretion system
TSA	Tryptic soy agar
WGS	Whole-genome sequencing

Chapter 1

General Introduction

1. Introduction

1.1 Infectious diseases

Infectious diseases have significantly shaped the course of human history, including everyday examples of individuals across the globe being affected through illness and death attributable to microbes, to more overt examples where the development of entire civilizations was affected. The presence of epidemic malaria hindered the European conquest of Old World tropical regions for centuries, Smallpox effectively destroyed both the Aztec and Inca empires during the Spanish conquest of Central America, and the Black Death of the 14th century decimated European populations (Morens et al., 2008; Wolfe et al., 2007).

The late 19th and early 20th centuries saw significant progress in humanity's battle against these communicable and endogenous diseases. Industrialized nations implemented significant improvements to sanitation and public health (Snowden, 2008). The struggle against infectious diseases also drove medical and scientific advances, leading to the establishment of the germ theory and identification of specific microorganisms as the causative agents of these human diseases (Morens et al , 2004). The implementation of food safety techniques such as pasteurization and refrigeration, along with the discovery of antibiotics (penicillin and streptomycin) and the development of effective immunizations led to the widespread belief that it was only a matter of time until infectious diseases were eradicated (Marth, 2001; Snowden, 2008).

However, this optimism was premature and by the 1990's it was evident that the threat from infectious diseases was far from over. Today, it is estimated that over

25% of all deaths worldwide are directly related to infectious diseases (Figure 1) (Morens et al., 2004). Additionally, it has been reported that the years between 1940 and 2004 saw the emergence of 335 infectious diseases (Jones et al., 2008). These diseases, including newly emerging diseases such as the HIV and Ebola viruses, re-emerging diseases such as cholera and plague, and developing antibiotic-resistant infections, demonstrate that humanity is far from conquering infectious diseases, if that is even possible given this astounding dynamism (Snowden, 2008). Over the past century, human civilization has undergone tremendous technological and societal transformations. These and future changes constantly alter factors including the environment, population demographics, medical practices, and global trade and travel, influencing exposure and susceptibility to potential human pathogens (Cohen, 2000). The way humans interact with each other and their environment is always changing, but so do the microbes that make us ill, in what seems like a never-ending game of cat and mouse.

1.2 Foodborne diseases

1.2.1 Incidence of foodborne diseases

Foodborne diseases have plagued mankind for centuries, affecting the course of human history like other infectious diseases. References to these illnesses can be found in some of the oldest remaining human records. Descriptions of cholera can be found on Sanskrit manuscripts from India dating back to the 5th century BC (Harris et al., 2012), and references to ergotism due to fungal contamination of food crops have been found on Assyrian tablets dated to 600 BC (De Costa, 2002).

Today, despite improvements in sanitation and public health, foodborne illnesses remain a significant cause of human morbidity and mortality worldwide (Figure 1).

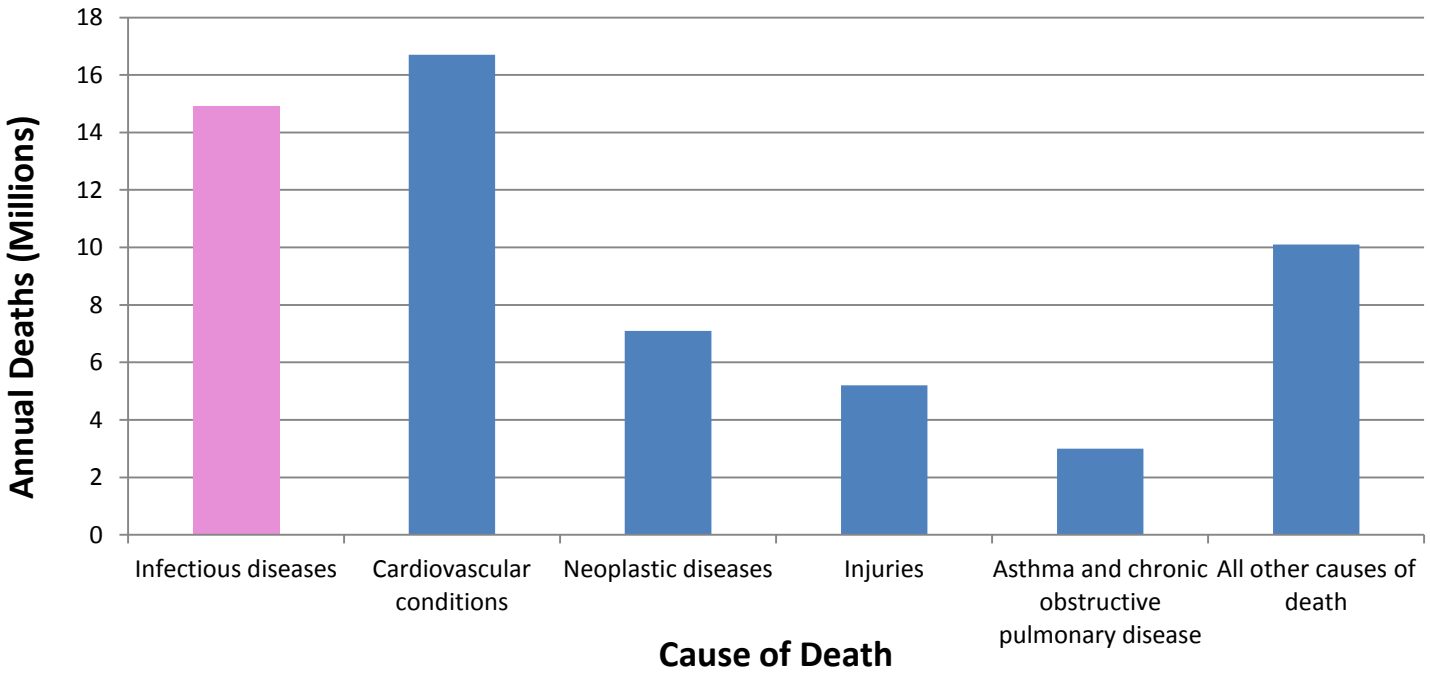
Whereas the exact burden of foodborne diseases is unknown, it is estimated that approximately 30% of all emerging infections that occurred during the past 60 years can be attributed to pathogens that are commonly transmitted through food (Jones et al., 2008). Globally, diarrheal diseases alone cause an estimated 2 billion cases yearly, resulting in 2.2 million deaths annually (World Health Organization, 2008). In the United States, foodborne diseases are responsible for approximately 48 million illnesses, 128,000 hospitalizations, and 3000 deaths annually (Centers for Disease Control and Prevention, 2012; Scallan et al., 2011), resulting in up to $83 billion in costs to the US economy (United States Food and Drug Administration, 2012). In Canada, it is estimated that there are 11 million cases of foodborne illness annually (CFIA, 2012).

1.2.2 Etiology of foodborne disease

Over 200 unique microbial, chemical, or physical agents have been identified that can cause illness when ingested (Newell et al., 2010). These agents include a broad spectrum of human microbial pathogens such as *Salmonella* spp., *Vibrio cholera*, *Escherichia coli*, and *Listeria monocytogenes*; viruses such as the hepatitis A virus and rotavirus; and foodborne parasites such as *Toxoplasma gondii*, *Cryptosporidium* spp., and various species of trematodes (Koopmans & Duizer, 2004; Robertson et al., 2013). Upon contamination of human food or water supplies these pathogens may cause illness when either they or their toxins are consumed.

Some agents of foodborne disease, such as *Shigella* spp. or norovirus, require a

Figure 1: Leading causes of death worldwide. Figure adapted from: (Morens et al., 2004)



Infectious diseases	Annual deaths (millions)
Respiratory infections	3.96
HIV/AIDS	2.77
Diarrhoeal diseases	1.80
Tuberculosis	1.56
Vaccine-preventable childhood diseases	1.12
Malaria	1.27
STDs (other than HIV)	0.18
Meningitis	0.17
Hepatitis B and C	0.16
Tropical parasitic diseases	0.13
Dengue	0.02
Other infectious diseases	1.76

human host as part of their life cycle. Others have a primary reservoir in the environment or in other animals, and human infection occurs incidentally during human contact. Whereas these zoonotic pathogens may be exclusively transmitted through food, others can be transmitted via several different routes, including fecal oral transfer or transmission on inanimate objects such as fomites (Tauxe, 2002). Before the domestication of animals, hunter-gatherer societies regularly encountered microbes and their animal reservoirs during the consumption of wild meat. Over time our society has become dependent on mass production food facilities, which are capable of rapidly distributing both food products and associated foodborne pathogens to consumers around the globe. Food products may be contaminated owing to inadequate processing techniques that fail to ensure removal of zoonotic microbes, or products may be contaminated by pathogens that had been previously deposited in the facility and continue to persist long after the original contaminating animal was removed. Ingestion of these organisms may result in a wide range of illness in humans, the most common of which is gastroenteritis characterized by diarrhea, vomiting, and abdominal pain or discomfort. Others may incur systemic infection and even death (Newell et al., 2010).

1.2.3 Emerging foodborne diseases

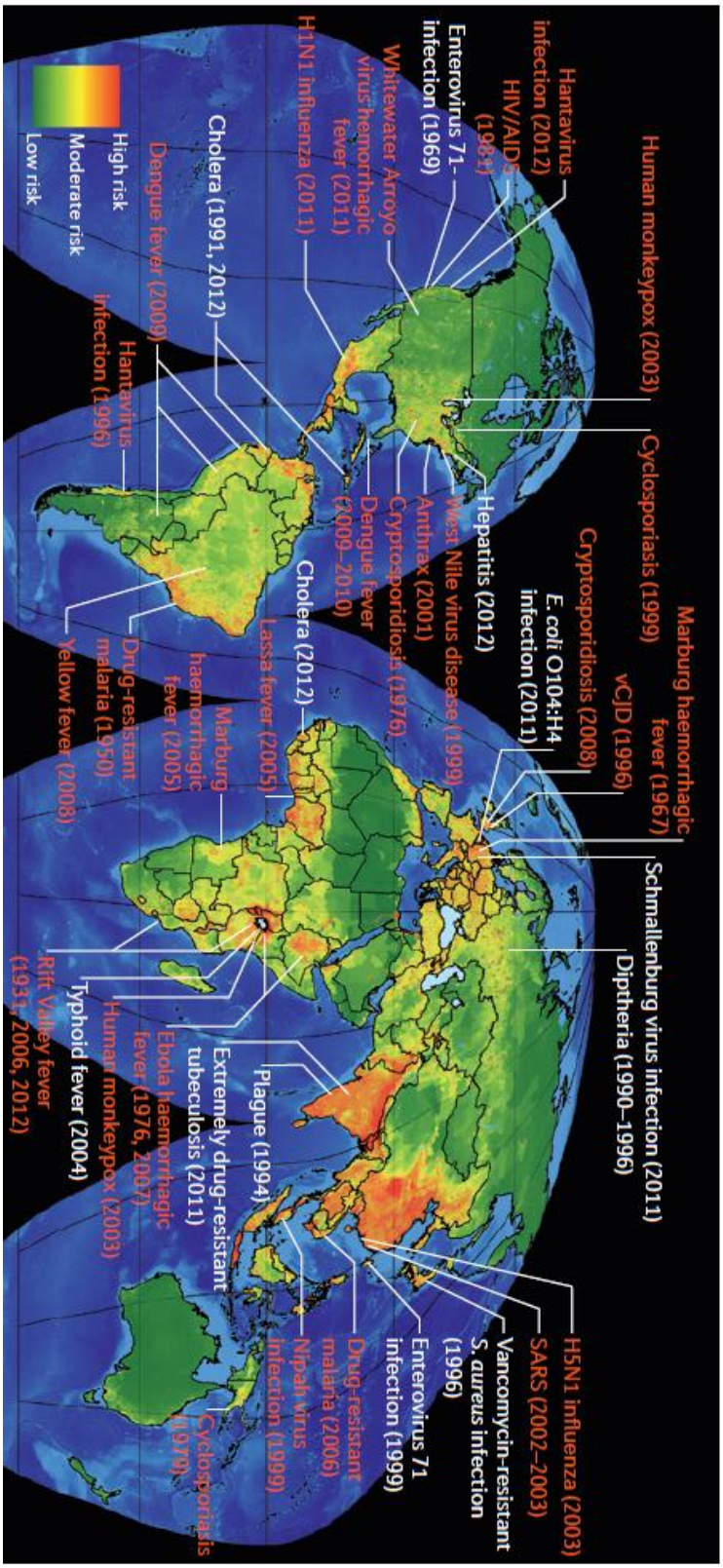
Patterns of foodborne diseases do not remain static; as human society advances and evolves, so does the spectrum of foodborne pathogens. These patterns of disease are the outcome of a complicated interrelationship of ever-changing genetic, biological, social, political, and economic factors (Morens et al., 2008). For example, the challenges to food safety in the early 20th century were extensive, including

unsafe water and poor sanitation, consumption of raw foods such as milk and shellfish, poor food preservation, and a lack of disease control in domesticated animals. As a result, foodborne outbreaks of hepatitis, cholera, typhoid, brucellosis, and staphylococcal food poisoning were common. Today, the incidence of these foodborne diseases has significantly decreased due to the implementation of food safety regulations, and technological advances such as refrigeration and pasteurization (Cohen, 2000). The nature of centralized processing of foods (and their subsequent broad distribution across the country) means that although contamination is rare, when it does happen, it has far-reaching impacts.

While many of these 'classical' foodborne diseases were in decline, new diseases began to emerge as important human pathogens, often through mutation or by moving into a new ecological niche (Figure 2) (Tauxe, 2002). Some, such as *E. coli* O157:H7, appear to have evolved recently and are genuinely new pathogens. *E. coli* O157:H7 was first recognized as a human pathogen in 1982 during two outbreaks of severe bloody diarrhea associated with consumption of undercooked hamburgers from a fast-food restaurant chain (Riley et al., 1983). This pathogenic strain of *E. coli* is thought to have evolved from a less virulent ancestor through the acquisition of Shiga toxins Stx1 and Stx2, along with a large virulence plasmid (Pennington, 2010).

Other foodborne diseases have become the focus of growing concern due to the development of antimicrobial resistance. The use of antimicrobial agents has been commonplace in both human medicine and veterinary/agricultural practice for more than 50 years, leading to the emergence of bacteria resistant to these agents

Figure 2: Locations of emerging and re-emerging/resurging infectious diseases. vCJD=variant Creutzfeldt-Jakob disease. Figure reproduced directly from (Lipkin, 2013)


Human monkeypox (2003)
Cyclosporiasis (1999)
Marburg haemorrhagic fever (1967)
Hantavirus infection (2012)
HIV/AIDS (198-)
vCJD (1996)
Cryptosporidiosis (2008)
E. coli O104:H4 infection (2011)
Schmallenburg virus infection (2011)
Diptheria (1990–1996)
Enterovirus 71 infection (1969)
Hepatitis (2012)
West Nile virus disease (1999)
H5N1 influenza (2003)
SARS (2002–2003)
Whitewater Arroyo virus haemorrhagic fever (2011)
Anthrax (2001)
Cryptosporidiosis (1976)
Dengue fever (2009–2010)
Vancomycin-resistant S. aureus infection (1996)
Enterovirus 71 infection (1999)
H1N1 influenza (2011)
Cholera (2012)
Lassa fever (2005)
Drug-resistant malaria (2006)
Nipah virus infection (1999)
Cholera (1991, 2012)
Marburg haemorrhagic fever (2005)
Plague (1994)
Extremely drug-resistant tubeculosis (2011)
Cyclosporiasis (1979)
Dengue fever (2009)
Ebola haemorrhagic fever (1976, 2007)
Hantavirus infection (1996)
Drug-resistant malaria (1950)
Human monkeypox (2003)
Yellow fever (2008)
Typhoid fever (2004)
Rift Valley fever (1931, 2006, 2012)
High risk
Moderate risk
Low risk

(Scott, 2005). The incidence of antibiotic resistance is increasing in numerous foodborne human pathogens. *Salmonella Typhimurium* phage type DT104 is a multidrug resistant (MDR) strain that has been isolated from human patients around the world (Briggs & Fratamico, 1999). Antibiotic resistance has also emerged in *Campylobacter jejuni*, and fluoroquinolone resistance is now common across the globe, and has been associated with increased virulence during human infection (Zhu et al., 2006).

1.3 The genus *Listeria*

Listeria monocytogenes is a unique emerging foodborne pathogen. Although it has been known to cause disease in humans since the early 20th century, it was identified as a foodborne pathogen only relatively recently (Schlech et al., 1983a). Unlike other infectious diseases, the genus *Listeria* was not named after its discoverer, but instead was named after Sir Joseph Lister, the pioneer of antiseptic surgery (Pirie, 1927). The genus was first discovered in 1924 by Murray, Webb, and Swann during an outbreak in young rabbits in the animal breeding section of the Department of Pathology in Cambridge, England (Murray et al., 1926). This bacterium had many designations, including *Bacterium monocytogenes* and *Listerella monocytogenes,* until finally *Listeria monocytogenes* was agreed upon (Pirie, 1940).

The genus *Listeria* consists of Gram-positive bacteria with low G+C content that are most closely related to other Gram-positive bacteria including *Bacillus*, *Clostridium*, *Staphylococcus*, *Streptococcus*, and *Enterococcus* (Glaser et al., 2001; Wilkinson & Jones, 1977). These bacteria form short, regular rods that measure

0.4-0.5 by 1-2 μm and occurring singly or in short chains. *Listeria* spp. are non-sporulating, facultatively anaerobic and motile with peritrichous flagella when cultured between the temperatures of 20 and 30°C, but are non-motile at 37°C (Wagner & McLauchlin, 2008).

The *Listeria* genus consists of 8 species: *L. monocytogenes, L. marthii, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi, and L. rocourtiae*; *L. marthii* and *L. rocourtiae* were only recently confirmed as species of *Listeria* (Graves et al., 2010; Leclercq et al., 2010). Previously, *L. denitrificans* was recognized as an additional species of *Listeria*, but was dropped in 1987 due to 16S rRNA cataloguing (Rocourt et al., 1987). Of these, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic, with *L. monocytogenes* causing illness in both humans and animals, while *L. ivanovii* is associated with illness in animals (Hain et al., 2007). However, rare cases of human *L. ivanovii* infections have occurred along with single human cases of *L. seegleri* (Rocourt et al., 1986) and *L. innocua* (Perrin et al., 2003).

1.4 *Listeria monocytogenes*

1.4.1 *L. monocytogenes* infection

Listeria spp. are the etiologic agents of listeriosis, a serious foodborne disease that occurs in various animals and humans. However, *L. monocytogenes* is responsible for almost all human cases, and is therefore the species of most concern for human health. The first human cases were described in Denmark in 1929, when *L. monocytogenes* was isolated from the blood cultures of patients that exhibited a mononucleosis-like infection (Nyfeldt, 1929). In 1936, it was determined that *L.*

monocytogenes was responsible for both sepsis in newborn infants and meningitis in adults (Gray & Killinger, 1966). After these initial discoveries, *L. monocytogenes* remained a relatively rare and obscure disease that attracted limited attention, and many bacteriology texts failed to mention the disease. While incidences of human listeriosis continued to occur in Europe, some of which reached epidemic levels, no epidemiological connections could be confirmed (Seeliger, 1988). *L. monocytogenes* was believed to be a zoonotic disease, with transmission resulting from direct animal contact or with contact with human carriers (Gray & Killinger, 1966).

Despite the lack of evidence, the concept of foodborne listeriosis had been considered previously. As early as 1926, Pirie stated: “Infection can be produced by subcutaneous inoculation or by feeding, and it is thought that by feeding that the disease is spread in nature.” (Pirie, 1927) However, foodborne transmission was not confirmed until 1981 when an outbreak of human listeriosis in Halifax, Nova Scotia was directly linked to coleslaw contaminated with *L. monocytogenes.* It was found that the coleslaw was made from cabbage harvested from fields that had been fertilized with manure obtained from infected sheep (Schlech et al., 1983b). There was a rise in both human and animal listeriosis outbreaks in the 1980s, including others linked to commercial food sources, such as milk (Fleming et al., 1985), soft cheeses (Linnan et al., 1988), and pâté (McLauchlin et al., 1991); these outbreaks confirmed that food is the primary route of *L. monocytogenes* transmission and spurred renewed interest in the emerging human pathogen.

Infection by *L. monocytogenes* can result in subclinical infection, febrile gastroenteritis, or invasive listeriosis, and as such are part of the bacterial identification algorithms of blood and CSF samples for front-line clinical microbiology laboratories. Outcomes of infection are highly dependent on three variables: 1) the number of bacteria ingested; 2) the virulence of the ingested strain; and 3) the immune status of the human host (Kuhn et al., 2008). Based on data from animal models and outbreaks, the consensus is that the infectious dose for healthy, immunocompetent individuals is $\geq 10^6$ colony forming units (CFUs), whereas $\leq 10^2$ CFUs can be safely consumed by an individual with decreased susceptibility (Bakardjiev et al., 2004; Smith et al., 2003). However, the immune status and underlying condition of the host is the most critical factor in determining the outcome of *L. monocytogenes* exposure. The majority of severe human listeriosis cases have a physiological or medical condition that results in an impaired ability to mount an effective cellular immune response (Kuhn et al., 2008). Therefore, those most at risk for invasive listeriosis are the elderly over the age of 60, neonates, pregnant women, individuals with cancer, and those undergoing immunosuppressing therapies (Vazquez-Boland et al., 2001).

In healthy, immunocompetent individuals, listeriosis presents as febrile gastroenteritis characterized by watery diarrhea, fever, abdominal pain, headache, chills, and myalgia after an incubation period of approximately 20 hours (Dalton et al., 1997; Ooi & Lorber, 2005). This illness is self-limiting and most patients recover without antimicrobial treatment (Drevets & Bronze, 2008). Infection by *L. monocytogenes* may also result in subclinical infections, but they are rarely

identified. It is likely that these subclinical infections occur on a relatively regular basis, contributing to antilisterial immunity and the relatively low incidence of listeriosis among healthy adults (Munk & Kaufmann, 1988; Vazquez-Boland et al., 2001).

Invasive listeriosis can be divided into fetomaternal/neonatal and non-pregnancy associated listeriosis. Fetomaternal and neonatal listeriosis occurs during *L. monocytogenes* infection of pregnant women, leading to placental transfer of the pathogen to the fetus, and can result in spontaneous abortion or stillbirth in 20% of cases (Mylonakis et al., 2002). Up to two thirds of surviving neonates develop neonatal listeriosis that presents as pneumonia, bacteremia, or meningitis. Infection in the mother is generally asymptomatic but may present as a mild flu-like illness 2 - 14 days prior to miscarriage (Vazquez-Boland et al., 2001).

Invasive listeriosis in non-pregnant adults has an incubation period of 20 - 30 days, but may last up to 70 days after consumption of contaminated foods, seriously confounding epidemiological investigations (Linnan et al., 1988). This form of listeriosis results in bacteremia in 15 - 50% of cases, with symptoms of fever, headache, and muscle pain leading to septicemia. This bacteremia can have a mortality rate as high as 70% in susceptible populations (Lorber, 1997). Invasive listeriosis is also associated with infections of the CNS in 50 - 70% of cases, including meningitis and encephalitis. Depending on the severity of infection and the underlying conditions of the patient, mortality from these CNS infections can range from 20 - 60%. Among the bacteria capable of causing meningitis, *L.*

monocytogenes is the third most common and has the highest average mortality rate at 22% (Lecuit, 2007).

One of the primary reasons for the research in *L. monocytogenes* is that most food-borne infections in humans, such as *Salmonella* serovar Enteritidis or *Campylobacter* spp., are characterized by a relatively high incidence with low morbidity and mortality. However, the opposite is true for *L. monocytogenes,* as the incidence of listeriosis is low yet results in potentially severe infections. Human listeriosis has a mortality rate of 30%, compared to 0.38% for *S.* Enteritidis and 0.02 to 0.1% for *Campylobacter* spp. (Kuhn et al., 2008), and is estimated to be the third leading cause of foodborne illness-related death in the United States (Mead et al., 1999; Scallan et al., 2011). The interest in *Listeria* is compounded further in that it can contaminate and be present in a variety of food products that consumers buy and consume on a regular basis. In addition, while *L. monocytogenes* is killed by cooking and pasteurization, it is unlike many other enteric pathogens because it can grow at refrigeration temperatures (see below).

1.4.2 Life cycles within the host and environment

L. monocytogenes is an opportunistic human pathogen, which means it harbors the ability to replicate both within animal hosts as well as within outside environments. Unlike obligate bacterial pathogens, which generally rely on the warmth and stability of their hosts to provide a suitable environment for replication, *L. monocytogenes* maintains a wide range of abilities to live in these highly disparate conditions. *L. monocytogenes* also differs from other soil pathogens, such as *Clostridium* spp. and *Bacillus anthracis*, in that it does not utilize resistant, long-lasting spores to survive

until conditions become favorable (Xayarath & Freitag, 2012). As a result, *L. monocytogenes* is capable of growth and survival under a wide range of harsh environmental conditions. It is capable of growth at temperatures ranging from 0°C to 45°C, and the ability to grow at refrigeration temperatures is of primary concern to food safety. The growth of *L. monocytogenes* at refrigeration temperatures, while relatively slow, can result in heavy contamination of food products during long-term storage. The human pathogen is also capable of growth in a pH range of 4.4 to 9.6 and in up to 10% NaCl, while able to survive pH's ranging from 3 to 12 and in up to 40% NaCl (Chaturongakul et al., 2008).

The limited experimental data available indicates that the other members of the *Listeria* spp. are capable of surviving the same environmental stresses as *L. monocytogenes* (Liu, 2008). As a result, *Listeria* spp. are found ubiquitously throughout the environment, where they are naturally found in soil, mud, fresh water, and wastewater (Rocourt & Seeliger, 1985); one study found that up to 20% of soil samples were positive for *Listeria* (Weis & Seeliger, 1975), while another found that 81% of fresh water samples contained *Listeria* spp. (Colburn et al., 1990). *Listeria* is also often found to be present on vegetation from both cultivated fields and naturally occurring forest. The free-living bacteria appear to thrive in these varied environments, filling the niche of saprophytes that live off dead plant matter (Gray & Killinger, 1966).

Due to the high incidence in soil, water, and vegetation, these bacteria are easily contracted by and transmitted within wild and domestic herd animals. *L. monocytogenes* has also been isolated from a wide range of wild and domesticated

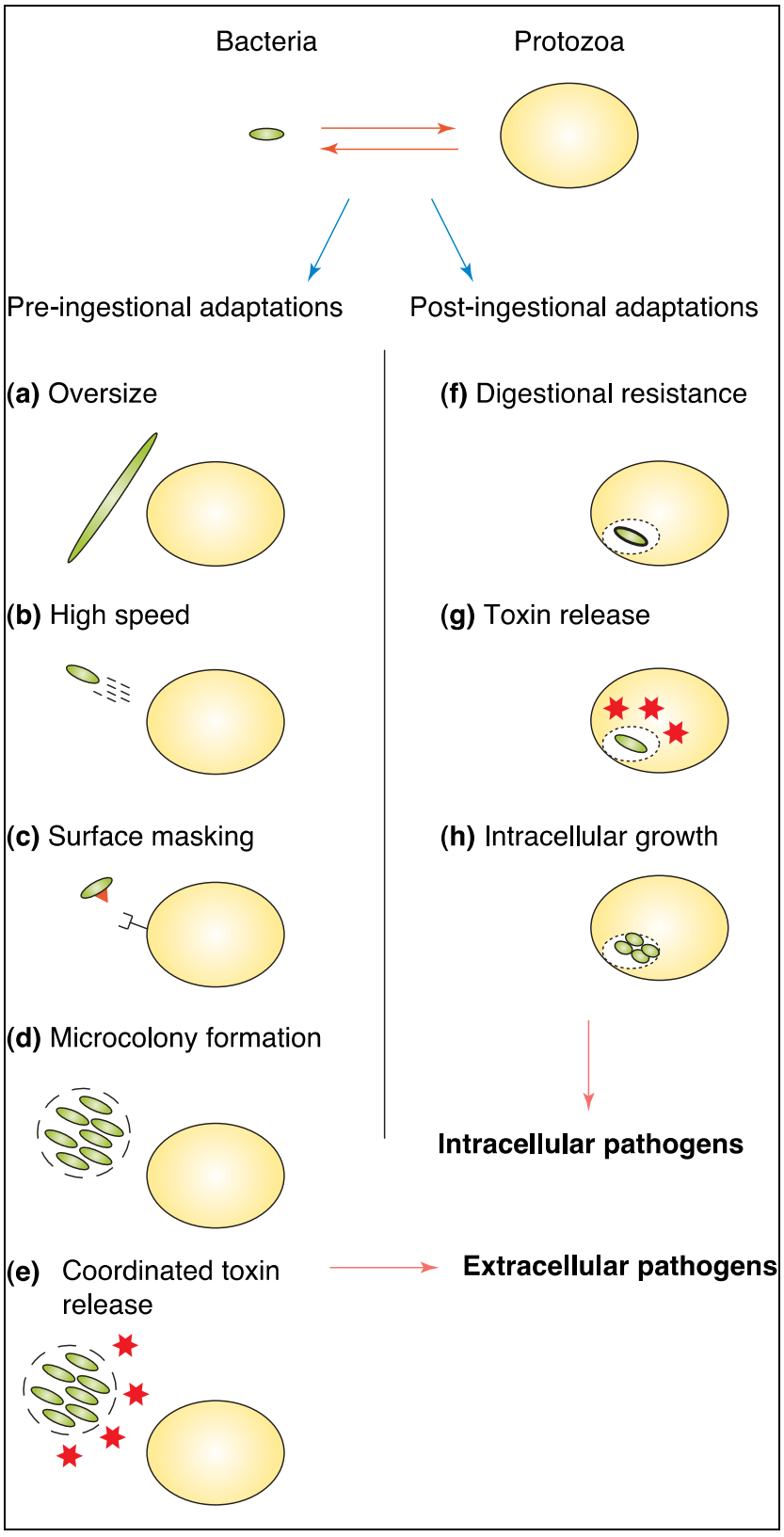
animals, including deer, cattle, sheep, horses, goats, chickens, rodents, and fish (Hutchison, 2004; Nightingale et al., 2004; Weis & Seeliger, 1975); Gray and Killinger isolated *L. monocytogenes* from the feces of 37 different mammals (Gray & Killinger, 1966). Both *L. ivanovii* and *L. monocytogenes* have been frequently isolated from wild and domestic animals, and have been shown to cause illness, including abortion and stillbirths. Other species of *Listeria*, including *L. grayi* and *L. innocua* also occur in animals but their role has yet to be confirmed (Liu, 2008).

A reservoir is defined as *'one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population'* (Haydon et al., 2002). It is likely that the farm environment, including soil, plant materials, and animal feed, serves as a reservoir for *L. monocytogenes*. Whereas the pathogen regularly causes illness in animals, *L. monocytogenes* can also survive for long periods of time within the intestines of ruminants, being constantly shed in feces while displaying no apparent signs of infection (Borucki et al., 2005; Nightingale et al., 2004; Winter et al., 2004). One study from Finland observed as high as 45% of dairy cows were fecal carriers of *L. monocytogenes* (Husu, 1990). Whereas cycles of transmission are clearly evident in farm environments, it is difficult to quantify the importance of these environments as sources of virulent strains of *L. monocytogenes* that enter the food chain and cause human infections.

Life as a soil saprophyte means that *L. monocytogenes* must also frequently encounter other microorganisms, and we are only beginning to understand the complex relationships between these organisms. Environmental bacteria live under

constant threat by bacterivorous predators, including free-living single-celled protozoa and nematodes. Consumption by these predators is considered to be a significant source of bacterial mortality in most freshwater, marine, and soil ecosystems. This constant evolutionary pressure is believed to help shape bacterial defense strategies and drive the establishment of new replicative niches (Hilbi et al., 2007). Protozoa are primordial phagocytes that feed on bacteria by engulfing them in a manner similar to mammalian phagocytes, such as macrophages. As a result, environmental bacteria have developed numerous strategies to deal with the threat of predation (Figure 3). One of these strategies is the formation of biofilms; the congregation of *Pseudomonas* spp. within biofilms has been shown to prevent engulfment by protozoa (Hahn et al., 2000). Other strategies include the development of virulence factors that allow the bacterial prey to survive ingestion by microorganism predators. These post-ingestional adaptations include the ability to survive and replicate within protozoa, as well as virulence mechanisms that kill the bacterial predator. For example, *Legionella pneumophila* uses a variety of virulence factors including type II secretion systems (T2SSs), type IV secretion systems (T4SSs), toxins, pili, and flagella to form Legionella-containing vacuoles to survive and replicate in protozoans such as *Acanthamoeba* spp. and *Tetrahymena* spp. The life cycle of *L. pneumophila* within these hosts very strongly resembles the life cycle

Figure 3: Routes of bacterial adaptation against predation by protozoa. (a-e) represent pre-ingestional strategies, while (f-h) represent post-ingestional adaptations emerging from bacteria-protozoa interactions. Figure reproduced directly from: (Matz & Kjelleberg, 2005)


Bacteria
Protozoa
Pre-ingestional adaptations
Post-ingestional adaptations
(a) Oversize
(b) High speed
(c) Surface masking
(d) Microcolony formation
(e) Coordinated toxin release
(f) Digestional resistance
(g) Toxin release
(h) Intracellular growth
Intracellular pathogens
Extracellular pathogens

of the opportunistic pathogen within mammalian phagocytes such as macrophages (Swanson & Hammer, 2000).

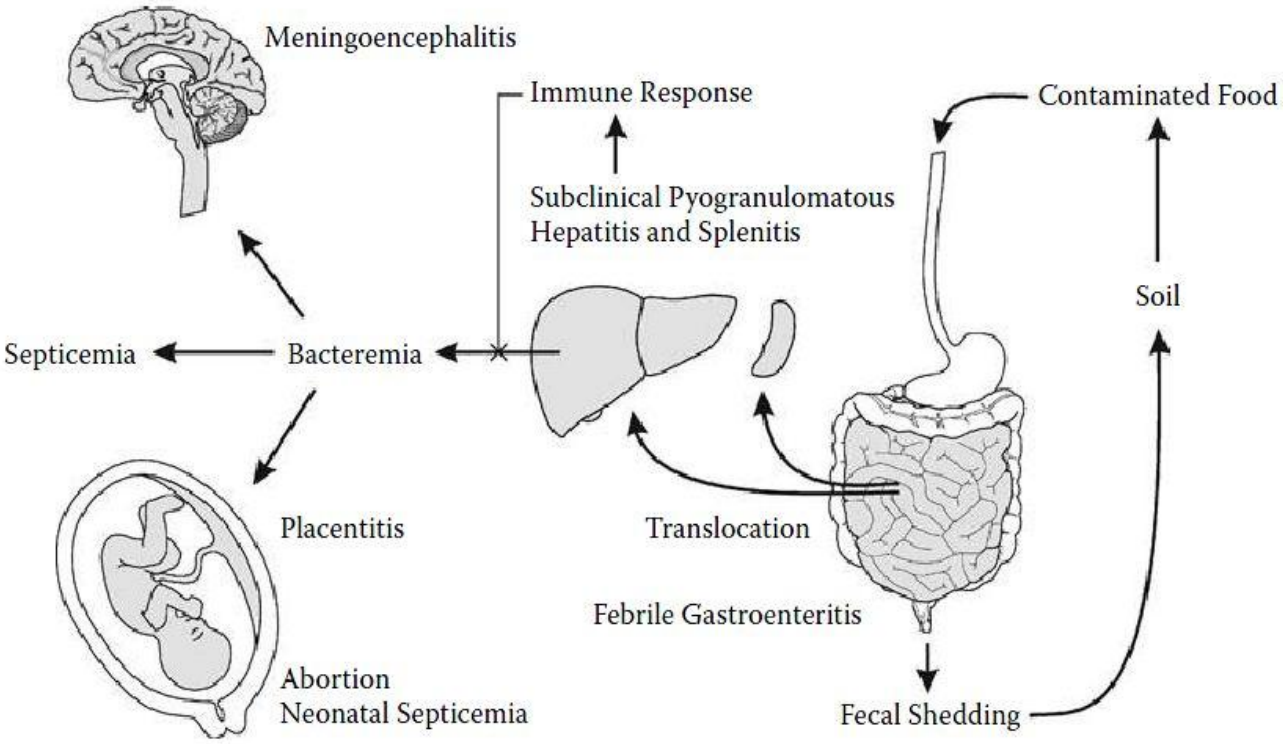
Experimental data has shown that *L. monocytogenes* is able to survive and multiply within protozoans, including *T. pyriformis* and *Acanthamoeba* spp. (Ly & Muller, 1990). *L. monocytogenes* has the ability to survive and replicate within a wide range of mammalian cells, including both phagocytes and non-phagocytic cells. Aside from the internalization step, the intracellular life cycle of *L. monocytogenes* within these two cell types is essentially identical (Kuhn et al., 2008). The fact that *L. monocytogenes* is able to survive within both predator protozoa and mammalian macrophages and that these cells use similar phagocytic mechanisms strongly supports that this *Listeria*-protozoan interaction may have been a driving force in the evolution of this opportunistic human pathogen (Matz & Kjelleberg, 2005).

1.4.3 Route of infection

The primary route of *L. monocytogenes* infection in humans and other mammals is oral ingestion (Figure 4) (Vazquez-Boland et al., 2001). However, the bacterial pathogen can also be transmitted vertically from mother to fetus in fetomaternal listeriosis, or transmitted directly via the skin during exposure to aborted fetuses and genital excretions of *L. monocytogenes* infections in ruminants. Infection in this manner generally results in a rash with pustules that is seen sporadically in farmers and veterinarians that are exposed to ruminants that are positive for the infection (McLauchlin & Low, 1994).

Following oral ingestion *L. monocytogenes* survives passage through the stomach, although it is thought that a significant number of the bacteria are killed;

Figure 4: Schematic representation of the physiopathology of *L. monocytogenes* infection. Figure reproduced directly from (Vazquez-Boland et al., 2001)


Meningoencephalitis
Immune Response
Contaminated Food
Subclinical Pyogranulomatous
Hepatitis and Splenitis
Soil
Septicemia
Bacteremia
Placentitis
Translocation
Febrile Gastroenteritis
Abortion
Neonatal Septicemia
Fecal Shedding

the use of antacids and drugs that decrease gastric acidity have been shown to be risk factors for listeriosis (Schuchat et al., 1992). After entry into the intestinal tract, *L. monocytogenes* crosses the intestinal barrier through two methods. The first method is by the direct invasion of the enterocytes of the intestinal epithelium and involves specific ligand-receptor interactions of listerial internalins. This method of invasion is thought to only occur in hosts that express susceptible receptor isoforms on intestinal cells, such as humans and guinea pigs (Lecuit, 2005; Schuchat et al., 1992). The second method of entry is less efficient and unspecific, involving phagocytosis by the M cells of Peyer's patches in hosts that lack required receptors (Corr et al., 2006; Marco et al., 1997). Gastroenteritis is thought to occur when relatively large amounts of bacteria are ingested, resulting in extensive invasion of the intestinal enterocytes by *L. monocytogenes* (Vazquez-Boland et al., 2001).

Within 24 hours of crossing the intestinal barrier, *L. monocytogenes* is carried by the blood or lymph to the mesenteric lymph nodes, spleen, and liver, where replication occurs. Most of the bacterial load accumulates in the liver, due to the high susceptibility of hepatocytes to internalization and intracellular replication by the bacterial pathogen (Conlan & North, 1992; Gaillard et al., 1996). It is at this point in immunocompetent hosts where the infection is usually contained by cytotoxic $CD8^+$ T lymphocytes, resulting in clearance after six or seven days after ingestion (Palmer, 2004). Exposure to *L. monocytogenes* in healthy hosts leads to the development of an adaptive immune response to the bacterium. This memory response results in rapid elimination of *L. monocytogenes* during subsequent infections. Munk and Kaufmann found that immunity to *L. monocytogenes* is commonly present in healthy

individuals in the human population, presumably due to chronic exposure to the pathogen during the consumption of contaminated food (Munk & Kaufmann, 1988).

However, if the immune response fails to eradicate the bacterium, it is subsequently released from the liver into the bloodstream. If this bacteremia is prolonged, it can progress to more serious forms of clinical listeriosis, including septicemia or localized infections. There is evidence that dissemination of *L. monocytogenes* throughout the body is aided by infected phagocytes in a Trojan horse-like mechanism (Drevets, 1999). In pregnant women, *L. monocytogenes* uses internalins to target and cross the placenta in a manner similar to that used to cross the intestinal barrier. After translocation across the placental endothelial barrier, the bacterium enters the fetal bloodstream, leading to generalized infection and death of the fetus (Vazquez-Boland et al., 2001). Invasive listeriosis is also frequently associated with infections of the CNS, resulting in meningitis and/or encephalitis. This is the result of *L. monocytogenes's* ability to cross the blood-brain barrier; the pathogen has been shown to achieve this in numerous ways, including invasion of CNS endothelial cells, using intracellularly-infected phagocytes, and direct infection of peripheral axons with migration to the brain stem (Drevets & Bronze, 2008).

1.4.4 Diagnosis and treatment

Currently, *L. monocytogenes* infection is diagnosed by obtaining a positive culture from a normally sterile site, such as blood, cerebrospinal fluid, or from amniotic fluid or the placenta in pregnant women. Specimens are directly plated on TSA with 5% blood, and incubated overnight at ambient temperature. Stool cultures are generally not considered useful for diagnosis, as 1 - 15% of the population represent

asymptomatic carriers of *L. monocytogenes* (Grif et al., 2003). Serological testing using commercial whole cell suspensions containing O and H antigens also have limited usefulness for diagnosis due to cross-reactivity between *Listeria* spp. and other Gram-positives including *Bacillus* spp., *Staphylococcus*, and *Enterococcus.* PCR assays have been developed that use targets specific to *L. monocytogenes*, and are in use within some laboratories as a method of rapid identification of infection, particularly when prior use of antimicrobial agents may have compromised a culture (Allerberger & Wagner, 2010).

L. monocytogenes is generally susceptible to a wide range of antibiotics, with the exception of resistance to older quinolones, mecillinam, aztreonam, and innate resistances to the cephalosporins. Treatment for this bacterial infection usually involves ampicillin and penicillin, often combined with gentamicin, although the latter is not prescribed for pregnant women due to possible teratogenic effects. Patients with allergies to β-lactams can be treated with trimethoprim-sulfamethoxazole or erythromycin (Hof, 2004).

1.4.5 Virulence factors

1.4.5.1 Regulators of virulence

As an opportunistic pathogen, *L. monocytogenes* must be capable of switching from a saprophytic lifestyle to that of an intracellular pathogen, effectively transitioning from a life in the soil to life within the cell. In order to make this transition, the bacterium must be able to detect cues from its environment that signal the need for this change, and express the proper gene products for each location (Freitag et al., 2009). This transition is mediated by a complex interplay of regulatory networks that

mediate the expression of genes related to both environmental stressors and virulence. However, two of the main transcriptional regulators of these two very different lifestyles are the alternative sigma factor β (σ^{β}) and the positive regulatory factor A (PrfA). σ^{β} is the central regulator of stress adaptation and is conserved in many Gram-positive bacteria, including *B. anthracis*, *S. aureus*, and *B. licheniformis* (Brody & Price, 1998; Fouet et al., 2000; Wu et al., 1996). It has been found to contribute to survival in these organisms during exposure to numerous environmental stresses, including acid, oxidative, temperature, and energy stresses (Oliver et al., 2010). σ^{β} is the main transcriptional regulator during the lifestyle in the external environment and gastrointestinal tract, while PrfA regulates intracellular infection.

σ^{β} has also been shown to positively regulate 105 and to negatively regulate 111 genes. Altogether, these account for 7.6% of all the genes in genome of *L. monocytogenes* (Hain et al., 2008). σ^{β} is required for rapid induction of genes required for survival in the gut, including conditions of low pH, elevated osmolarity, and the presence of bile salts (Gahan & Hill, 2005). In mouse and guinea pig models, loss of σ^{β} has been shown to decrease virulence during oral infection (Garner et al., 2006; Nadon et al., 2002). Additionally, many of the systems regulated by σ^{β} contribute to survival in the external environment and in the food matrix (Gahan & Hill, 2005). It is also evident that σ^{β} modulates expression of PrfA, and it appears that the alternative σ-factor is not only required for successful transit of the GI but that it also primes the pathogen for later stages of infection, such as cellular invasion (Ollinger et al., 2009).

PrfA is the master transcriptional regulator for virulence genes in *L. monocytogenes*, controlling nearly all the gene products required for cellular invasion, entry and growth in the cytosol, and intracellular motility. The core PrfA regulon consists of 10 genes directly controlled by the protein (Table 1), and evidence indicates that it may also modulate at least 145 additional genes (Scortti et al., 2007). Murine models have shown that PrfA mutants are unable to replicate inside cells and are 100,000 times less virulent compared to wild-type strains (Freitag et al., 1993). Temperature has been shown to be an important regulator of PrfA. Expression of PrfA is controlled by an RNA thermosensor that prevents translation of the protein at temperatures lower than 30°C (Freitag et al., 2009). However, it also appears that temperature is not the only control mechanism of PrfA and the protein does not achieve full activation until entry into the cytoplasm of host cells. Evidence indicates that full activation of the transcriptional regulator may be related to the carbon sources available in the host cell cytosol (Joseph et al., 2008).

1.4.5.2 Cellular invasion and replication

L. monocytogenes is capable of crossing numerous barriers within its many hosts, and the bacterium has the ability to invade and replicate inside a wide range of cells. The pathogen has been shown to readily invade and replicate in epithelial cells, macrophages, neutrophils, dendritic cells, hepatocytes, endothelial cells, and glial cells (Kuhn et al., 2008). Figure 5 illustrates the primary proteins required for each step during invasion of and replication within host cells. While macrophages actively engulf the bacterium spontaneously, internalization in non-phagocytic cells is mediated by the bacterium itself.

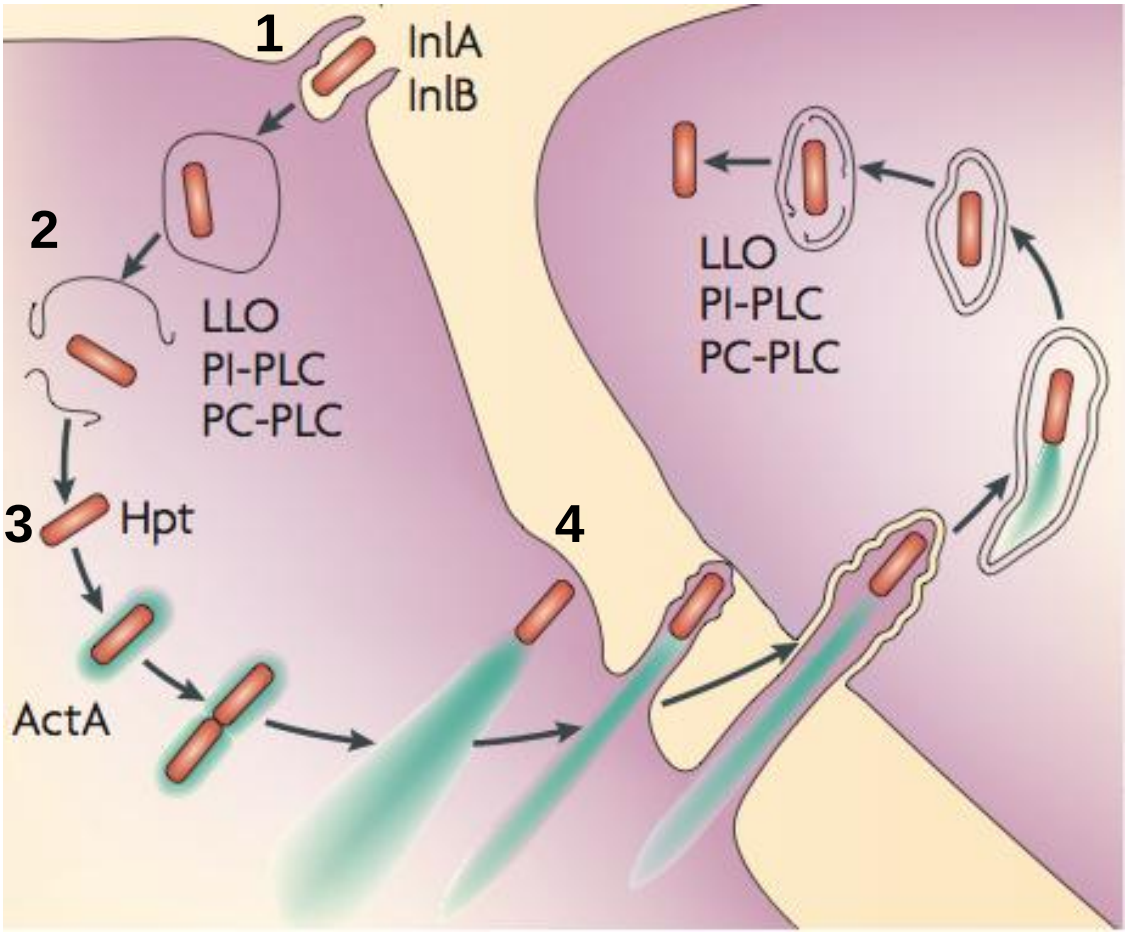
Table 1: *Listeria monocytogenes* gene products that are directly regulated by PrfA. Table reproduced directly from (Freitag, 2009)

Gene name	Protein product	Function
hly	Listeriolysin O (LLO)	A pore-forming cytolysin that is required for phagosome lysis
plcA	Phosphatidylinositol-specific phospholipase C (PI-PLC)	Aids in phagosome lysis
plcB	Phosphatidylcholine phospholipase C (PC-PLC)	A broad substrate specificity phospholipase that aids in phagosome lysis
mpl	Mpl	A zinc metalloprotease that processes the PC-PLC precursor to its mature form
actA	Actin assembly-inducing protein (ActA)	Stimulates actin-based intracellular bacterial motility
hpt	Hexose phosphate transporter (Hpt)	Required for optimal intracellular bacterial growth
inlA	Internalin A (InlA)	Contributes to host cell invasion
inlB	Internalin B (InlB)	Contributes to host cell invasion
inlC	Internalin C (InlC)	Contributes to bacterial virulence; exact role unknown
prfA	Positive regulatory factor A (PrfA)	Required for the expression of L. monocytogenes virulence factors

The internalins, InlA and InlB, are the main bacterial surface proteins required for invasion of host cells. InlA binds to E-cadherin which is found primarily on enterocyte-like epithelial cells and hepatocytes, invasion of which allows *L. monocytogenes* to cross the intestinal and placental barriers (Lecuit et al., 2001). InlB interacts with several host cell receptors, but primarily interacts with the hepatocyte growth factor receptor c-Met (Shen et al., 2000). Binding of either InlA or InlB to the corresponding host cell receptors triggers a signaling cascade that allows the invading bacterium to exploit the host cell's endocytic machinery and to be internalized (Figure 5). Once inside the phagosome, *L. monocytogenes* releases lysteriolysin O (LLO), a pore-forming cytolysin, as well as two phospholipases termed PI-PLC and PC-PLC (Marquis et al., 1995; Schnupf & Portnoy, 2007; Wadsworth & Goldine, 1999). These secreted molecules work together to break down the phagosome, releasing the pathogen into the cytosol. There *L. monocytogenes* replicates using nutrients located in the host's cytoplasm, including hexose phosphate sugars acquired using a hexose phosphate transporter (Hpt), as well as peptides and lipoic acids (Joseph & Goebel, 2007).

L. monocytogenes uses a fascinating mechanism to move within the host cell and to invade neighboring cells. Using the surface protein ActA, the bacterium is able to polymerize actin filaments from the cytoplasm to create an actin tail, which acts as a molecular motor and serves as a form of locomotion. ActA-based motility, also known as "actin rockets", is used to propel *L. monocytogenes* through the plasma membrane of the host cell into adjacent cells (Southwick & Purich, 1994). This allows the intracellular pathogen to spread cell-to-cell while aiding in evading

Figure 5: Major proteins involved in cellular invasion by *L. monocytogenes*. Once inside the host, PrfA becomes activated (PrfA*) and induces the expression of gene products that are needed for the stages of infection: 1) *host cell invasion* (internalins InlA and InlB); 2) *phagosome lysis* (listeriolysin O (LLO), phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine (PC)-PLC); 3) *intracellular growth* (hexose-6-phosphate transporter (Hpt)); and 4) *cell-to-cell spread* (actin assembly-inducing protein (ActA); actin polymerization is shown in turquoise). Figure adapted from (Freitag et al., 2009)


1
InlA
InlB
2
LLO
PI-PLC
PC-PLC
3
Hpt
ActA
4
LLO
PI-PLC
PC-PLC

the host's humoral immune system. It has also been recently shown that ActA promotes bacterial aggregation and biofilm formation *in vitro*. The same study also found that this ActA-dependent aggregation resulted in increased persistence of *L. monocytogenes* within the gut lumen of mice, as well as increased bacterial shedding (Travier et al., 2013). As a result, ActA is one of the major determinants of virulence in *L. monocytogenes*.

1.4.6 *L. monocytogenes* serotypes, lineages, and subtyping

Members of the genus *Listeria* can be classified into serotypes based on the reactions of specific antisera with the bacterial somatic (O-factor) and flagellar (H-factor) antigens. *L. monocytogenes* is comprised of the serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7. The majority of human illnesses worldwide are caused by a small number of serotypes, with isolates from 1/2a, 1/2b, 1/2c, and 4b accounting for at least 98% of human listeriosis (Wagner & McLauchlin, 2008).

Serotyping alone is often not sufficient for distinguishing isolates during epidemiological investigations. Therefore, other subtyping methods are used in conjunction with serotyping, including phage typing, ribotyping, multi-locus sequence typing (MLST), multi-locus variable-number tandem repeat analysis (MLVA), and pulsed-field gel electrophoresis (PFGE). Using these subtyping methods *L. monocytogenes* isolates can be classified into phylogenetic groups such as evolutionary lineages, epidemic clones, and clonal complexes. Subtyping methods also provide discriminatory power beyond serotyping, including sequence types and PFGE pattern types (Graves & Swaminathan, 2001; Pagotto, Ng et al., 2006; Sperry et al., 2008; Wiedmann, 2002).

Numerous phylogenetic and subtyping studies have shown that *L. monocytogenes* isolates cluster into four evolutionary lineages (Table 2): lineages I, II, III, and IV. Although the exact distribution of serotypes within these lineages is under debate, the majority of *L. monocytogenes* isolates cluster in lineages I and II (Orsi et al., 2011). The general consensus is that lineage I consists of serotypes 1/2b, 3b, 4b, 4d, 4e, while lineage II contains types 1/2a, 1/2c, 3a, and 3c.

There are considerable differences between serotypes and lineages, including their prevalence in different environments and outcomes of clinical illness. The majority of listeriosis outbreaks worldwide have been linked to serotype 4b isolates belonging to lineage I, although there have been major outbreaks associated with lineage I serotype 1/2b isolates and lineage II 1/2a serotypes (Aureli et al., 2000; Centers for Disease Control and Prevention, 2011; Gianfranceschi et al, 2003; Gilmour et al., 2010; Hong et al., 2007; Kiss et al., 2006; McLaughlin et al., 2004). Lineage II serotype 1/2a isolates appear to be more prevalent in Finland (Lukinmaa et al., 2004) and Sweden (Parihar et al., 2008), whereas isolates belonging to lineage I serotype 4b seem to be predominant in human cases in the United States (Orsi et al., 2011). Notably, two high profile listeriosis outbreaks, one in Canada in 2008 and the other in the US in 2011, were attributed to serotype 1/2a strains and are considered to be among the worst foodborne outbreaks in history (Gilmour et al., 2010; McCollum et al., 2013). Isolates from lineages III and IV are rare, and while occasionally isolated from cases of human illness, they have never been linked to a human listeriosis outbreak (Gray et al., 2006; Jeffers et al., 2001). However, there is evidence that isolates from lineages III and IV may be adapted to non-primate

Table 2: Summary of *L. monocytogenes* lineages and serotypes. Table adapted from (Orsi et al., 2011)

Lineage	Serotypes	Genetic Characteristics	Distribution
I	1/2b, 3b, 3c, 4b	Lowest diversity among the lineages; lowest levels of recombination among the lineages	Commonly isolated from various sources; overrepresented among human isolates
II	1/2a, 1/2c, 3a	Most diverse, highest recombination levels among the lineages	Commonly isolated from various sources; overrepresented among food and food-related as well as natural environments
III	4a, 4b*, 4c	Very diverse; recombination levels between those for lineage I and lineage II	Most isolates obtained from ruminants
IV	4a, 4b*, 4c	Few isolates analyzed to date	Most isolates obtained from ruminants

*****Serotype 4b generally classify into lineage I; lineage III and IV 4b isolates are considered atypical

mammalian hosts, and at least one outbreak of listeriosis in goats was caused by a lineage IV strain (Orsi et al., 2011; Wiedmann et al., 1999).

A number of studies have also found that isolates belonging to lineage II, such as 1/2a and 1/2c, are more frequently recovered from foods and food processing environments compared to lineage I isolates (Chen et al., 2009; Corcoran et al., 2006; De Cesare et al., 2007; Gianfranceschi et al., 2003; Gray et al., 2004; Handa et al., 2005; Hong et al., 2007; Klæboe et al., 2006; Lomonaco et al., 2009; Lukinmaa et al., 2004; Manfreda et al., 2005; Meloni et al., 2009; Norton et al., 2001; Praakle-Amin et al., 2006; Sauders et al., 2004; Trott et al., 1993; Zhou & Jiao, 2006). A number of the authors of these studies have speculated that the overrepresentation of lineage II isolates in food and food-associated environments may be due to an increased ability to grow and survive in these environments, including the ability to persist in these environments for long periods of time (Orsi et al., 2011).

There is also evidence that serotypes may exhibit differing levels of virulence. As stated previously, the majority of human listeriosis cases are linked to lineage I 4b isolates, while lineage II isolates are generally more prevalent in foods. Therefore, it is hypothesized that these 4b isolates are more pathogenic than lineage II isolates of serotype 1/2a and 1/2c. Outside of 4b isolates this has been attributed to carriage of a truncated version of the *inlA* gene due to premature stop codons, and this mutation results in decreased invasion efficiency in mammalian cell lines (Jacquet et al., 2004; Nightingale et al., 2005). Truncated forms of *inlA* have been shown to be present in >30% of *L. monocytogenes* isolated from food in both France

and the United States, while no serotype 4b isolates carrying truncated versions of the *inlA* gene have been found to date (Orsi et al., 2011).

1.4.7 Persistence in food-associated environments

Due to the ubiquitous nature of *L. monocytogenes* within soil and farm environments, as well the organism's ability to readily infect cattle and other food animals, entry into the human food chain is inevitable. Since the confirmation of *L. monocytogenes* as a foodborne disease, a wide variety of foods have been implicated in both sporadic cases and outbreaks of human listeriosis. However, many of these foods share a common characteristic: they are "ready-to-eat" (RTE) foods, meaning they are eaten without further cooking by the consumer (Norton & Braden, 2007). These foods are usually treated in some manner, such as cooking, curing, or pasteurization, to inactivate foodborne pathogens including *L. monocytogenes*. Despite considerable evidence that these methods are effective at killing *L. monocytogenes* in these food products, the pathogen is routinely isolated after these processes (Kornacki & Gurtler, 2007). Therefore, the primary source of contamination in these food products is the processing environment, with contamination occurring post-processing (Kathariou, 2002). The low infection CFU of *L. monocytogenes*, and the fact that a single cell that is carried over can replicate at refrigerator temperatures means there is very little tolerance for carryover of this bacterium relative to other pathogens. *Listeria* spp. have been isolated from nearly all food processing facilities involved in *Listeria*-related food recalls (Hitchins & Whiting, 2001; Kornacki & Gurtler, 2007). Although it is possible that *L. monocytogenes* can enter the human food chain at many different points (Figure 6),

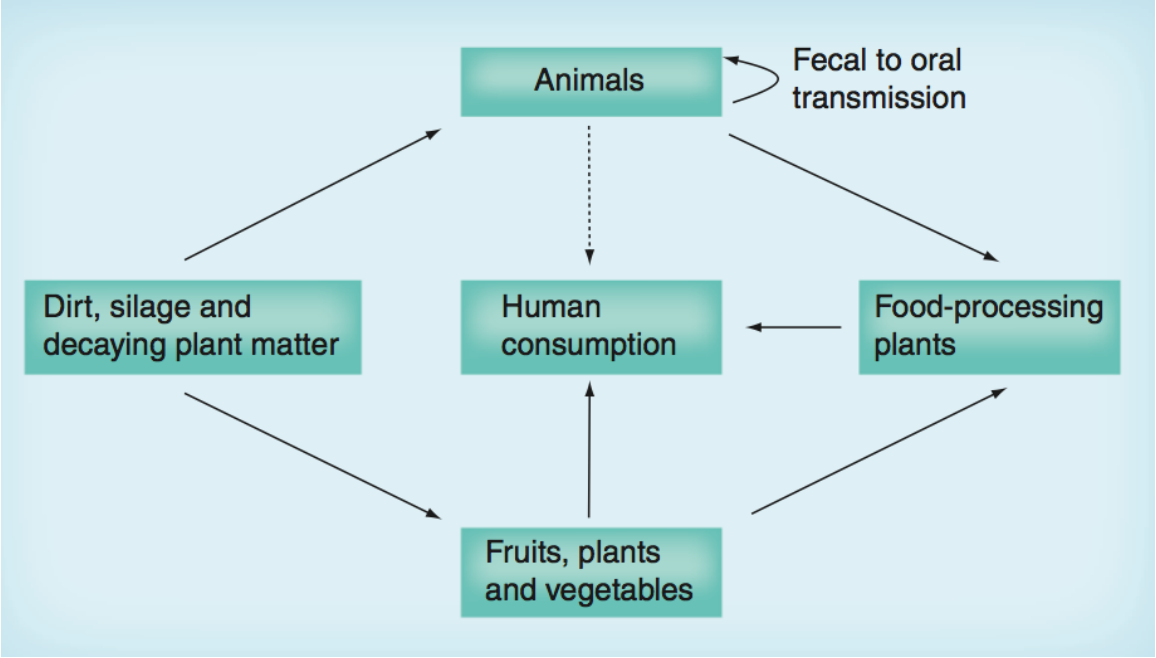
food processing environments have been shown to be of particular importance as sources of contamination (R. B. Tompkin, 2002).

As previously discussed, the evolution of genes in *L. monocytogenes* required for survival in the natural environment led to the ability to incidentally cause infections in mammals. It appears that certain elements of the saprophyte's genetic arsenal that allow the bacterium to thrive in nature are also key to its role as an opportunistic foodborne pathogen. These characteristics provide certain strains of *L. monocytogenes* with the ability to persist in food processing environments for long periods of time. Persistence in these environments may thereby allow a particular strain to contaminate food products repeatedly over a long period of time (Orsi et al., 2011). Persistence of *L.* monocytogenes lineage II isolates has been well-documented (Aarnisalo et al., 2003; Lappi et al., 2004; Lawrence & Gilmour, 1995; Nesbakken et al., 1996; Norton et al., 2001; Olsen et al., 2005; Rørvik et al., 2003; Senczek et al., 2000). One study found that a single strain responsible for a multi-state human listeriosis outbreak in the US had persisted in a turkey meat-processing facility for at least 12 years (Orsi et al., 2008). These facilities, which are often refrigerated, are moist nutrient-rich environments in which *L. monocytogenes* readily grows and survives. Due to the ubiquitous nature of the bacterium, entry into these facilities is difficult to prevent, and thus an outright and sustained elimination of the pathogen from these environments may be impossible (Tompkin et al., 1999).

1.4.8 Mechanisms of persistence

As previously discussed, strains of *L. monocytogenes* are capable of persisting within food processing environments for years at a time. However, not all facilities

Figure 6: The transmission cycle of *L. monocytogenes.* Figure reproduced directly from: (Xayarath & Freitag, 2012)


Animals
Fecal to oral transmission
Dirt, silage and decaying plant matter
Human consumption
Food-processing plants
Fruits, plants and vegetables

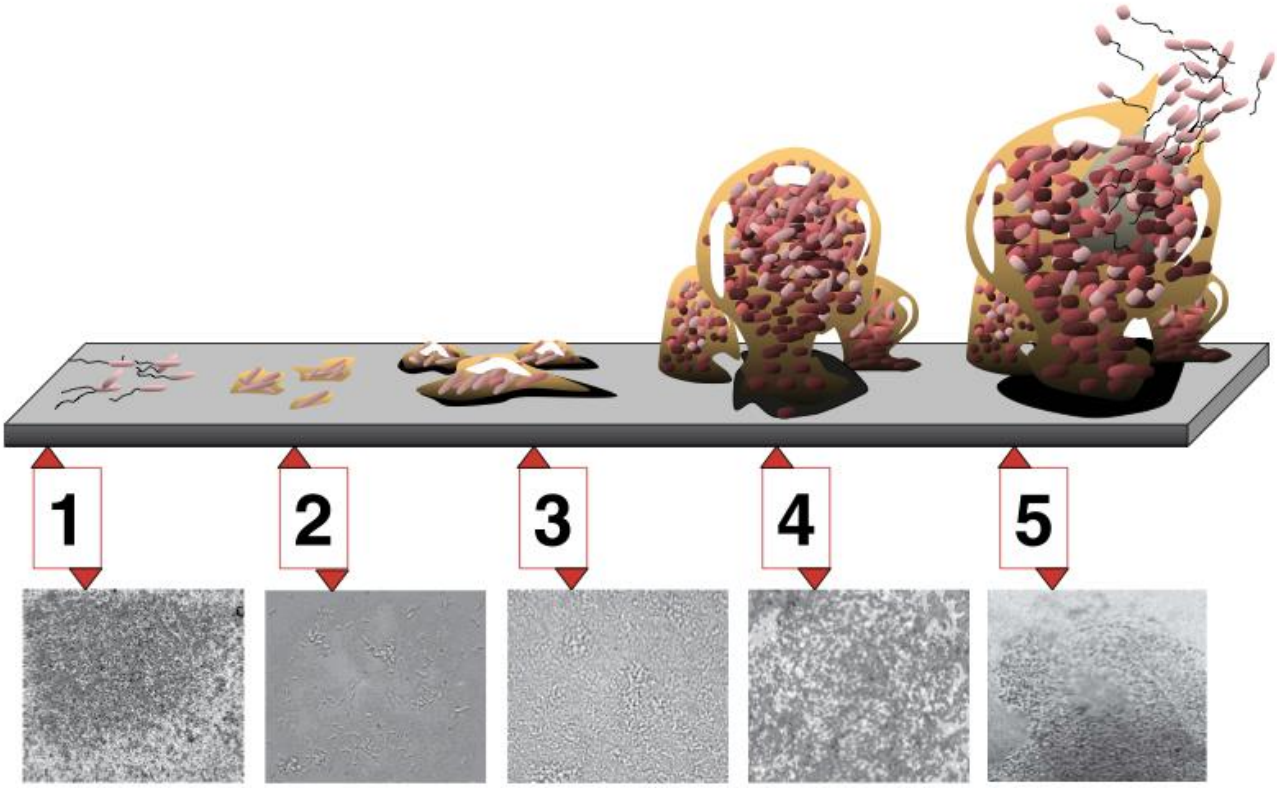
have been found to contain persistent strains, maintaining only a transient population of *L. monocytogenes* (Norton et al., 2001). As not all strains of *L. monocytogenes* persist in these environments, it is likely that those that do persist are phenotypically distinct from transient strains. A number of studies have indicated that biofilm formation and resistance to commonly-used antimicrobial compounds are characteristics that may contribute significantly toward persistence of *L. monocytogenes* (Møretrø & Langsrud, 2004).

1.4.8.1 Biofilm formation

A biofilm is an organized microbial community of cells enclosed in a self-produced polymeric matrix that is adhered to either an inert or living surface (Costerton et al., 1999). The general steps of biofilm formation are presented in Figure 7. Planktonic bacteria first encounter a surface resulting in a reversible adhesion to the surface; this occurs over minutes to a few hours (Bryers, 2000). Bacterial cells then become sessile and irreversibly adhere to the surface, producing extracellular compounds such as proteins and exopolysaccharides. The bacteria multiply and form thicker, multilayer biofilms with complex three-dimensional structures (Costerton et al., 1999). Eventually, motile planktonic forms of the bacteria can be released from the biofilm to spread and form new biofilms.

Bacterial cells that are living within biofilms can differ greatly from free-living planktonic forms. They are more difficult to remove from surfaces using physical cleaning methods (Holah & Gibson, 2000; Wirtanen et al., 2000). They often also have decreased growth rates, varied gene transcription, and decreased susceptibilities to antimicrobial compounds, including antibiotics and sanitizers

Figure 7: Stages of bacterial biofilm formation. 1) Reversible adsorption to surface 2) Irreversible attachment of bacteria 3) Growth and division, formation of microcolonies 4) Formation of mature biofilms with complex three-dimensional structures 5) Release of planktonic bacteria to form biofilms on other surfaces. Figure reproduced directly from (Monroe, 2007)


1
2
3
4
5

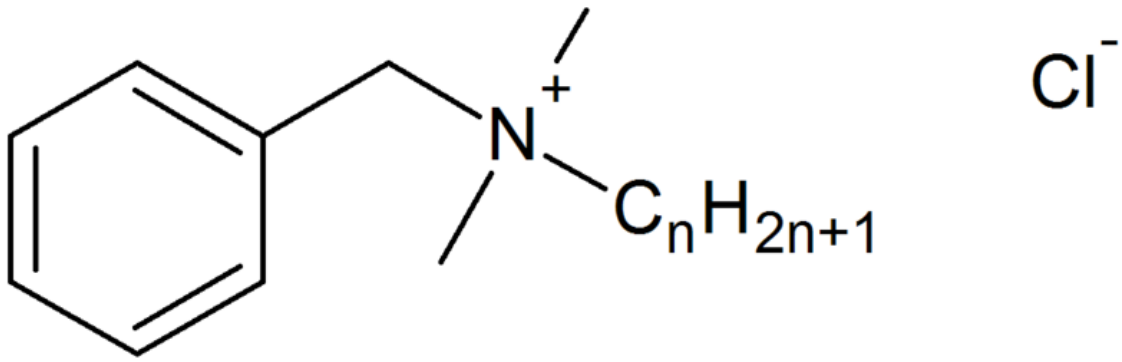
(Donlan, 2002; Gandhi & Chikindas, 2007; Robbins, Fisher et al., 2005). Increased resistances within biofilms are thought to occur due to the combination of chemical or enzymatic modification of the antibacterial agent, low diffusion through the extracellular matrix, physiological changes to the bacteria due to slow growth rates and starvation responses, and the induction of attachment-specific drug resistant physiologies (Costerton et al., 1999; Gilbert, 2002). Numerous pathogenic bacteria have been shown to form biofilms on inert surfaces in food processing facilities, including *L. monocytogenes*, *S. enteritidis*, *C. jejuni*, *E. coli* O157:H7, *B. cereus*, and *S. aureus* (Brooks & Flint, 2008; Chmielewski & Frank, 2003; Shi & Zhu, 2009).

The exact mechanisms behind biofilm formation by *L. monocytogenes* have yet to be fully elucidated; however, a recent study has revealed at least 24 chromosomal loci are involved in biofilm formation, some of which appear to be related to flagellar motility and quorum sensing (Chang et al., 2012). *L. monocytogenes* within biofilms are protected from a wide range of harmful environmental conditions, including toxic metals, acids, desiccation, salinity, ultraviolet rays, and antimicrobial drugs and sanitizers (Carpentier & Cerf, 2011). Biofilms often form on the surfaces of equipment in the production line, and are believed to be major causes of contamination of food products (Shi & Zhu, 2009).

1.4.8.2 Resistance to quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are a class of antibacterials that are commonly found in a wide range of commercial and domestic products. These include sanitizers and disinfectants commonly used in food processing facilities, agriculture settings, hospitals, and domestic cleaners. Members of this class of

Figure 8: Structure of benzalkonium chloride, a widely used quaternary ammonium compound.



n = 8, 10, 12, 14, 16, 18

molecules have antibacterial and antifungal activities (Buffet-Bataillon et al., 2012). QACs are also commonly found in pharmaceutical and cosmetic products, including shampoos, moisturizers, mouthwashes, sunscreen, facial cleansers, and hand sanitizers. These compounds (Figure 8) have the general structure of N+ R1R2R3R4… X-, with a positive amine group at one end of the molecule and a cation on the other, while the R groups are either plain alkyl groups or an alkyl group substituted with a different functional group. Of these compounds, benzalkonium chloride (BKC) is the most common QAC in use today, but other commonly-used QACs include stearalkonium chloride, cetrimonium chloride/bromide (cetrimide), cetylpyridinium chloride, and didecyldimethylammonium chloride (DDAC) (Buffet-Bataillon et al., 2012).

Like antibiotics, resistance to antibacterials such as QACs can be either an intrinsic property of an organism, or it can be acquired by via horizontal gene transfer (HGT) of transposons or plasmids (Russel & Chopra, 1996). Previous studies have shown that the most effective antibacterial formulations for *L. monocytogenes* contain chlorine (bleach) along with a QAC or anionic acid compounds (Earnshaw & Lawrence, 1998). However, factors such as insufficient cleaning before disinfection, improper dosage/concentration, inadequate rinsing post disinfection, and the presence of biofilms can significantly reduce the efficacy of antibacterial sanitizers and expose the bacteria to sublethal concentrations. Constant exposure to sublethal concentrations can lead to adaptation of initially susceptible bacteria through non-specific membrane changes, or promote selective acquisition of resistance genes (Soumet et al., 2005).

Studies have begun to elucidate the development of resistance to antibacterials in common use in food processing facilities and domestic settings. A number of studies have found strains of *L. monocytogenes* that exhibit reduced susceptibilities to QACs, a number of which have been associated with the food processing environment (Aase et al., 2000; Mullapudi et al., 2008; Soumet et al., 2005). As a result, a number of authors have suggested that resistance to QACs may be related to persistence of *L. monocytogenes* within these environments.

1.5 Horizontal gene transfer in *L. monocytogenes*

The ability of bacteria to transfer genes between different strains and species has been recognized for over 50 years (Toussaint & Chandler, 2012). This ability, known as lateral or horizontal gene transfer (HGT), is an important factor in the evolution of bacteria and the generation of genetic variability. HGT has also been shown to be a driving force in the development of microbial pathogenicity (Kelly et al., 2009b). Using various HGT mechanisms, pathogenic bacteria are capable of passing genes encoding toxins, antimicrobial resistances, and other virulence factors to previously non-pathogenic strains (Kelly et al., 2009a).

The first experimental evidence for the concept of HGT was obtained by Frederick Griffith in 1928 (Griffith, 1928). During his work to develop a vaccine for *Streptococcus pneumoniae*, he found that a non-pathogenic strain was transformed into a pathogenic strain through the uptake of genetic material, which later became known as transformation. Since then, numerous other mechanisms of HGT have been identified, including conjugative plasmids, transducing phages, transposons, integrons, and insertion sequence (IS) elements (Toussaint & Chandler, 2012).

Recent advances in genome sequencing technologies have led to significant increases in the number of completed bacterial genomes publically available. Using powerful bioinformatic tools, it is possible to determine the components of a bacterial genome that were obtained through HGT. Using comparative genomics, the analysis of G+C content, codon usage, and the presence of mobility genes, regions that were acquired through lateral gene movement can be identified (Hacker et al., 2004). HGT has been shown to play a major role in the development of multi-drug resistant (MDR) bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and β-lactamase-producing MDR *E. coli* (Scott, 2009). There is also concern that mechanisms of HGT could create highly virulent bacterial strains through the acquisition of novel toxin genes, as previously discussed regarding *E. coli* O157.

1.5.1 Bacteriophages in *L. monocytogenes*

Bacteriophages are viruses that infect and parasitize bacteria, and it is estimated that these viruses are the most abundant “life-form” in the biosphere (Brabban et al., 2005). Bacteriophages consist of a wide variety of viruses that are highly specific to their bacterial hosts. The bacteriophage-mediated transfer of genes between bacteria is known as transduction, and can be either generalized or specialized. The recent advances in whole-genome sequencing have revealed that bacteriophages play a significant role in the evolution of bacteria. For many of the gamma-proteobacteria and low-GC Gram-positives, 10 - 20% of their genomes are comprised of both intact or remnants of bacteriophages (Brabban et al., 2005). It is also known that many bacterial species contain prophages that encode various

virulence factors, including many toxins. For example, the Shiga toxin genes *stx1* and *stx2* are found in prophages within *E. coli*, and have been shown to be transferrable by bacteriophages (Wick et al., 2005).

To date, it does not appear that bacteriophages have been involved in the acquisition of any of the virulence genes currently present in *L. monocytogenes* (Buchrieser et al., 2003; Orsi et al., 2011). However, prophages and bacteriophage-related elements are commonly found within the genomes of *L .monocytogenes* isolates. Experimental evidence has demonstrated that transduction is possible between strains of *L monocytogenes* using listerial bacteriophages (Hodgson, 2000). These bacteriophages appear to have narrow host ranges, as transduction was serotype-specific, with no genes transferred between serotypes. This may indicate that generalized transduction is only possible between isolates of the same serotype, and transduction-associated HGT may be limited between serotypes (Orsi et al., 2011).

Notably, a study by Chen and Novick (2009) found that pathogenicity islands found in *S. aureus* are capable of transduction intergenerically to *L. monocytogenes*. SaPIs, highly mobile pathogenicity islands that encode the toxic shock toxin (TSST-1) were found to be transferred to *L. monocytogenes* at the same high frequencies that they are transferred within *S. aureus*. While bacteriophage-mediated HGT is thought to occur mainly within species, this study shows that it may be possible for genetic elements to be transferred between genera in the natural environment.

1.5.2 Insertion sequences and transposons in *L. monocytogenes*

Transposons were discovered in the 1940s by Barbara McClintock, when she noticed that the maize genome contained “jumping genes” that could cause insertions, deletions, and translocations in the DNA (McClintock, 2013). Soon after, similar genes were discovered within the genomes of bacteria and were found to be associated with antibiotic resistance (Toleman & Walsh, 2011). There can be considerable variation between these mobile elements and their classification and nomenclature can be rather complicated. Table 3 lists the main classes of transposons and insertion sequences, along with a description of each category. In prokaryotes, these elements are divided into two general categories: insertion sequences (ISs), which are mobile elements that generally just mobilize themselves, and transposons that carry additional genes (Toleman & Walsh, 2011).

Transposons can cause significant changes within the genome of bacteria. They can insert into a gene thereby disrupting its function, and when present in numerous copies they can catalyze recombination events that can lead to loss of large portions of the genome (Cole et al., 2001). Whereas the majority of these alterations are detrimental to the bacterial cell, transposition events can also result in the development of antibiotic resistance phenotypes. For example, the insertion of the transposon may disrupt a key porin or transporter involved in uptake of an antibiotic resulting in reduced susceptibility (Rodriguez-Martinez et al., 2009). Insertions can also inactivate DNA repair mechanisms, such as mismatch repair genes *uvrD, mutS,* or *mutL*, resulting in high mutation rates that allow for

Table 3: Categories of transposon-related elements. Table adapted from (Roberts et al., 2008)

Type of transposable element	Definition
Composite transposons	Flanked by IS elements. The transposase of the IS element is responsible for the catalysis of insertion and excision
Unit transposons	Typical unit elements encode an enzyme involved in excision and integration (DD(35)E or tyrosine) often a site-specific recombinase or resolvase and one or several accessory (e.g. resistance) genes in one genetic unit
Conjugative transposons (CTns)/integrative conjugative elements (ICEs)	The conjugative transposons (CTns), also known as integrative conjugative elements (ICEs), carry genes for excision, conjugative transfer and for integration within the new host genome. They carry a wide range of accessory genes, including antibiotic resistance
Mobilizable transposons (MTns)/integrative mobilizable elements (IMEs)	The mobilizable transposons (MTns), also known as integrative mobilizable elements (IMEs), can be mobilized between bacterial cells by other “helper” elements that encode proteins involved in the formation of the conjugation pore or mating bridge. The MTns exploit these conjugation pores and generally provide their own DNA processing functions for intercellular transfer and subsequent transposition
Mobile genomic islands	Some chromosomally integrated genomic islands encode tyrosine or serine site-specific recombinases that catalyze their own excision and integration but do not harbor genes involved in transfer. They carry genes encoding for a range of phenotypes. The name of a genomic island reflects the phenotype it confers, e.g. pathogenicity islands encode virulence determinants (toxins, adhesins, etc.)

spontaneous generation of enhanced resistance mechanisms (LeClerc & Cebula, 2000; Oliver et al., 2000). Additionally, if inserted adjacent to an already existing resistance gene, transposons can capture and incorporate the gene. Over time, the combination of numerous transpositional events can result in the accumulation of large regions of resistance and transposon-related genes.

There have been numerous families of transposons identified within bacteria, and the exact mechanisms of transposition can vary considerably between them (Roberts et al., 2008; Toussaint & Chandler, 2012). Generally, transposons use a transposase that catalyzes the excision of the element from the chromosome, as well as integration back into the chromosome. They also encode a resolvase that is required for integration. However, many transposons also code for accessory proteins that are either required for or aid in these functions. The specificity of target-sites for integration also varies among transposases, ranging from very low to high specificity (Toussaint & Chandler, 2012).

Transposons that simply move around a bacterial chromosome contribute very little to HGT. However, transposons may jump from the chromosome to conjugative plasmids, which can be transferred to a new host cell where the transposon can be integrated within the new chromosome. There are also forms of transposons that are self-transmissible, encoding machinery required for conjugative transfer to other cells. These conjugative transposons, also known as integrative and conjugative elements (ICEs), differ from plasmids in that they do not appear to be maintained in an extrachromosomal state and are incapable of autonomous replication (Wozniak & Waldor, 2010).

Examination of genome sequences of *L. monocytogenes* has shown that there are relatively few IS elements and transposons within its genome (Buchrieser et al., 2003; Kelly et al., 2009b; Nelson et al., 2004). Nelson et al. (2004) studied the genomes of four strains of *L. monocytogenes* (two serotype 1/2a strains and two serotype 4b strains) and found multiple copies of transposase ORFA of the IS3 family in homologous locations in all the strains. The study also found an intact IS element, termed ISLmo1, to be present in multiple copies in the serotype 1/2a strains. They also found that none of these insertions disrupted any chromosomal genes. Interestingly, a study by Morvan et al. (2010) that examined 4,816 human *L. monocytogenes* isolated between 1926 and 2007 found that 14 harbored tetracycline resistance on a conjugative transposon. The gene *tetM* was found to be associated with the integrase of the transposon Tn*916*-Tn*1545*, a family of conjugative transposons that are commonly found to carry tetracycline resistance.

Gilmour et al. (2010) performed genome sequencing on two outbreak-related isolates and discovered a novel *Listeria* genomic island, termed LGI1, which appeared to have been acquired by HGT. This 49.8 kbp region was bordered by inverted terminal repeats, contained a putative recombinase, and had a significantly altered G+C content than the flanking regions. Ziegler (2011) examined a panel of 126 Canadian *L. monocytogenes* isolates and found that 66 contained LGI1, including an isolate from 1988. However, despite the presence of putative genes related to conjugative transfer, LGI1 has not been experimentally demonstrated to be mobile.

1.5.3 Plasmids and conjugation in *L. monocytogenes*

The term plasmid was first coined by Joshua Lederberg in 1952, and is defined as an extrachromosomal DNA molecule that is capable of autonomous replication separate from the chromosomal DNA (Lederberg, 1952). Plasmids occur naturally in bacteria and are usually circular and double-stranded, can be either conjugative (horizontally and vertically transferred) or non-conjugative (vertically inherited). Non-conjugative plasmids partition between mother and daughter cells, and if appropriate *cis* traits are also present, can be mobilized to other cells in the presence of conjugative plasmids (Siefert, 2009).

Plasmids play an important role in HGT within bacteria. Horizontally transferred DNA that enters a bacterial cell through mechanisms such as transduction, natural transformation, or ICEs must be integrated into the host chromosome in order to permanently persist (Thomas & Nielson, 2005). Plasmids do not require integration to persist, and can replicate and express their genes in their extrachromosomal form. In addition, plasmids may also carry other mobile elements such as insertion sequences and transposons, providing a means for these non-conjugative elements to enter new cells (Thomas & Nielson, 2005).

Plasmids were first reported in *L. monocytogenes* in 1982 by Perez-Diaz et al. Since this report, various plasmids have been isolated from a wide range of *L. monocytogenes* isolates, with rates of plasmid isolation ranging from 0-79%, and an overall average of 30% (Kuenne et al., 2010). Two separate studies found that plasmids were overrepresented in food and environmental isolates compared to clinical isolates (Lebrun et al., 1992; McLauchlin et al., 1997). Another study by

Harvey and Gilmour (2001) found that plasmids were found more frequently (75%) within isolates from food and food processing environments than in sporadic isolates (35%) (Harvey & Gilmour, 2001). It has also been observed that isolates of lineage II (serotypes 1/2a and 1/2c) are more likely to harbor plasmids than lineage I (serotypes 1/2b and 4b) (Harvey & Gilmour, 2001; Orsi et al., 2011).

The recent advances in sequencing technologies have provided the full sequences of numerous *L. monocytogenes* plasmids. The evolutionary history of plasmids in *L. monocytogenes* is not well understood, but some analyses of the DNA sequences of these plasmids indicate that they share homologies to other Gram-positive plasmids, including pXO2 from *Bacillus anthracis* (Canchaya et al., 2010; Gilmour et al., 2010; Kuenne et al., 2010). Genome sequencing has also revealed that *L. monocytogenes* plasmids contain a large number of mobile genetic elements, including insertion sequences and transposons. In their analysis of the DNA sequences of 14 plasmids, Kuenne et al. (2010) found that between 6 and 24 genes per plasmid were putatively annotated as integrase, resolvase, transposase, or recombinase. Gilmour et al. (2010) found similar results on the newly-discovered plasmid pLM5578.

1.6 The 2008 Canadian listeriosis outbreak and WGS

In Canada in the fall of 2008, *L. monocytogenes* serotype 1/2a was the causative agent of a nationwide foodborne outbreak associated with RTE meat products that resulted in 57 laboratory confirmed human infections and 23 fatalities (Gilmour et al., 2010; Weatherill Report, 2009). During this outbreak, the Gilmour laboratory performed the first real-time application of high-throughput whole-genome

sequencing (WGS) on two representative outbreak strains (08-5578 and 08-5923). Whole-genome sequencing allowed the National Microbiology Laboratory (NML) to detect within these outbreak isolates a repertoire of genetic determinants involved in diversification and microevolution. The 2 primary outbreak isolates encoded collinear chromosomes, however 28 SNPs and three insertion/deletion (indels) events were observed (Gilmour et al., 2010). Isolate 08-5578 also encoded a 33 kbp prophage (ΦLMC1) that accounted for the difference in the *AscI* pulsed-field gel electrophoresis (PFGE) patterns of the isolates. Analysis of the full genetic data of these outbreak strains resulted in the discovery of novel genetic elements. Compared to the reference isolate *L. monocytogenes* EGDe (serotype 1/2a) that was used to assemble the genomes, these 2 outbreak isolates encoded a 49.8 kbp novel genomic island designated as the *Listeria* Genomic Island 1 (LGI1). Additionally, a newly-discovered 77 kbp plasmid termed pLM5578 (Figure 9) was identified in isolate 08-5578. Following the outbreak, the Gilmour laboratory obtained funding to sequence the genomes of 37 additional *L. monocytogenes* strains. These sequenced isolates were selected to represent significant listeriosis-related Canadian public health events from the last three decades (Table 4). Analysis of this extended genomic data led to the discovery of two previously undiscovered plasmids, termed pLM5026 and pLM0813.

1.7 The current investigation

Plasmids are frequently isolated from *L. monocytogenes* isolates, including those associated with human listeriosis outbreaks. Despite their presence, few studies have attempted to characterize *L. monocytogenes* plasmids. There has also been a

Figure 9: Genetic organization and predicted functions of pLM5578. Coding sequences are color-coded based on predicted functions. Figure reproduced directly from (Gilmour et al., 2010)

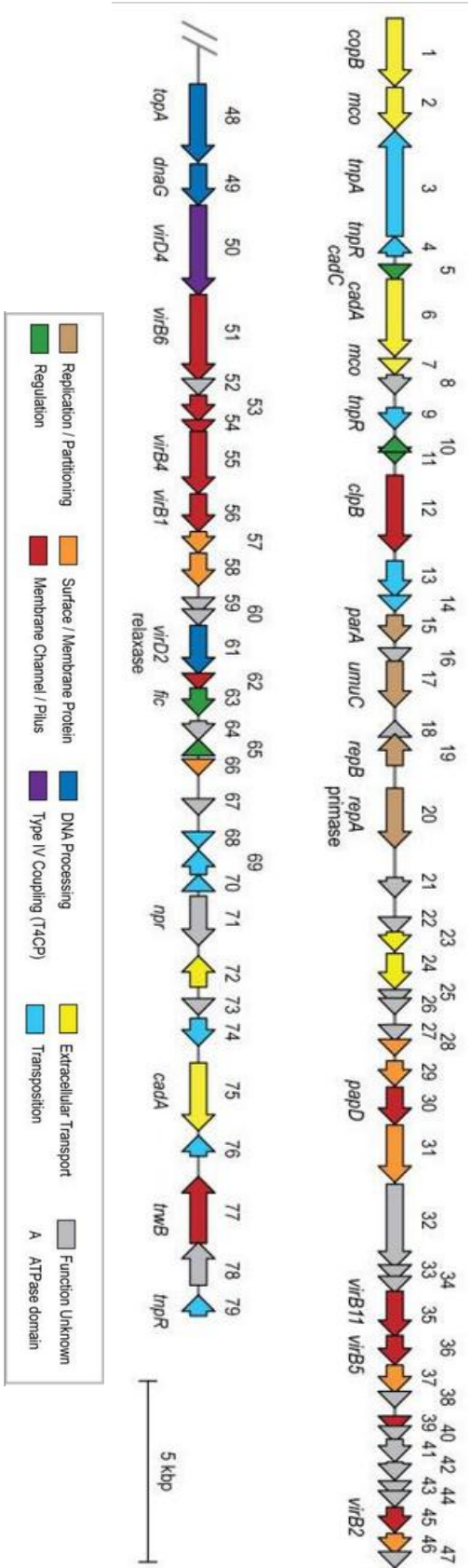

copB
mco
tnpA
tnpR
cadC
cadA
mco
tnpR
clpB
parA
umuC
repB
repA
primase
papD
virB11
virB5
virB2
topA
dnaG
virD4
virB6
virB4
virB1
virD2
relaxase
fic
npr
cadA
tnwB
tnpR
5 kbp
Replication / Partitioning
Regulation
Surface / Membrane Protein
Membrane Channel / Pilus
DNA Processing
Type IV Coupling (T4CP)
Extracellular Transport
Transposition
Function Unknown
A ATPase domain

Table 4: *L. monocytogenes* isolates on which whole-genome sequencing was performed by our laboratory. Table source: Gilmour laboratory

	NML #	Referring Lab	Source	Plasmid Present	Serotype	PFGE AscI	PFGE ApaI	Epidemiological Context
	Maritimes, coleslaw, 1981							
1	81-0861	PHL-NS	Coleslaw	No	4b	LMACI.0648	LMAAI.0449	Schlech et al 1983; First definitive link of listeriosis to food; at least 41 cases: 7 non-pregnant adults, 34 perinatal; at least 11 deaths
2	10-0809	BMH	Clinical	No	4b/not typable	LMACI.0160	LMAAI.0449	
3	81-0592	PHL-NS	Fetal Blood	No	4b	LMACI.0107	LMAAI.0449	
4	81-0558	PHL-NS	CSF	No	4b	LMACI.0107	LMAAI.0449	
	Ontario, imitation crab, 1996							
5	10-0810	BMH (ON)	Clinical	No	1/2b	LMACI.0046	LMAAI.0223	Farber et al 2000; 2 otherwise healthy adults: severe GI symptoms, 1 septic & hospitalized
6	10-0811	BMH (ON)	Imitation Crab	No	1/2b	LMACI.0046	LMAAI.0223	
	Manitoba, whipping cream, 2000							
7	10-0812	BMH (MB)	Whipping cream	Yes	1/2a	LMACI.0118	LMAAI.0005	Pagotto et al 2006; Church event, 5 relatively mild cases: 1 pos CSF, 5 pos stool
8	10-0813	BMH (MB)	Clinical	Yes	1/2a	LMACI.0118	LMAAI.0005	
	Quebec, heat-treated cheese, 2002					QC pulsotype 85		
9	10-4754	PHL-QC	CSF	No	1/2a	LMACI.0616	LMAAI.0818	Gaulin et al 2002; 17 confirmed cases: 11 hospitalized, no deaths?, 2 perinatal w/premature births
10	10-4758	PHL-QC	Cheese	No	1/2a	LMACI.0616	LMAAI.0818	
	Quebec & Ontario, cheese, 2008					QC pulsotype 93		
11	10-0933	PHL-QC	Blood	No	1/2a	LMACI.0149	LMAAI.0265	MAPAQ 2009; 39 confirmed cases: 32 non-pregnant adults, 7 perinatal cases; 1 adult death, 1 stillborn
12	10-0934	PHL-QC	Cheese	No	1/2a	LMACI.0149	LMAAI.0265	
	BC, Cheese, 2002							
13	02-1103	PHL-BC	CSF	?	4b	LMACI.0023	LMAAI.0140	Abbott Cheese; 47 cases: 2 meningitis, 2 perinatal bacteremia
14	02-1289	PHL-BC	Stool	?	4b	LMACI.0023	LMAAI.0140	
15	02-1792	PHL-BC	Cheese	?	4b	LMACI.0023	LMAAI.0140	
16	02-6679	PHL-BC	Stool	?	4b	LMACI.0082	LMAAI.0017	Little Qualicum Tiny Tomme Cheese
17	02-6680	PHL-BC	Cheese	?	4b	LMACI.0082	LMAAI.0017	86 febrile gastroenteritis cases
	Canada, nationally-distributed RTE meat, 2008							
18	08-5578	PHL-Ont	Blood	Yes	1/2a	LMACI.0040	LMAAI.0001	Gilmour et al 2010; Weatherill Report; At least 58 cases: 23 deaths, no perinatal cases
19	08-5923	PHL-Ont	Blood	No	1/2a	LMACI.0001	LMAAI.0001	
20	08-7669	PHL-SK	Blood	Yes	1/2a	LMACI.0001	LMAAI.0001	
21	10-0814	BMH (ON)	RTE meat	Yes	1/2a	LMACI.0001	LMAAI.0001	
22	10-0815	BMH (SK)	RTE meat	Yes	1/2a	LMACI.0040	LMAAI.0001	
23	08-6569	CFIA-Ont	Environment	Yes	1/2a	LMACI.0040	LMAAI.0001	
24	08-7374	CFIA-Ont	Envt/Food?	Yes	1/2a	LMACI.0001	LMAAI.0001	
25	08-6056	CFIA-Ont	Turkey meat	Yes	1/2a	LMACI.0040	LMAAI.0003	
26	08-6997	PHL-Ont	Blood	No	1/2a	LMACI.0040	LMAAI.0003	
	Ontario, prosciutto cotto cooked ham, 2010							
27	10-1046	PHL-Ont	Blood	No	1/2a	LMACI.0001	LMAAI.0001	2 confirmed cases
28	10-1047	PHL-Ont	CSF	Yes	1/2a	LMACI.0001	LMAAI.0001	
29	10-1321	PHL-Ont	Blood	Yes	1/2a	LMACI.0001	LMAAI.0001	
30	10-5024	CFIA-ON	prosciutto cooked ham	Yes	1/2a	LMACI.0001	LMAAI.0001	
	Food Industry							
31	10-5025	CFIA-ON	fully cooked bacon flakes	Yes	1/2c	LMACI.0036	LMAAI.0658	Relatively common PFGE pattern & serotype from food, but rarely causing clinical cases.
32	10-5026	CFIA-ON	processing env't (bacon flakes)	Yes	1/2c	LMACI.0036	LMAAI.0658	
33	10-5027	CFIA-ON	processing env't Swab	Yes	3c	LMACI.0036	LMAAI.0658	
	Additional ECV Isolates from Sporadic Cases							
34	88-0478	PHL-ON	Blood	Yes	1/2a	LMACI.0001	LMAAI.0001	sporadic cases of unknown epidemiology
35	95-0093	PHL-AB	Blood	Yes	1/2a	LMACI.0001	LMAAI.0001	
36	98-2035	PHL-ON	Blood	No	1/2a	LMACI.0040	LMAAI.0003	
37	99-6370	PHL-ON	Blood	No	1/2a	LMACI.0040	LMAAI.0003	
38	02-5993	PHL-ON	Blood	No	1/2a	LMACI.0001	LMAAI.0001	
39	04-5457	PHL-ON	Blood	No	1/2a	LMACI.0001	LMAAI.0001	

lack of studies to ascertain the role of plasmids in *L. monocytogenes* pathogenesis. There is evidence that plasmids harboured by *L. monocytogenes* may encode genes relating to resistance to antimicrobial compounds, as well as putative virulence factors. *Thus, I hypothesize that these plasmids have evolved and been selected for due to a contribution to pathogenesis and persistence of the host L. monocytogenes, and will therefore be more frequently found in outbreak-associated strains.*

In order to test this hypothesis, the first objective of this study was to determine the distribution of plasmids in *L. monocytogenes*. A panel of *L. monocytogenes* isolates was selected from environmental, food, and clinical sources, and screened using conventional PCR. This assay tested for the presence of gene targets identified on plasmids pLM5578, pLM5026, and pLM0813. Bioinformatic analyses were then used to predict the functions of individual genes encoded by each of the three newly-identified plasmids. These analyses were then used to direct experiments on functions and phenotypes conferred by carriage of these plasmids. *L. monocytogenes* strains were cured of plasmids in order to create plasmid-negative isogenic mutants for phenotypic comparisons. Phenotypic analyses included antimicrobial susceptibility testing, heavy metal resistance, biofilm formation, and invasive assays. Finally, WGS was performed on all isolates with plasmid screening patterns that indicated carriage of potential novel plasmids.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Bacterial isolates and growth conditions

A total of 147 *L. monocytogenes* isolates (Table 5) from human, food, and environmental sources were included in this study. A portion of these isolates were collected by Canadian public health laboratories and the Canadian Food Inspection Agency from 1981 to 2010 and submitted to the Enteric Diseases Program at the NML. Additional isolates associated with significant Canadian listeriosis outbreaks and also environmental and animal-associated isolates that had not been linked to any known outbreaks were obtained from the Bureau of Microbial Hazards (Health Canada, Ottawa, ON) and Cornell University, Department of Food Science (Ithaca, NY). The non-clinical isolates were selected to allow further study of *L. monocytogenes* serotypes that are less frequently encountered clinically, such as 1/2c and 1/2b, with regard to plasmid carriage.

The 2008 listeriosis outbreak isolate 08-5578 and the plasmid pLM5578 which it harboured were previously sequenced, and the full DNA sequences are available for both the chromosome (GenBank accession number CP001602) and the plasmid (CP001603). As such, this isolate and plasmid were used as the reference for subsequent experiments conducted in this study.

L. monocytogenes was grown in Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, Mississauga, ON) at 35°C, under aerobic conditions with shaking, or on BHI agar (Becton, Dickinson and Company) at 35°C.

Table 5: *L. monocytogenes* isolates included in this study. Isolates were selected to represent significant outbreak-associated strains from the last three decades. These include clinical isolates, as well as those isolated from food and food processing environments. PFGE pattern designations are using PulseNet Canada standardized nomenclature.

Isolate	Serotype	Source	Province[a]	PFGE Pattern	
				PFGE *Asc*I	PFGE *Apa*I
81-0558	4b	Human cerebral spinal fluid	NS	N/A	N/A
81-0592	4b	Human blood	NS	N/A	N/A
81-0861	4b	Coleslaw	NS	N/A	N/A
87-0192	1/2a	Human blood	ON	N/A	N/A
87-0426	1/2a	Human cerebral spinal fluid	NS	N/A	N/A
88-0286	1/2a	Cooked pork	ON	N/A	N/A
88-0478	1/2a	Human blood	ON	N/A	N/A
88-0702	1/2a	Human cerebral spinal fluid	ON	N/A	N/A
88-0868	1/2b	Human blood	NFLD	N/A	N/A
88-0876	1/2b	Food processing environment	ON	N/A	N/A
88-0913	1/2b	Human blood	ON	N/A	N/A
88-0928	1/2b	Smoked salmon	MB	N/A	N/A
88-0941	1/2b	Food	ON	N/A	N/A
88-0978	3a	Human cerebral spinal fluid	ON	N/A	N/A
88-1059	3b	Human blood	NFLD	N/A	N/A
90-0558	1/2a	Human cerebral spinal fluid	AB	N/A	N/A
90-0602	4b	Smoked salmon	QC	N/A	N/A
91-0145	1/2a	Human blood	ON	N/A	N/A
92-0366	1/2a	Human blood	ON	N/A	N/A
93-0024	1/2a	Human blood	QC	N/A	N/A
93-0407	1/2a	Human blood	ON	N/A	N/A
93-0638	1/2b	Human brain	BC	N/A	N/A
93-0827	3a	Human cerebral spinal fluid	NS	N/A	N/A
93-0839	1/2b	Human cerebral spinal fluid	ON	N/A	N/A
94-0096	1/2a	Human blood	BC	N/A	N/A
94-0447	1/2a	Human blood	ON	N/A	N/A
95-0012	1/2a	Human blood	ON	LMACI.0001	LMAAI.0003
95-0093	1/2a	Human blood	AB	LMACI.0001	LMAAI.0001
95-0151	1/2a	Human blood	ON	LMACI.0001	LMAAI.0003
96-0033	4b	Human blood	ON	LMACI.0038	LMAAI.0070
96-0215	4b	Human blood	BC	LMACI.0026	LMAAI.0038
96-0218	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001
96-0247	1/2a	Human blood	ON	LMACI.0058	LMAAI.0001
97-0456	1/2b	Non-human	PEI	N/A	N/A
97-0465	3a	Non-human	PEI	N/A	N/A
97-0466	1/2c	Non-human	PEI	N/A	N/A
97-0468	1/2c	Non-human	PEI	N/A	N/A
97-0817	1/2b	Human blood	ON	LMACI.0046	LMAAI.0047
97-1602	1/2a	Human urine	AB	LMACI.0001	LMAAI.0001
97-1636	1/2a	Human blood	SK	LMACI.0001	LMAAI.0001
98-0041	1/2b	Human blood	ON	LMACI.0028	LMAAI.0023
98-0163	3c	Unknown	USA NY	N/A	N/A
98-0291	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
98-2035	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003
99-3046	1/2a	Human blood	ON	LMACI.0002	LMAAI.0002
99-3048	1/2a	Human blood	ON	LMACI.0001	LMAAI.0003
99-6370	1/2a	Human blood	ON	LMACI.0040	LMAAI.0004
99-6871	1/2a	Human clinical	NFLD	LMACI.0001	LMAAI.0001
00-0346	1/2a	Human peritoneal fluid	ON	LMACI.0007	LMAAI.0014
00-1149	1/2a	Human clinical	QC	LMACI.0001	LMAAI.0001
00-2572	1/2b	Human blood	MB	LMACI.0037	LMAAI.0029
00-3767	1/2c	Pepper salami	BC (CFIA)	LMACI.0064	LMAAI.0560
00-3770	1/2c	Pepper salami	BC (CFIA)	LMACI.0064	LMAAI.0560

00-3853	1/2b	Food processing environment	BC (CFIA)	LMACI.0016	N/A
00-5136	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
01-1280	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
01-1465	1/2a	Human blood	ON	LMACI.0096	LMAAI.0081
01-1468	1/2a	Human brain	NFLD	LMACI.0098	LMAAI.0081
01-2417	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001
01-3506	1/2a	Human cerebral spinal fluid	ON	LMACI.0001	LMAAI.0003
01-5364	4b	Food processing environment	BC (CFIA)	LMACI.0126	LMAAI.0139
01-5373	1/2a	Human blood	ON	LMACI.0002	LMAAI.0001
01-5991	1/2a	Food	CFIA-AB	LMACI.0128	LMAAI.0002
01-7107	1/2a	Human blood	BC	LMACI.0122	LMAAI.0003
01-7209	1/2a	Liverwurst	BC (CFIA)	LMACI.0001	LMAAI.0097
01-7210	1/2a	Liverwurst	BC (CFIA)	LMACI.0040	LMAAI.0097
02-1115	4b	Human cerebral spinal fluid	ON	LMACI.0023	LMAAI.0140
02-2448	1/2a	Human blood	ON	LMACI.0122	LMAAI.0003
02-4056	1/2a	Human blood	ON	LMACI.0015	LMAAI.0024
02-5993	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
03-0402	1/2a	Human blood	AB	LMACI.0001	LMAAI.0001
03-5195	3a	Cow’s milk	BC (CFIA)	LMACI.0033	LMAAI.0212
03-5196	3a	Cow’s milk	BC (CFIA)	LMACI.0033	LMAAI.0212
03-5197	3a	Cow’s milk	BC (CFIA)	LMACI.0033	LMAAI.0212
03-5473	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
03-5833	1/2a	Human blood	AB	LMACI.0002	LMAAI.0214
03-7258	3a	Human blood	NS	LMACI.0168	LMAAI.0217
03-7262	1/2a	Human blood	NS	LMACI.0001	LMAAI.0001
04-3697	1/2b	Bean curd	BC (CFIA)	LMACI.0181	LMAAI.0229
04-5455	4b	Human blood	ON	LMACI.0003	LMAAI.0008
04-5457	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
04-5458	4b	Human blood	ON	LMACI.0009	LMAAI.0234
04-7425	1/2b	Human blood	ON	LMACI.0190	LMAAI.0248
05-2144	4b	Human blood	ON	LMACI.0054	LMAAI.0053
05-4745	4b	Human blood	MB	LMACI.0003	LMAAI.0270
05-5920	3a	Human blood	BC	LMACI.0118	LMAAI.0120
06-3527	1/2b	Human blood	BC	LMACI.0046	LMAAI.0067
06-6391	1/2b	Human peritoneal fluid	ON	LMACI.0045	LMAAI.0287
06-6833	1/2a	Human blood	QC	LMACI.0007	LMAAI.0068
06-6837	1/2a	Human blood	QC	LMACI.0001	LMAAI.0001
06-6840	1/2a	Human blood	QC	LMACI.0001	LMAAI.0001
06-6863	1/2a	Human blood	QC	LMACI.0001	LMAAI.0001
06-6865	3a	Human blood	QC	LMACI.0226	LMAAI.0169
06-6909	1/2a	Human blood	QC	LMACI.0033	LMAAI.0122
06-6934	1/2a	Human blood	QC	LMACI.0004	LMAAI.0013
06-6956	1/2a	Human blood	QC	LMACI.0044	LMAAI.0074
07-1873	1/2c	Salami	BC (CFIA)	LMACI.0036	LMAAI.0028
07-1874	1/2c	Salami	BC (CFIA)	LMACI.0036	LMAAI.0028
07-6082	1/2a	Human blood	NS	LMACI.0040	LMAAI.0001
07-6083	1/2a	Human blood	NS	LMACI.0001	LMAAI.0001
08-3083	4b	Human blood	BC	LMACI.0030	LMAAI.0450
08-3347	1/2b	Human blood	BC (CFIA)	LMACI.0028	LMAAI.0287
08-5375	1/2a	Human blood	ON	LMACI.0002	LMAAI.0214
08-5379	4b	Human blood	ON	LMACI.0060	LMAAI.0542
08-5496	1/2b	Cheese	ON (CFIA)	LMACI.0028	LMAAI.0287
08-5578	1/2a	Human blood	ON	LMACI.0040	LMAAI.0001

08-5597	4b	Coleslaw	ON	LMACI.0060	LMAAI.0019
08-5923	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
08-6056	1/2a	Turkey meat	ON	LMACI.0040	LMAAI.0003
08-6337	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003
08-6569	1/2a	Food processing environment	ON	LMACI.0040	LMAAI.0001
08-6997	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003
08-7374	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
08-7669	1/2a	Human blood	SK	LMACI.0001	LMAAI.0001
08-8526	1/2c	Roast beef	ON (CFIA)	LMACI.0036	LMAAI.0658
08-8748	1/2c	Chicken breast	ON (CFIA)	LMACI.0036	LMAAI.0658
08-8749	1/2c	Pork loin chops	ON (CFIA)	LMACI.0036	LMAAI.0658
08-8750	1/2c	Pastrami	ON (CFIA)	LMACI.0036	LMAAI.0658
08-8751	1/2c	Cheddar mini sausage	ON (CFIA)	LMACI.0036	LMAAI.0658
08-8809	1/2b	Blue cheese	QC (CFIA)	LMACI.0045	LMAAI.0317
10-0809	N/A	Human clinical	ON	N/A	N/A
10-0810	1/2b	Human clinical	ON	LMACI.0046	LMAAI.0223
10-0811	1/2b	Imitation crab meat	ON	LMACI.0046	LMAAI.0223
10-0812	1/2a	Whipping cream	MB	LMACI.0118	LMAAI.0005
10-0813	1/2a	Human clinical	MB	LMACI.0118	LMAAI.0005
10-0814	1/2a	RTE Meat	ON	LMACI.0001	LMAAI.0001
10-0815	1/2a	RTE Meat	SK	LMACI.0040	LMAAI.0001
10-0933	1/2a	Human blood	QC	LMACI.0149	LMAAI.0265
10-0934	1/2a	Cheese	QC	LMACI.0149	LMAAI.0265
10-1046	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
10-1047	1/2a	Human cerebral spinal fluid	ON	LMACI.0001	LMAAI.0001
10-1321	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
10-1542	1/2c	Salmon nuggets	BC	N/A	N/A
10-1581	1/2c	Food processing environment	BC	N/A	N/A
10-1582	3a	Food processing environment	BC	N/A	N/A
10-1583	3a	Food processing environment	BC	N/A	N/A
10-1601	4b	Food processing environment	BC	N/A	N/A
10-1633	1/2b	Food processing environment	BC	N/A	N/A
10-2174	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001
10-3180	4b	Blood	ON	LMACI.0107	LMAAI.0104
10-4754	1/2a	Human cerebral spinal fluid	QC	LMACI.0616	LMAAI.0818
10-4758	1/2a	Cheese	QC	LMACI.0616	LMAAI.0818
10-5024	1/2a	Cooked ham	ON	LMACI.0001	LMAAI.0001
10-5025	1/2c	Cooked bacon flakes	ON	LMACI.0036	LMAAI.0658
10-5026	1/2c	Food processing environment	ON	LMACI.0036	LMAAI.0658
10-5027	3c	Food processing environment	ON	LMACI.0036	LMAAI.0863
10-6746	1/2c	Human blood	BC	LMACI.0036	LMAAI.0658
EGDe	1/2a	Rabbit tissue	UK	LMACI.0661	LMAAI.0944

a – Province: AB, Alberta; BC, British Columbia; MB, Manitoba; NB, New Brunswick; NF, Newfoundland; NS, Nova Scotia; ON, Ontario; PEI, Prince Edward Island; QC, Quebec; SK, Saskatchewan; USA NY, United States of America state of New York; CFIA, Canadian Food Inspection Agency; N/A, not available

Serotyping was performed by the NML Enterics department using slide agglutination with antisera prepared at the NML according to Seeliger and Höhne (1979). PFGE was performed by the NML PulseNet section according to the PulseNet standardized protocol using the restriction enzymes *AscI* (New England Biolabs, Pickering, ON) and *ApaI* (Roche Diagnostics, Indianapolis, IN). PFGE patterns were designated using BioNumerics software (Applied Maths, Austin, TX) after comparison to the PulseNet Canada database.

2.2 Bioinformatic analyses

Gene annotation analysis of the *L. monocytogenes* plasmids pLM5026 and pLM0813 sequences were performed using the GenDB version 2.2 (Meyer et al., 2003). Similarity searches were performed by using BLASTN and BLAST2P (Altschul et al., 1990) against the non-redundant nucleotide and protein databases, respectively. A BLAST2P search was performed against the databases nr, SWISS-PROT (UniProt Consortium, 2010) and KEGG-Genes (Kanehisa et al., 2010); the protein family databases Pfam (Finn et al., 2008) and TIGRFAM (The TIGRFAMs database of protein families.); predictive signal peptide, Signal P (Peterson et al., 2011) and transmembrane helix analysis, TMHMM (Krogh et al., 2001). A manual annotation of each predicted gene was performed using these tools. Artemis was used to view sequences, perform feature manipulations and to add additional custom annotations (Rutherford et al., 2000).

GView Server was used to create a pan-genome visualization of all genes present on plasmids identified in this study, using pLM5578 as the seed genome

(Petkau et al., 2010). Plasmid DNA alignments were performed using progressive Mauve v1.4.1 with the default settings (Darling et al., 2010).

2.3 DNA template preparation

DNA template used for PCR in this study was prepared according to the boiled cell method. Briefly, a loopful of bacterial culture was resuspended in 0.5 mL 1xTE buffer (Sigma-Aldrich, St. Louis, MO) (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and heated over a boiling water bath for 15 minutes to lyse the cells. Alternatively, 1 to 2 mL of liquid bacterial culture was pelleted by centrifuging at 10,000xg for 1 minute, the supernatant discarded, and the pellet resuspended in 1xTE buffer followed by boiling. The boiled resuspensions were then centrifuged at 16,000xg for 5 minutes at 4°C to pellet cell debris. The supernatant containing the DNA was transferred to a new tube and used in PCR reactions.

2.4 Plasmid isolation

Wild-type plasmids from this study had unknown antibiotic resistance phenotypes, and therefore selective media was not used for plasmid enrichment. Owing to the relatively large size (>50 kbp) and low copy number of these plasmids, commercial plasmid isolation kits, including the Qiagen plasmid midi kit (Qiagen, Mississauga, ON) were unable to produce sufficient plasmid yields. Instead, a modified alkaline lysis protocol was used to isolate *L. monocytogenes* wild-type plasmids (Birnboim & Doly, 1979). A single colony was inoculated in 5 mL of BHI broth and grown overnight at 35°C with shaking. Two mL of culture was pelleted by centrifugation at 16,000xg for 2 minutes. Supernatant was discarded and the cells were resuspended in 200 µL of glucose (Sigma-Aldrich) Tris-HCL-EDTA (GTE) buffer (50

mM glucose, 10 mM EDTA, 25 mM Tris-HCL). Freshly prepared lysozyme (Roche Diagnostics) was added to a final concentration of 2 mg/mL, along with RNase (Qiagen) for a final concentration of 20 ug/mL, and incubated for 30 minutes at 37°C. 300 µL of freshly prepared alkaline lysis buffer (Sigma-Aldrich) (0.2 NaOH, 1% SDS) was then added and mixed by inverting the tube multiple times. Note that mixing was not done by vortexing throughout the protocol as it can result in shearing of plasmid DNA, as well as incomplete removal of chromosomal DNA. After no more than 5 minutes, clearing occurred and the lysate was incubated on ice for 5 minutes. Following incubation, 300 µL of neutralization solution (Sigma-Aldrich) (3 M sodium acetate, pH 4.5) was mixed and incubated on ice for 15 minutes, after which a white precipitate formed. Cellular debris was then removed by centrifuging at 14,000xg for 10 minutes at room temperature, and the supernatant transferred to a fresh tube. An additional RNase step was required to remove remaining RNA. Following the RNase step, a phenol-chloroform clean up was performed on the isolated plasmid DNA in 1.5 mL snap lock tubes (Eppendorf, Mississauga, ON). 400 µL phenol-chloroform (Sigma-Aldrich) was added to the solution, mixed by inversion, and then spun down at 10,000xg for 2 minutes. The aqueous top layer was extracted and transferred to a new tube. Following a second phenol-chloroform extraction, the supernatant was extracted twice by 400 µL chloroform (Sigma-Aldrich). Plasmid DNA was then precipitated by adding an equal volume of 100% isopropanol (Fischer Scientific, Fair Lawn, NJ) and centrifuged at 14,000xg for 10 minutes at room temperature. Isopropanol supernatant was then discarded and the plasmid DNA pellet washed with 500 µL of 70% ethanol (Commercial Alcohols, Brampton, ON),

followed by centrifugation at 14,000xg at 4°C for 30 minutes. The ethanol was removed and the pellet resuspended in 25 µL double distilled water (ddH_2O) after a brief air dry.

2.5 Plasmid PCR screening

A conventional PCR assay was designed using Primer3 targeting eight genes unique to *L. monocytogenes* plasmids (Untergrasser et al., 2012) (Table 6; Figure 10). Two of these gene targets, p50-*traG* (*virD4*) and p62-*par* (*fic*) were designed by Gilmour et al. (2010) to screen for genes found on pLM5578. Additional gene targets were selected to represent various functional units and operons distributed across pLM5578. Following annotation of pLM5026 and pLM0813, two additional gene targets, p33-*yfiS* and *bcrBC*, were identified as targets to screen for the presence of pLM5026. The assay was performed using Invitrogen Platinum *Taq* Polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and with 0.5 µM of each oligonucleotide primer. The thermocycling parameters used were: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; followed by a final extension at 72°C for 7 minutes. Amplicons were visualized by capillary electrophoresis on a QIAxcel instrument (Qiagen Inc.) using the QIAxcel high-resolution kit, QX DNA size marker 100-2500 bp and QX alignment markers 15/3000 bp according to the manufacturer's recommendations.

2.6 Wild-type plasmid curing

To determine phenotypes associated with plasmid carriage, isogenic mutants were created in which the plasmids were removed but were otherwise genetically

Table 6: Oligonucleotide primers used for screening of *L. monocytogenes* wild-type plasmids. The gene target is identified in the primer name, and predicted amplicon sizes are indicated.

Primer Name	Sequence (5' - 3')	Product Size (bp)
p02-*mcoF*	GGTTAAACAAGAGGCTGCTA	442
p02-*mcoR*	TTCTTTTCTTGCTGAAGGAG	
p17-*umuCF*	ATGTACCGGAAACTGTATGG	467
p17-*umuCR*	TTCAAGAAAGTAGGGGACAA	
p50-*traGF*	ATTCTGGTACGGCAAAACTA	504
p50-*traGR*	TATCAATCCGCTTGTCTCTT	
p62-*parF*	GAACAAGCCTTTGCTTATGT	509
p62-*parR*	TCTACCCGTTCTTTTTCTTG	
p74-*cadAF*	CCGGATAGAGAGCAAGTATG	508
p74-*cadAR*	TGTACTGAAGGCTGAAGGTT	
p08-*ydhKF*	AGCTTGTTCAACTGGTAACGAAG	480
p08-*ydhKR*	TCGTCAGTTGTCATCCATTTATG	
p33-*yfiSF*	ATTGCCAGCGCTGCTTATAG	630
p33-*yfiSR*	TACCACAAGCCCTTGTTGTTC	
p63-*bcrB*	CGTGTCAGCAGATCTTTGATTAAG	637
p64-*bcrC*	TTGGCGCAATCTTATTTGAAG	

Figure 10: The pLM5578 genes selected as targets for the PCR plasmid screening assay.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47
copB mco tnpA tnpR cadC cadA mco tnpR clpB parA umuC repB repA primase papD virB11 virB5 virB2
48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79
topA dnaG virD4 virB6 virB4 virB1 virD2 relaxase fic npr cadA trwB tnpR
5 kbp
Targets for PCR Screening Assay

identical to wild-type isolates. Plasmid curing was performed using high-temperature treatment (Lebrun et al., 1992): a single colony was inoculated into Brain Heart Infusion (BHI) (Becton, Dickinson, and Company) broth and incubated overnight at 43°C with shaking. A loopful of culture was then streaked for single colonies on BHIA (Becton, Dickinson, and Company) plates and incubated at 35°C for 48 hours. 10 random colonies were picked and tested for the presence of plasmids using the conventional PCR plasmid screening method previously described. Plasmid isolation was also performed to confirm the creation of plasmid-negative mutant straiins.

2.7 Vertical transmission plasmid stability testing

To estimate the rate at which pLM5578 was lost during vertical transmission to daughter cells, isolate 08-5578 was passaged under varied growth conditions for 120 days. A sample of each culture was periodically sampled and screened for the presence of pLM5578. The growth conditions under test for passaging were: growth on BHIA at 35°C; growth on Columbian blood agar base (CBAB; Becton, Dickinson, and Company) plates at 35°C, and growth in a custom liquid growth media that was designed to mimic growth in a contaminated food processing environment. This liquid media contained autoclaved processed turkey deli meat obtained from a commercial grocery retailer along with a sub-lethal concentration of benzalkonium chloride (BKC; Sigma-Aldrich) (5g/L ground and autoclaved turkey, 10 µg/mL BKC 1% saline solution) passaged at 4°C and 22°C. A loopful of cultures grown at 35°C and 22°C were passaged to fresh media 3 times a week, while those grown at 4°C were passaged once a week. Every 10 passages, each of the bacterial cultures

were tested for the presence of pLM5578 by the PCR screening assay; liquid cultures were first streaked for single colonies onto BHIA and allowed to grow for 48 hours before colonies were picked.

2.8 Heavy metal susceptibility testing

As previously discussed, resistance to heavy metals including arsenic and cadmium is a common characteristic of *L. monocytogenes* isolates. Resistances were determined in study isolates for the heavy metal salts cadmium chloride ($CdCl_2$), manganese sulfate ($MgSO_4$), zinc chloride ($ZnCl_2$) (all from Fischer Scientific), copper (II) sulfate ($CuSO_4$) (Sigma-Aldrich), and magnesium chloride ($MgSO_4$) (Sigma-Aldrich). The MICs for cadmium were determined for the full panel of wild-type and plasmid-cured strains, whereas the MICs for the remainder of the heavy metals were determined solely for isolates 08-5578 and 10-5026 and their corresponding plasmid-cured strains.

Notably, heavy metal susceptibility testing for *L. monocytogenes* is not standardized in a manner similar to the CLSI's guidelines for antibiotic susceptibility testing. For example, there are no standardized breakpoints used to label an isolate as susceptible or resistant, and most studies involving the susceptibility of *L. monocytogenes* to cadmium report their breakpoints on MIC (minimum inhibitory concentration) values determined by Lebrun et al. (1992), with growth in concentrations of cadmium greater than 64 - 75 µg/mL considered as "resistant". Breakpoints for heavy metals other than cadmium were based on MICs as determined by Margolles et al. (2001).

The heavy metal MICs were determined in duplicate on Mueller-Hinton agar (MHA) (Becton, Dickinson, and Company) plates by the agar dilution method (Clinical and Laboratory Standards Institute [CLSI], 2009) using two-fold serial dilutions. For further comparison of plasmid-cured to wild-type strains, concentrations were increased by stepwise increments to obtain more accurate comparisons. Cultures were inoculated using the spotting method (CLSI, 2009). Briefly, bacterial cultures were grown overnight on BHIA, then multiple cultures were picked and suspended in 0.7% saline to an optical density equivalent to a 0.5 McFarland standard: absorbance was measured at 0.8-0.13 at 625 nm using a Microscan Turbidity Meter (Dade Behring, Deerfield, Illinois, USA), equivalent to 10^8 CFU/mL. The resultant bacterial culture was diluted 1 in 10 to achieve 10^7 CFU/mL, and 1 to 2 µL was spotted onto the surface of the agar containing heavy metal for a final concentration of approximately 10^4 CFU/spot. Growth was documented after 24, 48, and 76 hours incubation at 35°C.

2.9 Benzalkonium chloride MIC assays

The MICs for the sanitizer benzalkonium chloride (BKC) toward the panel isolates and their corresponding plasmid-cured strains were determined by stepwise dilution in increments of 5 µg/mL from 0 – 40 µg/mL. Using spot inoculations as previously described, bacterial cultures were spotted onto MHA plates supplemented with 2% sheep's blood and incubated at 4°C, 25°C, and 35°C. The 25°C and 35°C plates were incubated for 24 and 48 hours, while 4°C plates were incubated for 35 days, and all plates were run in triplicate.

2.10 Antibiotic susceptibility testing

The antibiotic susceptibilities were compared between strains 08-5578, 10-5026, and their corresponding plasmid-cured strains using Sensititre STP6F MIC Susceptibility 96-well plates (TREK Diagnostic Systems Inc., Cleveland, OH) according to the manufacturer's instructions. The isolate *S. pneumoniae* ATCC 49619 was used as a control organism, as specified by manufacturer instructions. Briefly, bacteria were emulsified in isotonic sterile saline to achieve a turbidity equivalent to the 0.5 McFarland standard and 10 - 100 µL of this diluted culture was added to the supplied tube of cation-adjusted Mueller-Hinton broth containing lysed horse blood. Subsequently, 100 µL of this solution was added to each well of the Sensititre STP6F MIC Susceptibility 96-well plate (TREK Diagnostic Systems Inc.). The plates were sealed and incubated at 37°C for 24 hours. Following incubation, plates were read for visible growth of a bacterial pellet or no growth in each well to determine the MIC as per CLSI interpretive criteria. A serial dilution of the cation-adjusted MH broth containing lysed horse blood culture was performed up to a concentration of 10^{-3}, 10 µL of each dilution was spread onto a CBAB plate and the plates were incubated overnight at 35°C. Colony counts were performed to ensure the initial inoculum concentrations were between $1x10^5$ - $1x10^6$ CFU/mL.

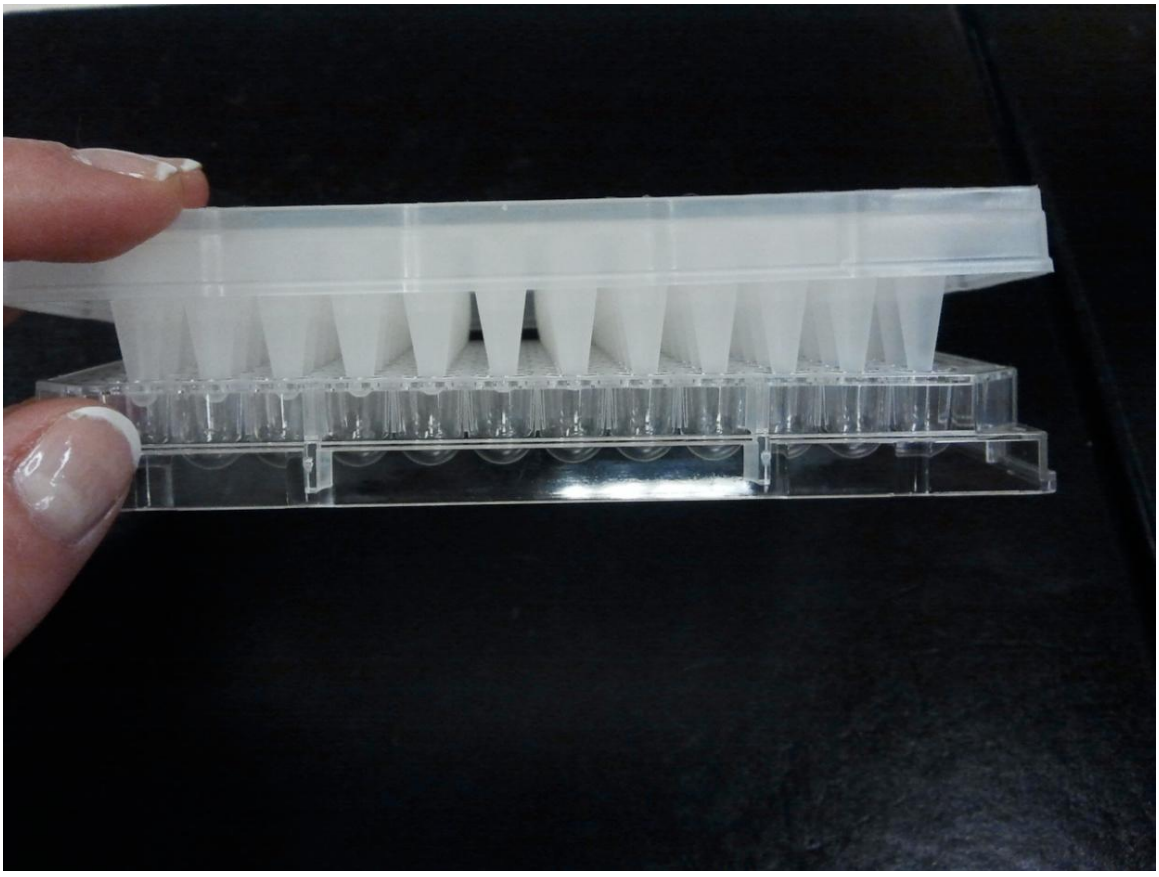
2.11 Biofilm testing

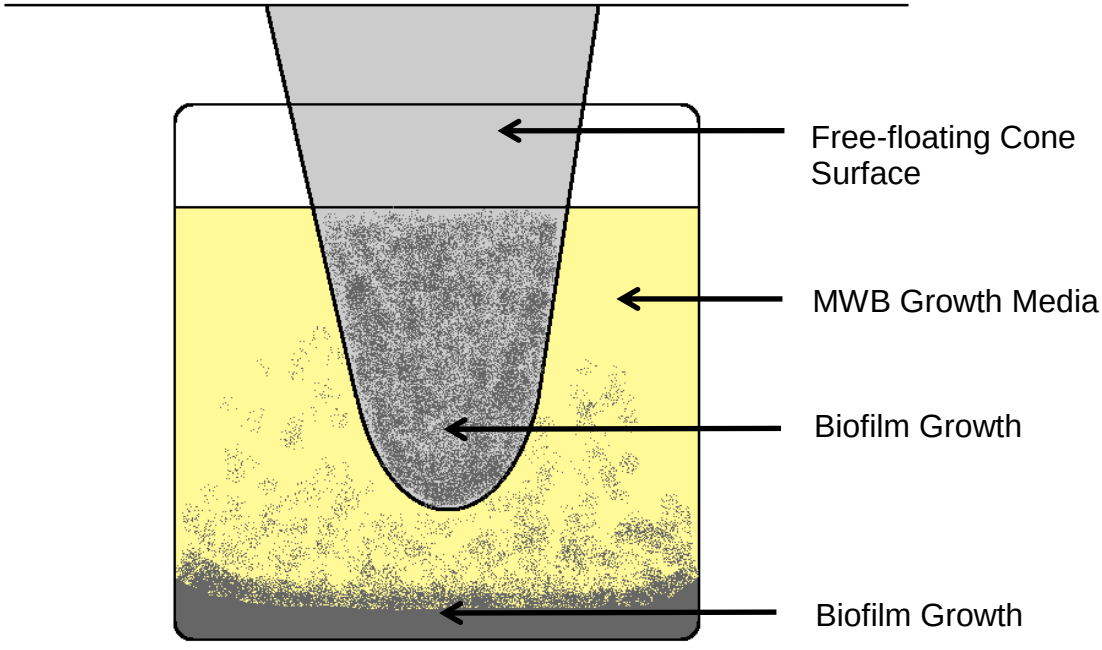
The biofilm forming ability of the full panel of wild-type and plasmid-cured isolates was determined according to a method developed by combining two well established protocols: the Calgary Biofilm Device protocol (Ceri et al., 1999) and the microtitre plate assay protocol (Djordjevic et al., 2002). Isolates were grown overnight on BHI

agar at 35°C. A single colony was used to inoculate BHI broth and the culture was incubated overnight at 35°C with shaking at 200 rpm. The culture was diluted 1:40 in Modified Welshimer's broth lacking iron additives (MWB, made in-house) and 150 µL of culture was added to each well of a sterile round bottom 96-well tissue culture plate (Thermo Scientific). A row of 8 wells was filled with 170 µL MWB to serve as a negative control. A sterile 96-well MicroAmp® PCR plate (Life Technologies) was placed inside the wells of the cylindrical round bottom tissue culture plate so that the biofilm could grow on the outside of the conical PCR plate wells (Figure 11). Motility is a factor in biofilm formation and allows the bacteria to travel to the biofilm formation site. Accordingly, studies have shown that *L. monocytogenes* biofilm formation is hindered when motility is suppressed, such as at a temperature of 37°C (Lemon et al. 2007). Therefore, a subset of the panel was incubated at 20°C, 25°C, 30°C, and 35°C to test biofilm formation at different temperatures. The optimal temperature for the strongest biofilm formation was determined to be 30°C.

The full panel of mutants and wild-type isolates were tested at 30°C for 48 hours, as well as at 4°C for 75 days to test growth temperatures similar to those found in food processing environments. The PCR plates were then removed from the tissue culture plates and the wells inspected for turbidity in the sample wells, as well as a lack of turbidity in negative control wells. The PCR plate was washed in a series of 4 water baths with shaking for 30 seconds to remove loosely adhered cells, followed by drying for 30 minutes. To stain the PCR plate, 200 µL of sterile 1% crystal violet (Sigma-Aldrich) was added to each well of a flat bottom 96-well

Figure 11. The set-up of the biofilm plate assay with the conical 96-well PCR plate placed inside the 96-well round bottom tissue culture plate. The media containing *L. monocytogenes* was incubated within the round bottom tissue culture plate and the conical PCR plate was placed inside this round-bottom plate. Biofilms formed on the exterior of the conical wells of the PCR plate and were inferred using a crystal violet staining method. Photograph provided by J. Ziegler. Artwork original to this thesis.




Free-floating Cone Surface
MWB Growth Media
Biofilm Growth
Biofilm Growth

to the biofilm formed on the outside of the PCR wells. The PCR plate was placed inside the immunoassay plate and incubated at room temperature for 30 minutes. Following incubation, the PCR plate was again washed in a series of 4 water baths with shaking for 30 seconds to remove unbound crystal violet. The plate was then dried for 30 minutes and visual results recorded. To de-stain the PCR plate, 150 µL of 95% ethanol (Sigma-Aldrich) was added to a sterile, round-bottom 96-well tissue culture plate (Thermo Scientific) and the PCR plate was placed inside. The plates were incubated at 4°C for 30 minutes. The optical density at 580 nm (the absorbance wavelength of crystal violet) was then determined using a µQuant plate reader (Bio-Tek Instruments, Inc., Winooski, VT). The optical density corresponded to the concentration of crystal violet remaining in each well following de-stain, therefore a higher optical density measurement was inferred to indicate greater thickness of biofilm or enhanced biofilm formation on the inverted PCR plate cone.

2.12 Swarming motility testing

The motility of a select panel of wild-type *L. monocytogenes* strains and their corresponding plasmid-cured strains was compared using swarming assays. Isolates were grown overnight in BHI broth at 30°C with no shaking, as well as in BHI broth plus 0.5 µg/mL $CdCl_2$ A plastic inoculating needle (Simport, Beloeil, QC), was dipped in each culture and used to stab inoculate BHI semi-soft agar (3%) in a total of 5 replicates. The plates were incubated at 30°C for 72 hours and the diameter of each resulting colony was measured manually with a millimeter ruler. The swarming ability of the selected isolates was inferred in three independent experiments.

2.13 Whole-genome sequencing (WGS)

WGS was used to obtain DNA sequences of plasmids identified using PCR plasmid screening. Sample libraries were prepared using an Illumina TruSeq DNA Sample Prep Kit V2 (Illumina, San Diego, California). 100 bp paired-end sequencing was performed on an in-house Illumina GAIIx instrument using TruSeq SBS Kit V5. Data was *de novo* assembled using Edena v3 (development version 120626), and the contigs were ordered to the 08-5578 reference genome using ABACAS (Assefa et al., 2009). Plasmid contig gaps were closed using custom PCR with inward-facing primers and Sanger sequencing. Assembly and annotation of plasmid sequences was completed as described in section 2.2.

2.14 Sanger sequencing

PCR and amplicons were purified using the Montage PCR Centrifugal Filter Device kit (Millipore) according to manufacturer's instructions. DNA was quantified on a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE) and diluted to 50 ng/µl for use as the sequencing template. Sequencing was conducted by the DNA Core at the NML using an ABI 3730XL Genetic Analyzer (Applied Biosystems) with the primers used to generate the amplicon. Sequence data was analyzed using SeqMan II^{TM} (DNAStar Inc. Madison, WI).

2.15 Statistical analysis

The effects of plasmid carriage on biofilm formation in *L. monocytogenes* was assessed by Student *t*-tests. *P*-values that were less than 0.005 were considered significantly different.

Chapter 3

Results

3. Results

3.1 Identification of *L. monocytogenes* isolates that harboured plasmids

Of the 147 *L. monocytogenes* isolates screened using the conventional PCR assay, 75 (51%) of the isolates were positive for at least one plasmid gene targets (Table 7). It was found that 39/95 (41%) of the human clinical isolates, 33/47 food or food environment isolates (70%), and 2/4 (50%) of non-human isolates presumptively harboured plasmids.

The distribution of *Listeria* evolutionary lineages within the screening panel included 106 isolates belonging to lineage II, 58 (55%) of which were plasmid-positive by PCR (Table 8). Forty-one isolates belonged to lineage I, and 14 (34%) of these tested positive for the presence of plasmid genes. Screening results by serotype for lineage I were as follows: serotype 1/2b, 13/22 (59%) plasmid-positive; serotype 4b, 1/18 (6%) plasmid-positive; serotype 3b, 0/1 plasmid-positive. Lineage II screening results were: serotype 1/2a, 40/77 (52%) plasmid-positive; serotype 1/2c, 14/16 (88%) plasmid-positive; serotype 3a, 5/11 (45%); serotype 3c, 2/2 (100%) plasmid-positive. Of the 40 plasmids identified in serotype 1/2a isolates, 25 (63%) had all the same plasmid gene targets as pLM5578 in isolate 08-5578 (positive for 6 of eight plasmid gene targets).

3.2 Annotation of plasmids pLM5026 and pLM0813

The plasmid pLM5578 was originally discovered during WGS of the serotype 1/2a isolate 08-5578 during the 2008 RTE listeriosis outbreak. Following this outbreak, WGS was performed on 37 additional outbreak-associated *L. monocytogenes* isolates, and this resulted in the discovery of pLM5026 and pLM0813. The DNA

Table 7: Results of the conventional PCR plasmid screening assay on the panel of 147 isolates used in this study. “NA”; not available.

Strain	Serotype	Source	p02 mco	p17 umuC	p50 traG	p62 par	p74 cadA	p08 ydhK	p33 yfiS	bcrB-bcrC	LGI1
81-0558	4b	Human cerebral spinal fluid	-	-	-	-	-	-	-	-	-
81-0592	4b	Human blood	-	-	-	-	-	-	-	-	-
81-0861	4b	Coleslaw	-	-	-	-	-	-	-	-	-
87-0192	1/2a	Human blood	-	-	-	-	-	-	-	-	-
87-0426	1/2a	Human cerebral spinal fluid	-	-	-	-	-	-	-	-	-
88-0286	1/2a	Cooked pork	-	+	-	-	-	-	-	-	-
88-0478	1/2a	Human blood	-	-	-	-	-	-	-	-	+
88-0702	1/2a	Human cerebral spinal fluid	-	-	-	-	-	-	-	-	-
88-0868	1/2b	Human blood	+	+	-	+	-	-	-	-	-
88-0876	1/2b	Food processing environment	-	-	-	+	-	-	-	-	-
88-0913	1/2b	Human blood	-	-	-	-	-	-	-	-	-
88-0928	1/2b	Smoked salmon	-	-	-	-	-	-	-	-	NA
88-0941	1/2b	Food	-	-	-	-	-	-	-	-	NA
88-0978	3a	Human cerebral spinal fluid	-	-	-	-	-	-	-	-	NA
88-1059	3b	Human blood	-	-	-	-	-	-	-	-	+
90-0558	1/2a	Human cerebral spinal fluid	+	+	-	+	+	-	-	-	-
90-0602	4b	Smoked salmon	-	-	-	-	-	-	-	-	NA
91-0145	1/2a	Human blood	-	-	-	-	-	-	-	-	-
92-0366	1/2a	Human blood	-	+	+	+	-	+	-	-	-
93-0024	1/2a	Human blood	-	-	-	-	-	-	-	-	+
93-0407	1/2a	Human blood	-	-	-	-	-	-	-	-	-
93-0638	1/2b	Human brain	+	+	-	+	+	-	-	-	-
93-0827	3a	Human cerebral spinal fluid	-	-	-	-	-	-	-	-	NA
93-0839	1/2b	Human cerebral spinal fluid	-	+	-	+	+	-	-	-	-
94-0096	1/2a	Human blood	-	-	-	-	-	-	-	-	-
94-0447	1/2a	Human blood	-	-	-	-	-	-	-	-	-
95-0012	1/2a	Human blood	+	+	+	+	+	+	-	-	+
95-0093	1/2a	Human blood	+	+	+	+	+	+	-	-	+
95-0151	1/2a	Human blood	+	+	+	+	+	+	-	-	+
96-0033	4b	Human blood	-	-	-	-	-	-	-	-	NA
96-0215	4b	Human blood	-	-	-	-	-	-	-	-	NA
96-0218	1/2a	Human blood	+	+	+	+	+	+	-	-	+
96-0247	1/2a	Human blood	-	-	-	-	-	-	-	-	+
97-0456	1/2b	Non human	-	-	-	-	-	-	-	-	NA
97-0465	3a	Non human	-	-	-	-	-	-	-	-	NA
97-0466	1/2c	Non human	+	+	-	+	+	-	-	-	-
97-0468	1/2c	Non human	+	+	-	+	+	-	-	-	-
97-0817	1/2b	Human blood	-	-	-	-	-	-	-	+	-
97-1602	1/2a	Human urine	+	+	+	+	+	+	-	-	+
97-1636	1/2a	Human blood	+	+	+	+	+	+	-	-	+
98-0041	1/2b	Human blood	+	+	-	+	+	-	-	-	-
98-0163	3c	Unknown	+	+	-	+	+	-	-	-	-
98-0291	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
98-2035	1/2a	Human blood	-	-	-	-	-	-	-	-	+
99-3046	1/2a	Human blood	+	+	+	+	+	-	-	-	-
99-3048	1/2a	Human blood	+	+	+	+	+	+	-	-	+
99-6370	1/2a	Human blood	-	-	-	-	-	-	-	-	+
99-6871	1/2a	Human clinical	-	-	-	-	-	-	-	-	+
00-0346	1/2a	Human peritoneal fluid	+	+	+	+	+	-	-	-	-
00-1149	1/2a	Human clinical	-	-	-	-	-	-	-	-	NA
00-2572	1/2b	Human blood	-	-	-	+	+	-	+	-	-
00-3767	1/2c	Pepper salami	-	-	-	-	-	-	-	-	NA
00-3770	1/2c	Pepper salami	-	-	-	-	-	-	-	-	NA
00-3853	1/2b	Food processing environment	+	+	-	+	+	-	-	+	-
00-5136	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
01-1280	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
01-1465	1/2a	Human blood	+	+	+	+	+	+	-	-	+
01-1468	1/2a	Human brain	-	-	-	-	-	-	-	-	+
01-2417	1/2a	Human blood	+	+	+	+	+	+	-	-	+
01-3506	1/2a	Human cerebral spinal fluid	+	+	+	+	+	+	-	-	+
01-5364	4b	Food processing environment	-	-	-	-	-	-	-	-	NA
01-5373	1/2a	Human blood	+	+	+	+	+	+	-	-	-
01-5991	1/2a	Food	+	+	+	+	+	-	-	-	-
01-7107	1/2a	Human blood	+	+	+	+	+	-	-	-	-
01-7209	1/2a	Liverwurst	+	+	+	+	+	+	-	-	+
01-7210	1/2a	Liverwurst	+	+	+	+	+	+	-	-	+
02-1115	4b	Human cerebral spinal fluid	-	-	-	-	-	-	-	-	NA
02-2448	1/2a	Human blood	+	-	+	+	+	-	-	-	-
02-4056	1/2a	Human blood	-	-	-	-	-	-	-	-	-
02-5993	1/2a	Human blood	-	-	-	-	-	-	-	-	+
03-0402	1/2a	Human blood	-	-	-	-	-	-	-	-	+
03-5195	3a	Cow's milk	+	+	-	+	+	-	-	-	-
03-5196	3a	Cow's milk	+	+	-	+	+	-	-	-	-
03-5197	3a	Cow's milk	+	+	-	+	+	-	-	-	-
03-5473	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
03-5833	1/2a	Human blood	+	+	+	+	+	-	-	-	-

03-7258	3a	Human blood	-	-	-	-	-	-	-	-	NA
03-7262	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
04-3697	1/2b	Bean curd	-	-	-	+	+	-	-	-	-
04-5455	4b	Human blood	-	-	-	-	-	-	-	-	NA
04-5457	1/2a	Human blood	-	-	-	-	-	-	-	-	+
04-5458	4b	Human blood	-	-	-	-	-	-	-	-	NA
04-7425	1/2b	Human blood	-	-	-	-	-	-	-	-	NA
05-2144	4b	Human blood	-	-	-	-	-	-	-	-	NA
05-4745	4b	Human blood	-	-	-	-	-	-	-	-	NA
05-5920	3a	Human blood	-	-	-	-	-	-	-	-	NA
06-3527	1/2b	Human blood	-	-	-	-	-	-	-	-	NA
06-6391	1/2b	Human peritoneal fluid	-	-	-	-	-	-	-	-	NA
06-6833	1/2a	Human blood	+	+	-	+	+	-	-	-	-
06-6837	1/2a	Human blood	-	-	-	-	-	-	-	-	+
06-6840	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
06-6863	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
06-6865	3a	Human blood	-	-	-	-	-	-	-	-	-
06-6909	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
06-6934	1/2a	Human blood	-	+	+	+	+	-	-	-	-
06-6956	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
07-1873	1/2c	Salami	+	+	-	+	+	+	-	-	-
07-1874	1/2c	Salami	+	+	-	+	+	+	-	-	-
07-6082	1/2a	Human blood	+	+	+	+	+	+	-	-	+
07-6083	1/2a	Human blood	+	+	+	+	+	+	-	-	+
08-3083	4b	Human blood	-	-	-	-	-	-	-	+	-
08-3347	1/2b	Human blood	+	+	-	+	+	-	-	-	-
08-5375	1/2a	Human blood	+	+	+	+	+	-	-	-	-
08-5379	4b	Human blood	-	-	-	-	-	-	-	-	NA
08-5496	1/2b	Cheese	+	+	-	+	+	-	-	-	-
08-5578	1/2a	Human blood	+	+	+	+	+	+	-	-	+
08-5597	4b	Coleslaw	-	-	-	-	-	-	-	-	NA
08-5923	1/2a	Human blood	-	-	-	-	-	-	-	-	+
08-6056	1/2a	Turkey meat	+	+	+	+	+	+	-	-	+
08-6337	1/2a	Human blood	-	-	-	-	-	-	-	-	NA
08-6569	1/2a	Food processing environment	+	+	+	+	+	+	-	-	+
08-6997	1/2a	Human blood	-	-	-	-	-	-	-	-	+
08-7374	1/2a	Human blood	+	+	+	+	+	+	-	-	+
08-7669	1/2a	Human blood	+	+	+	+	+	+	-	-	+
08-8526	1/2c	Roast beef	-	-	-	+	+	-	+	+	-
08-8748	1/2c	Chicken breast	-	-	-	+	+	-	+	+	-
08-8749	1/2c	Pork loin chops	-	-	-	+	+	-	+	+	-
08-8750	1/2c	Pastrami	-	-	-	+	+	-	+	+	-
08-8751	1/2c	Cheddar mini sausage	-	-	-	+	+	-	+	+	-
08-8809	1/2b	Blue cheese	-	-	-	-	+	-	-	+	-
10-0809	4b	Human clinical	-	-	-	-	-	-	-	-	-
10-0810	1/2b	Human clinical	-	-	-	-	-	-	-	-	-
10-0811	1/2b	Imitation crab meat	-	-	-	-	-	-	-	-	-
10-0812	1/2a	Whipping cream	-	+	-	+	+	-	-	-	-
10-0813	1/2a	Human clinical	-	+	-	+	+	-	-	-	-
10-0814	1/2a	RTE Meat	+	+	+	+	+	+	-	-	+
10-0815	1/2a	RTE Meat	+	+	+	+	+	+	-	-	+
10-0933	1/2a	Human blood	-	-	-	-	-	-	-	-	-
10-0934	1/2a	Cheese	-	-	-	-	-	-	-	-	-
10-1046	1/2a	Human blood	-	-	-	-	-	-	-	-	+
10-1047	1/2a	Human cerebral spinal fluid	+	+	+	+	+	+	-	-	+
10-1321	1/2a	Human blood	+	+	+	+	+	+	-	-	+
10-1542	1/2c	Salmon nuggets	-	-	-	+	+	-	-	+	-
10-1581	1/2c	Food processing environment	-	-	-	+	+	-	-	+	-
10-1582	3a	Food processing environment	-	+	+	-	-	-	-	+	-
10-1583	3a	Food processing environment	-	+	+	-	-	-	-	+	-
10-1601	4b	Food processing environment	-	-	-	-	-	-	-	-	NA
10-1633	1/2b	Food processing environment	+	+	-	+	+	-	-	-	-
10-2174	1/2a	Human blood	+	+	+	+	+	+	-	-	+
10-3180	4b	Blood	-	-	-	-	-	-	-	-	NA
10-4754	1/2a	Human cerebral spinal fluid	-	-	-	-	-	-	-	-	-
10-4758	1/2a	Cheese	-	-	-	-	-	-	-	-	-
10-5024	1/2a	Cooked ham	+	+	+	+	+	+	-	-	+
10-5025	1/2c	Cooked bacon flakes	-	+	-	+	+	-	+	+	-
10-5026	1/2c	Food processing environment	-	+	-	+	+	-	+	+	-
10-5027	3c	Food processing environment	-	+	-	+	+	-	+	+	NA
10-6746	1/2c	Human blood	-	-	-	+	+	-	+	-	-
EGDe	1/2a	Rabbit tissue	-	-	-	-	-	-	-	-	-

Table 8: Summary of the conventional PCR plasmid screening results: A) organized by source; B) organized by serotype.

A

Source	Number of Isolates Screened	Isolates PCR-Positive for Plasmid Genes
Human Clinical	95	39 (41%)
Food/Food processing environment	47	33 (70%)
Non-human	4	2 (50%)
Unknown	1	1 (100%)
Total	**147**	**75 (51%)**

B

	Serotype	Number of Isolates Screened	Isolates PCR-Positive for Plasmid Genes
Lineage II	1/2a	77	40 (52%)
	1/2c	16	14 (88%)
	3a	11	5 (45%)
	3c	2	2 (100%)
Lineage I	4b	18	1 (6%)
	1/2b	22	13 (59%)
	3b	1	0 (0%)
	Total	**147**	**75 (51%)**

sequences of pLM5578, pLM5026, and pLM0813 were annotated for predicted functional *cis* features in order to direct research into plasmid-conferred phenotypes. Plasmid pLM5578 was previously annotated by the Gilmour laboratory (Figure 9), and was used as a reference for transitive annotation of pLM5026 and pLM0813 (Tables 9 and 10; Figures 12 and 13). Not all putative coding sequences were assigned functions during annotation, as a majority of these CDS could only be identified as “conserved hypothetical” proteins - those that are found in organisms from several phylogenetic lineages but have not been functionally characterized (Galperin & Koonin, 2004). For investigative purposes, a number of these conserved hypothetical coding regions were annotated based on predicted gene functions that had quality scores considerably below those considered acceptable for publication.

Both plasmids pLM5026 and pLM0813 were found to encode regions pertaining to replication and plasmid partitioning. Both plasmids also carried *cadA* and *cadC*, genetic elements that are related to resistance to heavy metal salts containing divalent cations, such as zinc and cadmium. They also contained a number of genetic elements involved in surviving environmental stresses: *ltrC*, which may be involved in growth at low temperatures; *clpB*, which contained homology to chaperone proteins involved in heat-shock responses; *npr*, a putative peroxidase that may be involved in surviving oxidative stresses; and an unnamed ABC transporter involved in surviving oxidative stresses. Notably, plasmid pLM5026 also carried the BKC resistance cassette *bcrABC*, which was recently characterized in another plasmid isolated from *L. monocytogenes* (Elhanafi et al., 2010).

Table 9: Annotation of all predicted coding sequences of pLM5026 from isolate 10-5026.

CDS Location (bp Start-stop)	Gene Name	Functional Annotation	Putative/Predicted Function
224 - 1270	*repB*	ATPase involved in chromosome partitioning; likely controls plasmid copy number	Replication/Partitioning
1248 - 1544	*repC*	Replication copy and control-associated protein	Replication/Partitioning
2875 - 1595	*umuC*	Nucleotidyltransferase involved in DNA replication, DNA polymerase V	Replication
3207 - 2863		Conserved hypothetical protein	Unknown
4045 - 3365		Transposase	Transposition
4775 - 4350		Homologies to DNA/RNA helicases	Unknown
7768 - 4853	*tnpA*	Transposase for transposon Tn1546	Transposition
8326 - 7772	*tnpR*	Transposon resolvase	Transposition
8600 - 8965	*cadC*	Cadmium efflux system accessory protein	Extracellular transport
8965 - 11100	*cadA*	Cadmium transporting ATPase	Extracellular transport
12242 - 11643		Homologies to recombinases; invertase from transposon Tn552	Transposition
12401 - 13837		Transposase for transposon Tn552	Transposition
14333 - 15013		Putative transposase	Transposition
15077 - 16690	*ltrC*	Involved in growth at low temperatures	Regulation
17481 - 18080	*bcrA*	Transcriptional regulator of the *bcrABC* cassette responsible for resistance to the QAC benzalkonium chloride	Regulation
18068 - 18409	*bcrB*	Small multidrug efflux pump of the *bcrABC* cassette	Extracellular transport
18424 - 18771	*bcrC*	Small multidrug efflux pump of the *bcrABC* cassette	Extracellular transport
18971 - 19330		Transposase resolvase	Transposition
19343 - 19546	*resP3*	Resolvase from transposon Tn1546	Transposition
20449 - 21312		Homologies to a triphenylmethane reductase (TMR) complex; triphenylmethane are environmental pollutants	Stress response
22321 - 23265		MoxR-like ATPase, widespread in prokaryotes, often involved in modulation of stress response pathways	Regulation
23866 - 24894		Catalytic integrase region of transposon	Transposition
25515 - 24886		Homologies to a protein involved in bacteriophage resistance by abortive infection	Stress response
26780 - 25662	*clpB*	High homology to a chaperone protein involved in heat-shock responses	Stress response
26948 - 27628		Transposase from Insertion sequence 1297	Transposition
29631 - 27748	*cadA*	Heavy metal translocating P-type ATPase	Extracellular transport
30858 - 30094		Transposase	Transposition
31729 - 32598		Glycine-betaine binding ABC transporter involved in surviving osmotic stresses	Stress response
34208 - 32844	*npr*	NADH peroxidase that may be involved in surviving oxidative stresses	Stress response
34400 - 34801		Transposase with homology to IS1216	Transposition
34832 – 35512		Transposase with homology to IS1216	Transposition

35537 - 36199		Protein of unknown function DUF190	Unknown
36978 - 38216	*enoA*	Enolase (Phosphopyruvate dehydratase); important enzyme in glycolysis pathway	Stress response
38282 - 39517	*kefB*	Homology to a glutathione-regulated potassium-hydrogen antiporter efflux system protein KefB; possibly part of a detoxification pathway for methylgyoxal	Extracellular transport
40690 - 39905		High homology to the ISChy4 transposase helper protein	Transposition
41897 - 40671		Transposase IS712A from *Listeria grayi*	Transposition
42486 - 43676	*yfiS*	Efflux transporter with homology to macrolide efflux proteins	Extracellular transport
45997 - 43751	*copB*	Copper-translocating P-type ATPase	Extracellular transport
46679 - 46248	*copY*	Transcriptional copper transport repressor	Regulation
50496 - 47374		Putative site-specific deoxyribonuclease of type III restriction enzyme	DNA processing
52363 - 50531		DNA methyltransferase of DNA modification system	DNA processing
52923 - 52366		Hypothetical protein	Unknown
56118 - 52924		Helicase domain-containing protein	DNA processing
56429 - 56154		Hypothetical protein	Unknown
56792 - 57679	*tnpB*	Putative transposase with homology to tnpB from ISLL6 transposase	Transposition
59232 - 58243		Hypothetical protein	Unknown
60178 - 59609		Hypothetical protein	Unknown
62692 - 62090		Putative transposase with homology to integrase	Transposition
63554 - 62769		High homology to the ISChy4 transposase helper protein	Transposition
64761 - 63535		Transposase IS712A	Transposition
65304 - 65693		Hypothetical protein	Unknown
65696 - 66613		Hypothetical protein	Unknown
67011 - 67250		Hypothetical protein	Unknown
67262 - 67702		Hypothetical protein	Unknown
67695 - 69035		Conserved Hypothetical protein	Unknown
69046 - 69417		Hypothetical protein	Unknown
69437 - 70201		Homology to protein family related to filamentation induced by cAMP and death-on-curing family	Regulation
70315 - 70845		Hypothetical protein	Unknown
71793 - 73976		Putative adenine-specific DNA methylase	DNA processing
74148 - 75374		High homology to transposase Tnp712	Transposition
75355 - 76140		High homology to transposase ISChy4	Transposition
76217 – 76819		Putative transposase	Transposition
77608 - 76853		Hypothetical protein	Unknown
79517 - 77883	*repA*	Plasmid replication protein	Replication

Figure 12: Linear map of predicted coding sequences of pLM5026, colour coded by putative function (inset). Generated using Gview server. Scale bar in kbp.

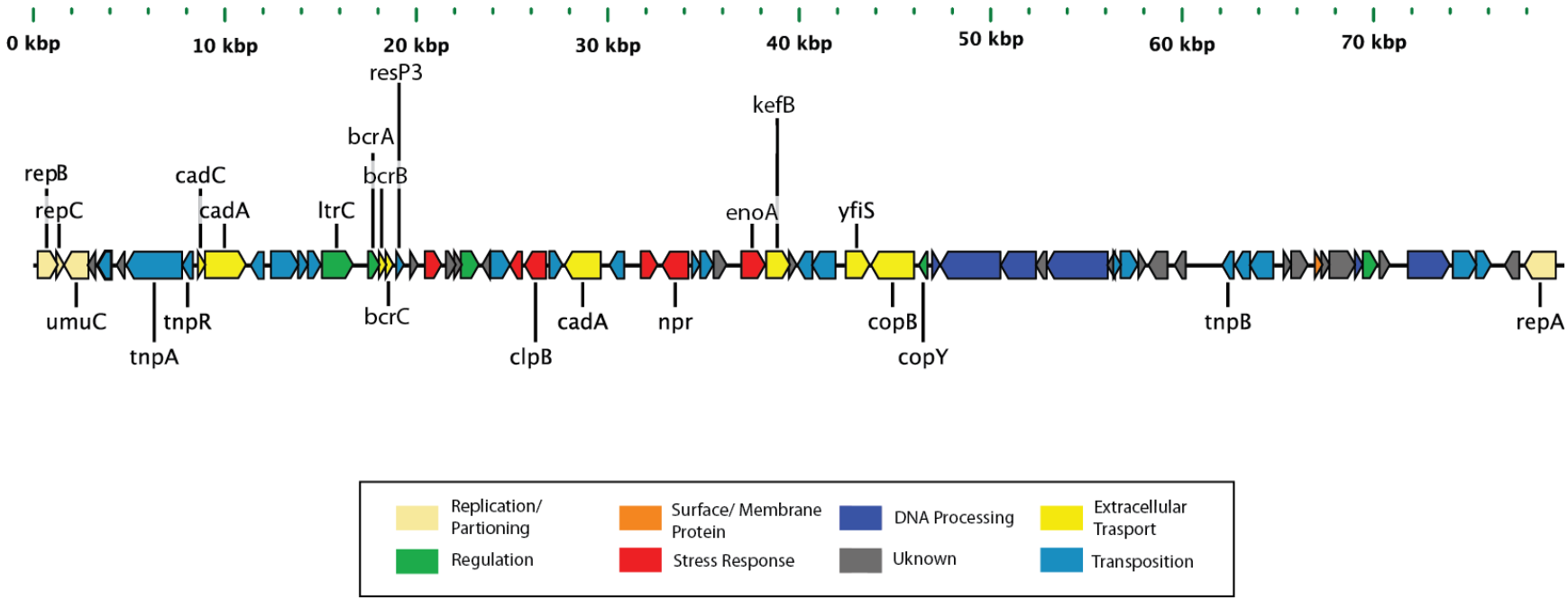
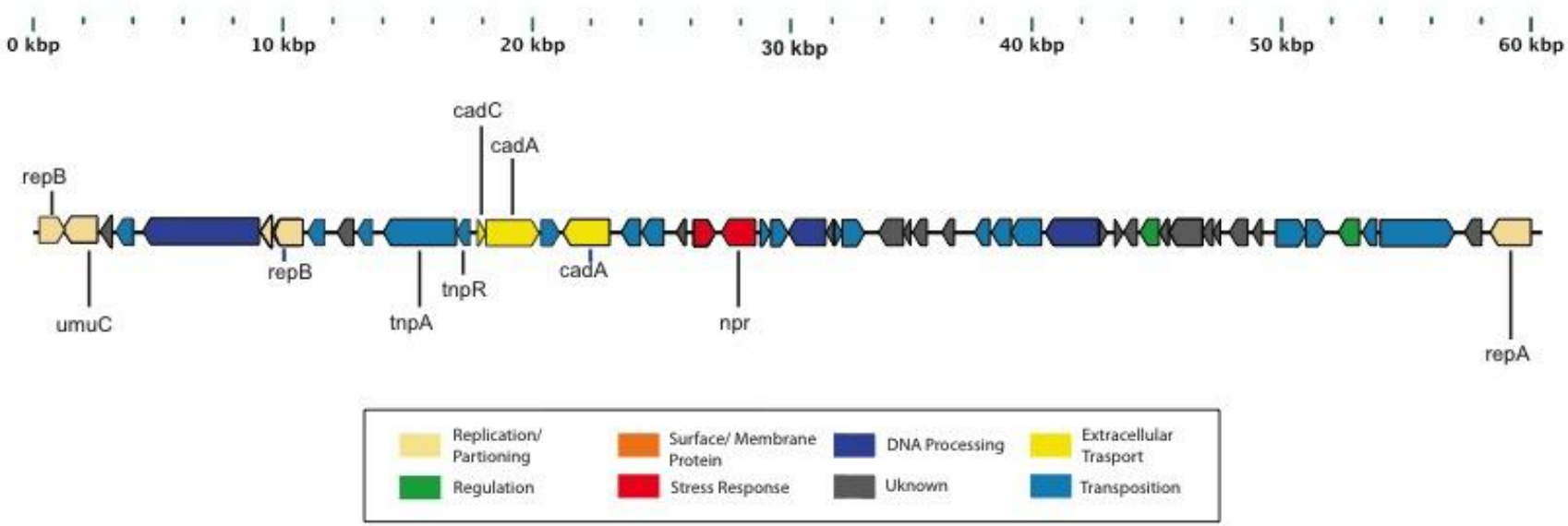

0 kbp
10 kbp
20 kbp
30 kbp
40 kbp
50 kbp
60 kbp
70 kbp
resP3
bcrA
bcrB
kefB
repB
repC
cadC
cadA
ltrC
enoA
yfiS
umuC
tnpA
tnpR
bcrC
clpB
cadA
npr
copB
copY
tnpB
repA
Replication/ Partioning
Regulation
Surface/ Membrane Protein
Stress Response
DNA Processing
Uknown
Extracellular Trasport
Transposition

Table 10: Annotation of all predicted coding sequences of pLM0813 from isolate 10-0813.

CDS Location (Bp Start-stop)	Gene Name	Functional Annotation	Putative/Predicted Function
224 - 1270	*repB*	ATPase involved in chromosome partitioning; likely controls plasmid copy number	Replication/Partitioning
2875 - 1595	*umuC*	Nucleotidyltransferase involved in DNA replication, DNA polymerase V	Replication
3207 - 2863		Conserved hypothetical protein	Unknown
4045 - 3365		Transposase	Transposition
9002 - 4350		Helicase/methyltransferase of a type III restriction modification system	DNA processing
9545 - 9213		Protein with homology to replication machinery in other plasmids	Replication
10705 - 9545	*repB*	Replication machinery	Replication
11665 - 10985		Putative transposase	Transposition
12826 - 12179		Conserved hypothetical protein	Unknown
13585 - 12971		Transposase resolvase	Transposition
16935 - 14020	*tnpA*	Transposase for transposon Tn1546	Transposition
17493 - 16939	*tnpR*	Transposon resolvase	Transposition
17773 - 18132	*cadC*	Cadmium efflux system accessory protein	Extracellular transport
18132 - 20267	*cadA*	Cadmium transporting ATPase	Extracellular transport
20407 - 21087		K04798 transposase	Transposition
23090 - 21207	*cadA*	Heavy metal translocating P-type ATPase	Extracellular transport
24317 - 23553		Transposase	Transposition
25249 - 24380		Integrase region of transposase	Transposition
26139 - 25768		Hypothetical protein	Unknown
26448 - 27317		Glycine-betaine binding ABC transporter involved in surviving osmotic stresses	Stress response
28927 - 27563	*npr*	NADH peroxidase that may be involved in surviving oxidative stresses	Stress response
29119 - 29520		Putative transposase with high homology to IS1216	Transposition
29551 - 30231		Transposase	Transposition
31737 - 30250		DNA-dependent ATPase; superfamily II DNA/RNA helicases	DNA processing
32048 - 31773		Conserved hypothetical protein	Unknown
32106 - 32399		K07483 transposase	Transposition
32411 - 33298	*tnpB*	Putative transposase with homology to tnpB from ISLL6 transposase	Transposition
34851 - 33862		Conserved hypothetical protein	Unknown
35118 - 34858		Hypothetical protein	Unknown
35797 - 35228		Conserved hypothetical protein	Unknown
36891 - 36397		Hypothetical protein	Unknown
38311 - 37709		Putative transposase	Transposition
39173 - 38388		Putative transposase	Transposition
40380 - 39154		Putative transposase	Transposition
42735 - 40552		Putative adenine-specific DNA methylase	DNA processing
43137 - 42739		Conserved hypothetical protein	Unknown
43311 - 43646		Conserved hypothetical protein	Unknown
44213 - 43683		Conserved hypothetical protein	Unknown

45091 - 44327		Conserved hypothetical protein; homology to family of proteins related to filamentation induced by cAMP	Regulation
45482 - 45111		Conserved hypothetical protein	Unknown
46833 - 45493		Conserved hypothetical protein	Unknown
47266 - 46826		Conserved hypothetical protein	Unknown
47517 - 47278		Conserved hypothetical protein	Unknown
48832 - 47915		Conserved hypothetical protein	Unknown
49224 - 48835		Conserved hypothetical protein	Unknown
49767 - 50993		Transposase IS712A	Transposition
50974 - 51759		Transposase helper protein	Transposition
53124 - 52279		Transcriptional activator	Regulation
53813 - 53238		Transposon resolvase	Transposition
53961 - 56927		Transposase	Transposition
58089 - 57334		Hypothetical protein	Unknown
59998 - 58364	*repA*	Plasmid replication protein	Replication

Figure 13: Linear map of predicted coding sequences of pLM0813, colour coded by putative function (inset). Generated using Gview server. Scale bar in kbp.


0 kbp
10 kbp
20 kbp
30 kbp
40 kbp
50 kbp
60 kbp
cadC
cadA
repB
repB
umuC
tnpA
tnpR
cadA
npr
repA
Replication/ Partioning
Surface/ Membrane Protein
DNA Processing
Extracellular Trasport
Regulation
Stress Response
Uknown
Transposition

3.3 Heat curing of wild-type *L. monocytogenes* plasmids was successful in 57/75 (76%) of plasmid-positive isolates

In total, 57 of the screening panel's 75 (76%) plasmid-positive isolates were cured of their replicated plasmid. The heat curing method was unable to remove plasmids from every *L. monocytogenes* isolate in the panel. This may be in part be owing to a number of isolates that exhibited very poor growth at 43°C. Other strains appeared to be much more resistant to plasmid heat curing, and the method was modified to passage these isolates multiple times, screening as many as 50 single colonies with each attempt. If a plasmid-negative isolate was not obtained after 3 distinct attempts, it was deemed 'non-curable' for this study and removed from further downstream phenotypic testing and comparisons (as plasmid-harbouring and plasmid-cured strains were required for the comparative analyses). However, non-curable isolates (totaling 18) were sequenced along with the other plasmid-positive members of the screening panel.

3.4 Plasmid pLM5578 is highly stable during vertical transmission under various growth conditions over 120 days

Stability of pLM5578 during vertical transmission was measured in various growth conditions, including growth during standard lab conditions (35°C on BHIA), growth in blood (35°C on CBAB), and conditions similar to those found in a food processing environment (25°C and 4°C in custom turkey-10 µg/mL BKC liquid media). For cultures grown at 35°C, 10 colonies were screened for the presence of pLM5578 after every 10 passages, whereas 4°C growth was tested every 5 passages (Table 11). For each of the growth conditions, there was no decrease in carriage of

pLM5578 over time (120 days), for a total of 50 passages. Plasmid loss was only observed in 1 of the 10 tested colonies during passage 30 for the BHIA plate passage, and during passage 50 on the CBAB plate passage, and for the 4°C food processing environment passage. However, loss of pLM5578 was not observed in further passages, and there were no patterns of loss to indicate further plasmid loss during subsequent passages.

3.5 74% of plasmid-cured strains had lower cadmium MICs than their corresponding wild-type strains

In order to determine the contribution of *L. monocytogenes* plasmids to cadmium resistance, the susceptibility to the heavy metal cadmium was compared between wild-type strains and their corresponding plasmid-cured mutants. Overall, 40 of the 57 (70%) wild-type strains were found to be resistant to cadmium (Table 12). When comparing wild-type strains to their plasmid-cured mutants, 42 of the 57 (74%) plasmid-cured strains had lower MICs than their corresponding wild-type strain. These differences in MICs are most likely due to plasmid-encoded cadmium resistance determinants. Alternatively, nine isolates had MICs that were identical to the corresponding plasmid-cured mutant, and it is most likely that genetic elements related to cadmium resistance (if any) were contained within the chromosomes of these strains.

3.6 BKC resistance determinants are carried on the plasmids of 21% of plasmid-positive isolates

The MIC for BKC was determined for the panel of 57 wild-type and corresponding plasmid-cured strains at 4°C, 22°C, and 35°C. An MIC to BKC greater than

Table 11: Results of pLM5578 stability testing after passaging through various media for 120 days.

Growth Condition	Colonies Positive for pLM5578					
	Day 0 Passage 0	**Day 24 Passage 10**	**Day 48 Passage 20**	**Day 72 Passage 30**	**Day 96 Passage 40**	**Day 120 Passage 50**
35°C BHI plate (Lab passage)	10/10	10/10	10/10	9/10	10/10	10/10
35°C CBAB plate (Blood passage)	10/10	10/10	10/10	10/10	10/10	9/10
22°C Custom turkey-BKC media (Food processing environment passage)	10/10	10/10	10/10	10/10	10/10	10/10
	Passage 0	**Passage 5**	**Passage 10**	**Passage 15**	**Passage 20**	**Passage 25**
4°C Custom turkey-BKC media (Food processing environment passage)	10/10	9/10	10/10	10/10	10/10	10/10

15 µg/mL was considered “BKC resistant” in this study. Thirty-eight of 57 (67%) wild-type isolates were found to be resistant to BKC (Table 12), of which 12 of these 38 (32%) wild-type strains lost their resistance to BKC upon removal of their plasmids. This plasmid-mediated resistance was found in all strains that were positive for the *bcrBC* genes in PCR screens. Twenty-six (46%) of the isolates tested had MICs identical to their plasmid-negative mutants, indicating that the resistance to BKC in these isolates was instead likely chromosomally-encoded. The BKC MIC results acquired were similar for the three different temperatures, with the exception of 5 wild-type strains (08-5826, 08-8748, 08-8749, 08-8750, and 08-8751) that had MICs 5 µg/mL higher at 22°C than at 30°C.

3.7 Antibiotic susceptibility testing did not reveal any significant antibiotic resistance

Antibiotic susceptibility testing was performed on the wild-type and corresponding plasmid-cured strains of isolates 08-5578 and 10-5026 to determine if any of the proteins of unknown function encoded on their plasmids were related to resistance to antibiotic compounds. There were no differences in MIC values between the wild-type strains and their plasmid-negative mutants for the panel of antibiotics tested (Table 13). The results of this testing did not reveal resistances to antibiotics that is not normally found in *L. monocytogenes*; most isolates appeared to have an intrinsic resistance or reduced susceptibility to cephalosporins (Morvan et al., 2010; Troxler et al., 2000). *L. monocytogenes* is almost always susceptible to penicillin and trimethoprim/sulfamethoxazole, and these susceptibilities were also observed in this study.

Table 12: Summary of results of cadmium chloride and benzalkonium chloride (BKC) susceptibility testing. Resistance to cadmium was denoted when growth was measured in concentrations of 64 µg/mL or higher cadmium. For BKC, an MIC greater than 15 µg/mL was considered resistant in this study.

Number of Isolates	Isolates Resistant to Cadmium (Cd^R)	Isolates with Plasmids Increasing Cd^R	Isolates with Chromosomal Cd^R
57	40 (70%)	42 (74%)	9 (41%)

Number of Isolates	Isolates Resistant to BKC (BKC^R)	Isolates with Plasmids Increasing BKC^R	Isolates with Chromosomal BKC^R
57	37 (67%)	12 (21%)	26 (46%)

Table 13: Summary of antibiotic susceptibility testing (minimum inhibitory concentration) on 08-5578, 10-5026, and their corresponding plasmid-negative strains.

Antibiotic	08-5578	pCure* 5578	10-5026	pCure 5026
Moxifloxacin (µg/mL)	≤1.0	≤1.0	≤1.0	≤1.0
Penicillin (µg/mL)	0.25	0.25	0.25	0.25
Levofloxacin (µg/mL)	1.0	1.0	1.0	1.0
Meropenem(µg/mL)	≤0.25	≤0.25	≤0.25	≤0.25
Azithromycin(µg/mL)	0.5	0.5	0.5	0.5
Tetracycline(µg/mL)	≤1.0	≤1.0	≤1.0	≤1.0
Ertapenem (µg/mL)	≤0.5	≤0.5	≤0.5	≤0.5
Erythromycin (µg/mL)	≤0.25	≤0.25	≤0.25	≤0.25
Cefuroxime (µg/mL)	>4.0	>4.0	>4.0	>4.0
Amoxicillin/clavulanic acid 2:1 ratio (µg/mL)	≤2/1	≤2/1	≤2/1	≤2/1
Trimethoprim/ sulfamethoxazole (µg/mL)	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5
Ceftriaxone (µg/mL)	>2.0	>2.0	>2.0	>2.0
Linezolid (µg/mL)	1.0	1.0	1.0	1.0
Vancomycin (µg/mL)	1.0	1.0	1.0	1.0
Cefotaxime (µg/mL)	>4.0	>4.0	>4.0	>4.0
Clindamycin (µg/mL)	>1.0	>1.0	>1.0	>1.0
Daptomycin(µg/mL)	2.0	2.0	2.0	2.0
Cefepime (µg/mL)	>8.0	>8.0	>8.0	>8.0
Chloramphenicol (µg/mL)	8.0	8.0	8.0	8.0
Tigecyline (µg/mL)	0.06	0.06	0.06	0.06

*** - pCure = stands for the plasmid-negative mutant created by heat-curing the wild-type strain**

3.8 Loss of plasmid pLM5578 results in decreased biofilm formation

The biofilm forming ability of 57 wild-type strains and their corresponding plasmid-cured strains were compared at both 4°C and 30°C. The assay did not detect any measurable amount of biofilm formation after 75 days of growth at 4°C. When comparing biofilm formation at 30°C, significant differences were observed between wild-type and plasmid-cured mutants (Figure 14), and significance persisted when using a more stringent p-value cutoff of 0.005 (99.5% confidence level). Thirty-five of 57 (61%) wild-type isolates had significantly increased biofilm formation compared to their corresponding plasmid-cured mutants. The opposite was observed in 12 of the 57 isolates (21%), with plasmid-cured mutants exhibiting significantly increased biofilm formation than their corresponding wild-type strains. For the remaining 10 isolates there was no measured effect on biofilm formation related to plasmid carriage.

The observed patterns of correlation between plasmid carriage and biofilm formation were strongly linked to serotype (Table 14 and Figure 15): 28 of the 36 (78%) isolates for which plasmid-carriage resulted in enhanced biofilm formation belonged to serotype 1/2a. None of the 1/2a strains exhibited stronger biofilm when cured of their plasmids, and curing had no effect in only 3 of the 1/2a strains. Twenty-four of the serotype 1/2a isolates tested had a plasmid PCR screening pattern that matched pLM5578, of which 23 (96%) had stronger biofilm formation when carrying plasmids. Plasmid loss had no effect on only one of these pLM5578-like strains (08-7374) (Table 15). Two 1/2a strains, 10-0813 and 10-0812, were found to both harbour the plasmid pLM0813, and also exhibited decreased biofilm

Figure 14: Comparison of biofilm formation at 30°C between wild-type (blue) and plasmid-cured mutant (orange) strains. ‘ * ‘ indicates statistical significance with a p-value < 0.005 (0.5%) using a Student`s *t*-test.

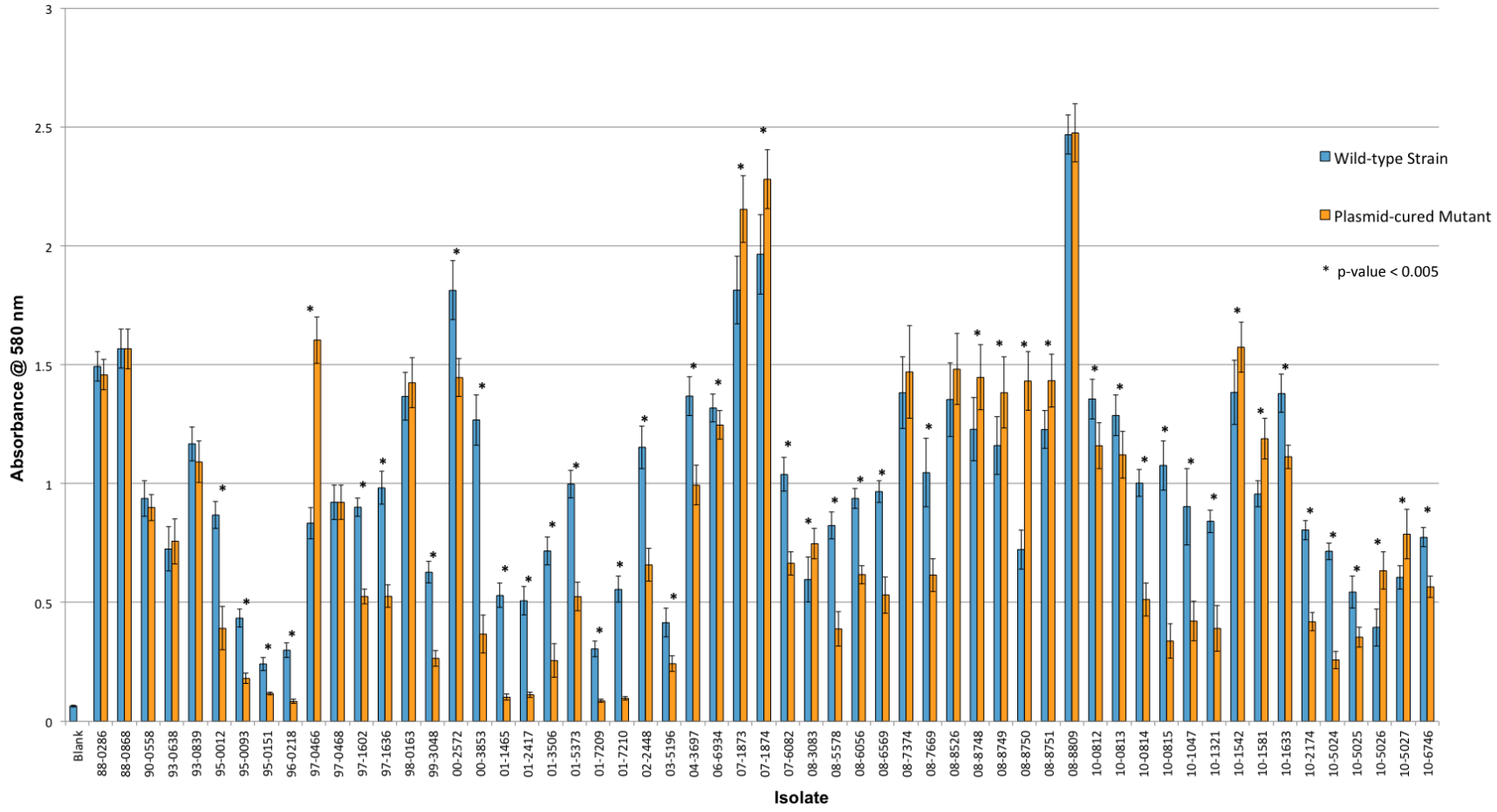
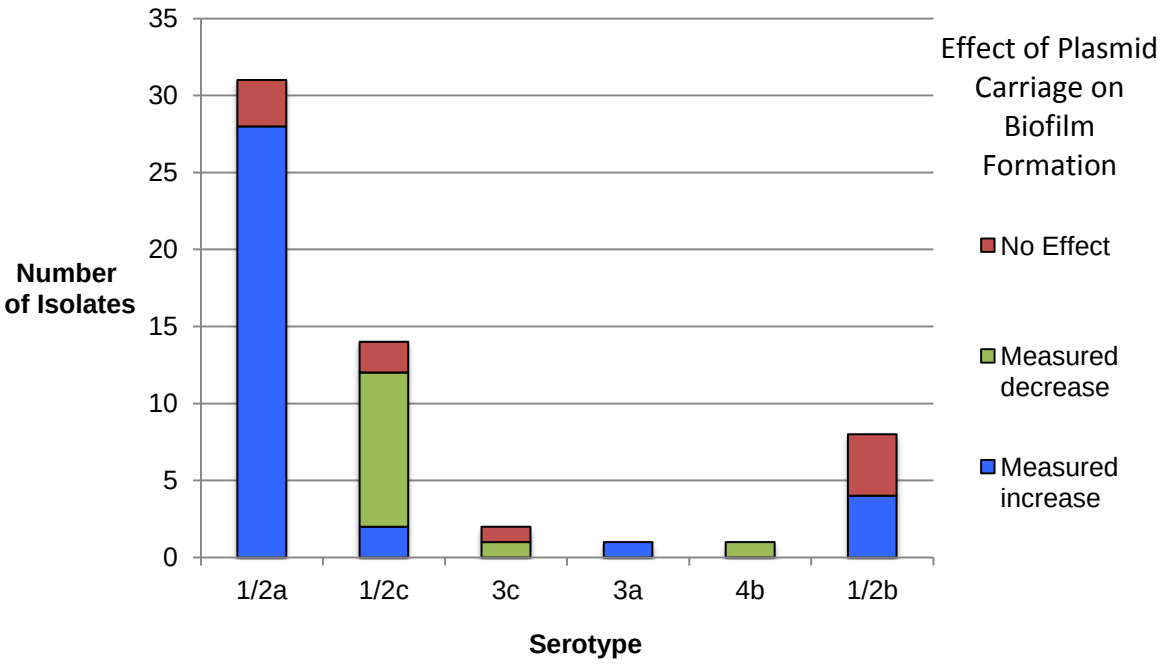

Absorbance @ 580 nm
3
2.5
2
1.5
1
0.5
0
Wild-type Strain
Plasmid-cured Mutant
* p-value < 0.005
Blank
88-0286
88-0868
90-0558
93-0638
93-0839
95-0012
95-0093
95-0151
96-0218
97-0466
97-0468
97-1602
97-1636
98-0163
99-3048
00-2572
00-3853
01-1465
01-2417
01-3506
01-5373
01-7209
01-7210
02-2448
03-5196
04-3697
06-6934
07-1873
07-1874
07-6082
08-3083
08-5578
08-6056
08-6569
08-7374
08-7669
08-8526
08-8748
08-8749
08-8750
08-8751
08-8809
10-0812
10-0813
10-0814
10-0815
10-1047
10-1321
10-1542
10-1581
10-1633
10-2174
10-5024
10-5025
10-5026
10-5027
10-6746
Isolate

Table 14: Summary of results for the 30°C biofilm assay sorted by *L. monocytogenes* lineage and serotype.

Serotype	Effect of Plasmid Carriage on Biofilm Formation:			Total
	Increased Biofilm Formation *	Decreased Biofilm Formation *	No Effect	
1/2a	28	0	3	**31**
1/2c	2	10	2	**14**
3c	0	1	1	**2**
3a	1	0	0	**1**
Lineage II	**31**	**11**	**6**	**48**
4b	0	1	0	1
1/2b	4	0	4	8
Lineage I	**4**	**1**	**4**	**9**
Total	**35 (61%)**	**12 (21%)**	**10 (18%)**	**57 (100%)**

* Significant at $p < 0.005$

Figure 15: Summary of results of the 30°C biofilm assay sorted by *L. monocytogenes* serotype.


35
30
25
20
15
10
5
0
Number of Isolates
1/2a
1/2c
3c
3a
4b
1/2b
Serotype
Effect of Plasmid Carriage on Biofilm Formation
No Effect
Measured decrease
Measured increase

formation when cured of this plasmid. Additionally, two 1/2a isolates (02-2448 and 06-6934) with unique plasmids also exhibited decreased biofilm formation when cured of their plasmids.

In serotypes other than 1/2a, plasmid-carriage was associated with varied biofilm production. 4 serotype 1/2b (lineage II) isolates were found to have increased biofilm production when carrying plasmids, while 4 appeared to have no effect when compared to plasmid-cured mutants. Examination of WGS data (discussed further in section 3.9) revealed that each of these isolates harbored unique plasmids of varied sizes. 10 of the 14 (71%) serotype 1/2c isolates (lineage II) tested were found to produce weaker biofilms when carrying plasmids, while 2 produced stronger biofilms and 2 appeared to have no effect. WGS data revealed that 6 of these isolates carried pLM5026, 2 (10-1542 and 10-1581) carried a unique plasmid, while the 2 others (07-1873 and 17-1874) carried an additional unique plasmid. Finally, the only plasmid-positive serotype 4b isolate was also found to carry a plasmid that was associated with decreased biofilm formation when lost.

3.9 Whole-genome sequencing revealed the presence of 24 unique plasmids in the panel of isolates

Analysis of WGS data led to the identification of 24 unique plasmid sequences (Table 15). 8 (33%) of these plasmids were present in multiple isolates; pLM5578 was the most common plasmid and was present in 25 isolates. All but 2 of the 24 plasmids were associated with a single serotype. Plasmid pLM0558 was found in 12 isolates from various lineage I and lineage II serotypes, including 1/2a, 1/2b, 1/2c,

Table 15: Unique plasmids identified in this project using plasmid screening and whole-genome sequencing, and summary of their corresponding contribution to biofilm formation.

Plasmid Name	Size (kb)	Sequencing Status	# of Isolates Plasmid Observed	Serotype of Host Strain	Measured Effect on Biofilm Formation	Plasmid-mediated Cd^R	Plasmid-mediated BKC^R
pLM5578	77	Closed	25	1/2a	23 Stronger 1 No effect	+	-
pLM0286	31	1 Contig	1	1/2a	No Effect	-	-
pLM0558	57.5	Closed	12	1/2a, 1/2b, 1/2c, 3a, 3c	Varied[a]	+	-
pLM3046	92	Closed	6	1/2a	Stronger	+	-
pLM2448	86	Closed	1	1/2a	Stronger	+	-
pLM6934	66	2 Contigs	1	1/2a	Stronger	+	-
pLM0813	60	Closed	2	1/2a	Stronger	+	-
pLM0868	42	1 Contig	1	1/2b	No Effect	+	-
pLM0638	31	Closed	1	1/2b	No Effect	+	-
pLM0839	29	Closed	1	1/2b	No Effect	+	-
pLM2572	74	3 Contigs	1	1/2b	Stronger	+	-
pLM3853	160	3 Contigs	1	1/2b	Stronger	+	*-
pLM3697	51	1 Contig	1	1/2b	Stronger	-	-
pLM8809	90	1 Contig	1	1/2b	Weaker	+	+
pLM1633	165	4 Contigs	1	1/2b	Stronger	+	-
pLM1873	58	1 Contig	2	1/2c	Weaker	+	-
pLM1582	66	2 Contigs	2	3a	NA	NA	NA
pLM5026	80	Closed	8	7 1/2c, 1 3c	6 Weaker 2 No effect	-	+
pLM1542	66	4 Contigs	2	1/2c	Weaker	+	+
pLM6746	63	3 Contigs	1	1/2c	Stronger	NA	-
pLM3083	69	Closed	1	4b	Weaker	-	+
pLM0817	81	Closed	1	1/2b	NA	NA	NA
pLM0366	77	Closed	1	1/2a	NA	NA	NA
pLM0876	37	Closed	1	1/2b	NA	NA	NA
Total of 24 Unique Plasmids			75		9 Stronger 4 Weaker 4 No Effect 3 Varied 4 No data available	15 Cd^R	4 BKC^R

* = Screened positive for *bcrBC* genes and had BKC^R phenotype, but resistance cassette was not present on the plasmid

[a] = Results of pLM0558 carriage: 90-0558 (1/2a), no effect; 97-0466 (1/2c), weaker; 97-0468, (1/2c), no effect; 03-5196 (3a), stronger; 98-0163 (3c), no effect

3a, 3c, while pLM5026 was determined to be present in 7 isolates belonging to serotype 1/2c and 1 of serotype 3c.

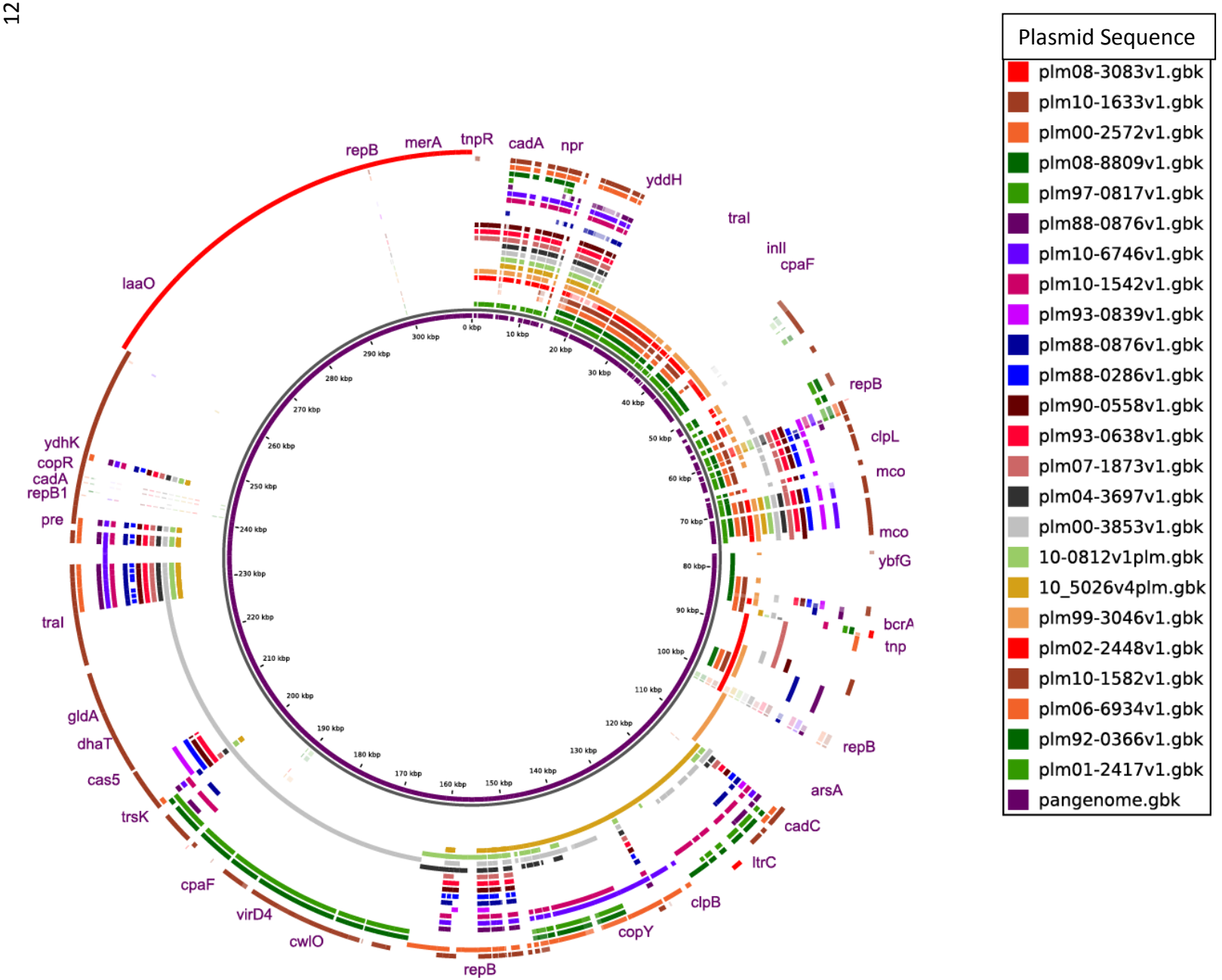
Of the 24 unique plasmids, 15 were associated with increased resistance to cadmium, 4 were found to confer resistance to BKC, and only 2 (pLM8809 and pLM1542) provided increased resistances to both cadmium and BKC. Nine were associated with enhanced biofilm formation, 4 were observed to have no effect, while 4 were associated with decreased biofilm formation. Three of the unique plasmids produced varied results in different strains of *L. monocytogenes*.

3.10 Bioinformatic analyses revealed that the *Listeria* plasmids in this study are chimeric in nature and are composed of many unique genes

Following annotation, plasmid DNA sequences were compared using a variety of bioinformatic programs, including Mauve, Gview, and OrthoMCL. Cumulatively, the 24 plasmids identified in this study (Figure 16) encoded a total of 402 non-redudant putative coding DNA sequences (CDS) with an average length of 857 bp. There were no genes found to be universally present on every plasmid. However, various genetic elements were shared on the majority of plasmids. These shared genes were primarily related to transposition, although they also included heavy metal transport and DNA polymerases.

Alignments of the 24 plasmids (Figure 17) revealed that plasmids are chimeric in nature, and are composed of gene groups that are bordered by elements involved in transposition. While some plasmids share a number of the same transposon gene

Figure 16: Visual representation of all non-redundant CDSs encoded by the 24 unique plasmids. The figure was produced using the GView server (https://server.gview.ca/), and depicts regions of DNA shared between plasmids.


repB
merA
tnpR
cadA
npr
yddH
traI
inlI
cpaF
laaO
repB
clpL
mco
mco
ybfG
bcrA
tnp
repB
arsA
cadC
ltrC
clpB
copY
repB
cwlO
virD4
cpaF
trsK
cas5
dhaT
gldA
traI
pre
repB1
cadA
copR
ydhK
Plasmid Sequence
plm08-3083v1.gbk
plm10-1633v1.gbk
plm00-2572v1.gbk
plm08-8809v1.gbk
plm97-0817v1.gbk
plm88-0876v1.gbk
plm10-6746v1.gbk
plm10-1542v1.gbk
plm93-0839v1.gbk
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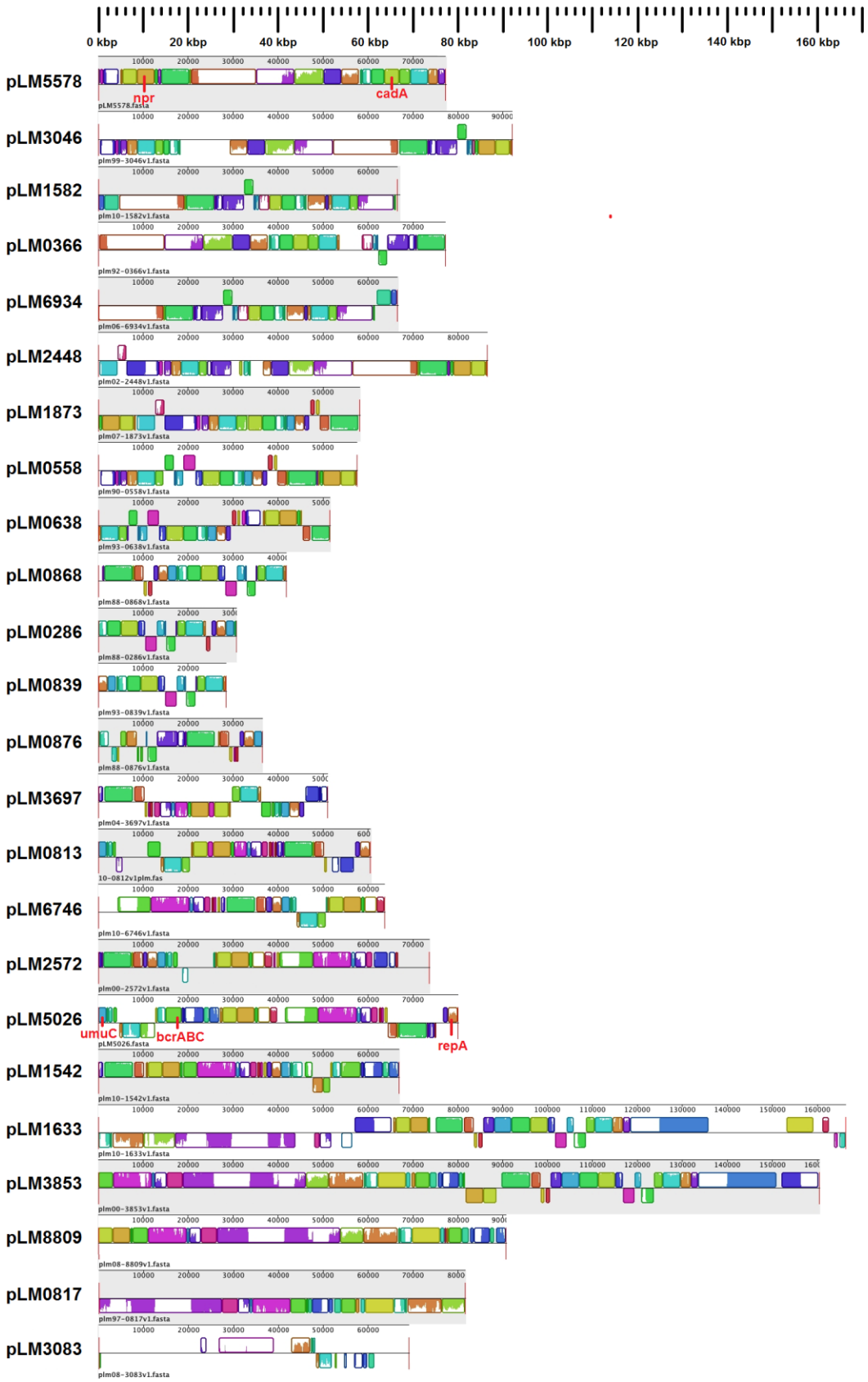
10_5026v4plm.gbk
plm99-3046v1.gbk
plm02-2448v1.gbk
plm10-1582v1.gbk
plm06-6934v1.gbk
plm92-0366v1.gbk
plm01-2417v1.gbk
pangenome.gbk

Figure 17: Alignment of unique plasmids identified in this study created using progressive Mauve alignment tool. “Blocks’ that are the same colour represent shared regions (local collinear blocks, LCBs) between plasmids.


0 kbp
20 kbp
40 kbp
60 kbp
80 kbp
100 kbp
120 kbp
140 kbp
160 kbp
pLM5578
npr
cadA
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pLM1582
pLM0366
pLM6934
pLM2448
pLM1873
pLM0558
pLM0638
pLM0868
pLM0286
pLM0839
pLM0876
pLM3697
pLM0813
pLM6746
pLM2572
pLM5026
umuC
bcrABC
repA
pLM1542
pLM1633
pLM3853
pLM8809
pLM0817
pLM3083

groups, others such as pLM3083 share almost no genes with other plasmids. For example, pLM5578 and pLM5026 share 4 gene regions that are flanked by transpositional elements (Figure 18). The results of comparisons between the shared gene content between plasmids is displayed in Figure 19. This tree is not a phylogeny indicative of the geneology of plasmid sequences, but instead is based on an estimate of shared gene content between plasmids. Colours were used to indicate the serotype of the isolates that harbour the plasmids. While a number of plasmids (pLM5578, pLM3046, pLM6934, pLM0366, and pLM2448) grouped together with others of the same serotype, there was considerable mixing of serotypes within the clades. Further examination of the plasmid DNA sequences within these clades (Figures 20 – 22) revealed that these plasmids are not 100% identical to each other.

Figure 18: Annotated plasmid pLM5026 with regions that are shared with pLM5578 indicated by green blocks. Figure was generated using Gview server and depicts coding sequences encoded by the plasmid.

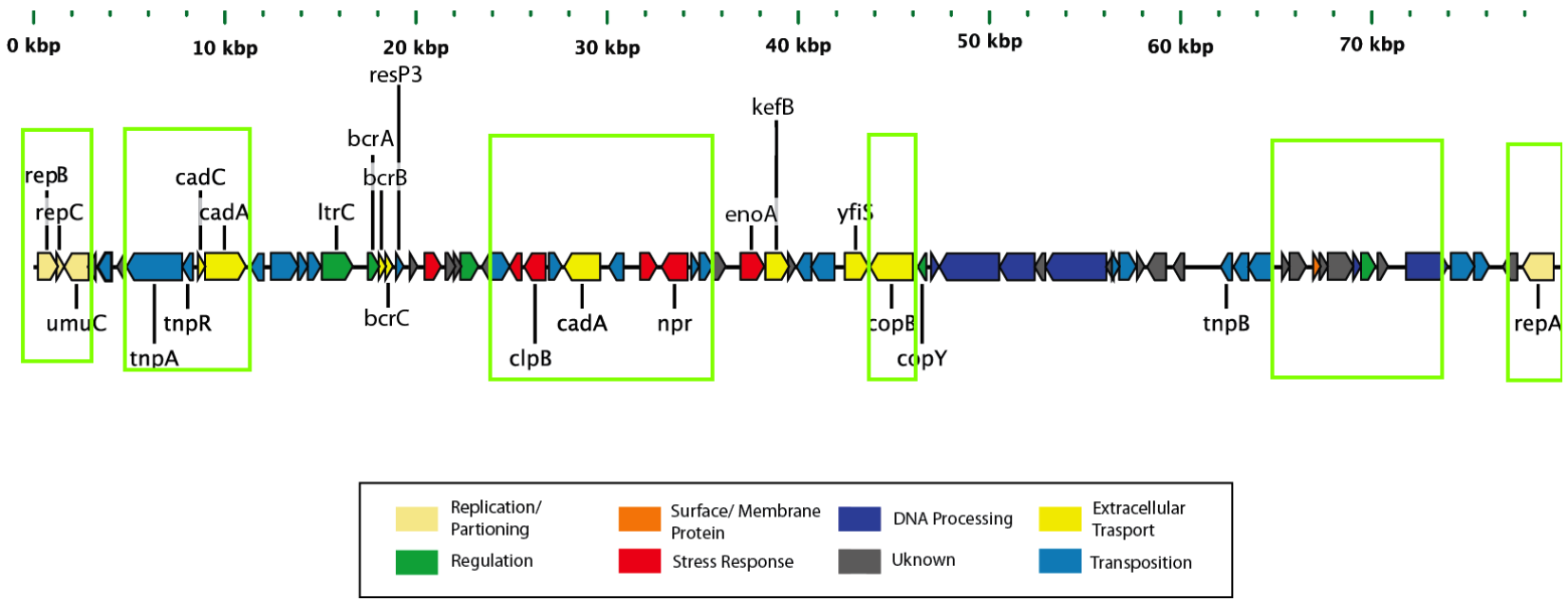

0 kbp
10 kbp
20 kbp
30 kbp
40 kbp
50 kbp
60 kbp
70 kbp
repB
repC
umuC
cadC
cadA
tnpR
tnpA
ltrC
resP3
bcrA
bcrB
bcrC
clpB
cadA
npr
kefB
enoA
yfiS
copB
copY
tnpB
repA
Replication/ Partioning
Regulation
Surface/ Membrane Protein
Stress Response
DNA Processing
Uknown
Extracellular Trasport
Transposition

Figure 19: Relation of plasmids identified in this study based on amounts of shared gene content. Colours indicate the serotype of isolates in which plasmids were carried. Figure was generated using Progressive Mauve's draw tree function.

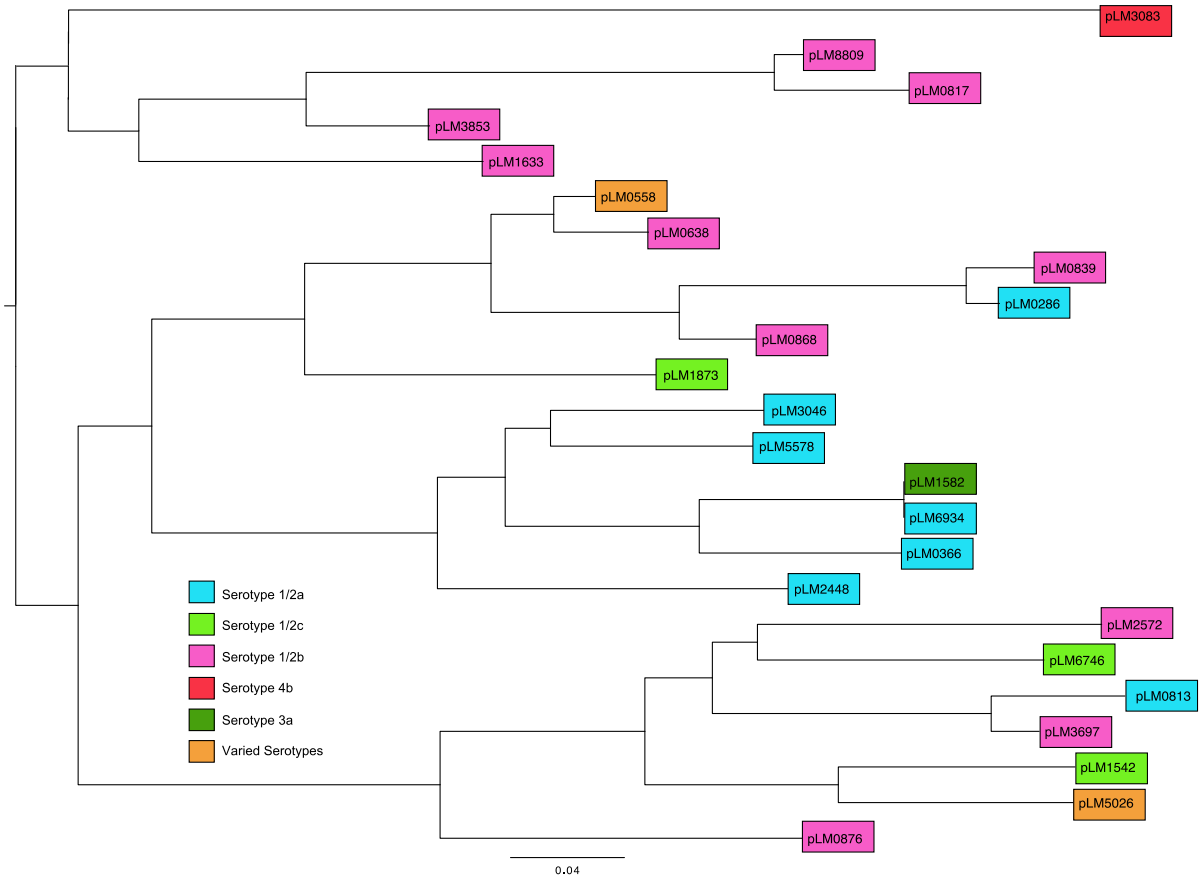

pLM3083
pLM8809
pLM0817
pLM3853
pLM1633
pLM0558
pLM0638
pLM0839
pLM0286
pLM0868
pLM1873
pLM3046
pLM5578
pLM1582
pLM6934
pLM0366
pLM2448
pLM2572
pLM6746
pLM0813
pLM3697
pLM1542
pLM5026
pLM0876
Serotype 1/2a
Serotype 1/2c
Serotype 1/2b
Serotype 4b
Serotype 3a
Varied Serotypes
0.04

Figure 20: BLAST atlas of annotated plasmid DNA sequences from the clade containing pLM5026 in Figure 19, depicting putative genes shared between these plasmids. Figure was generated using the Gview server.

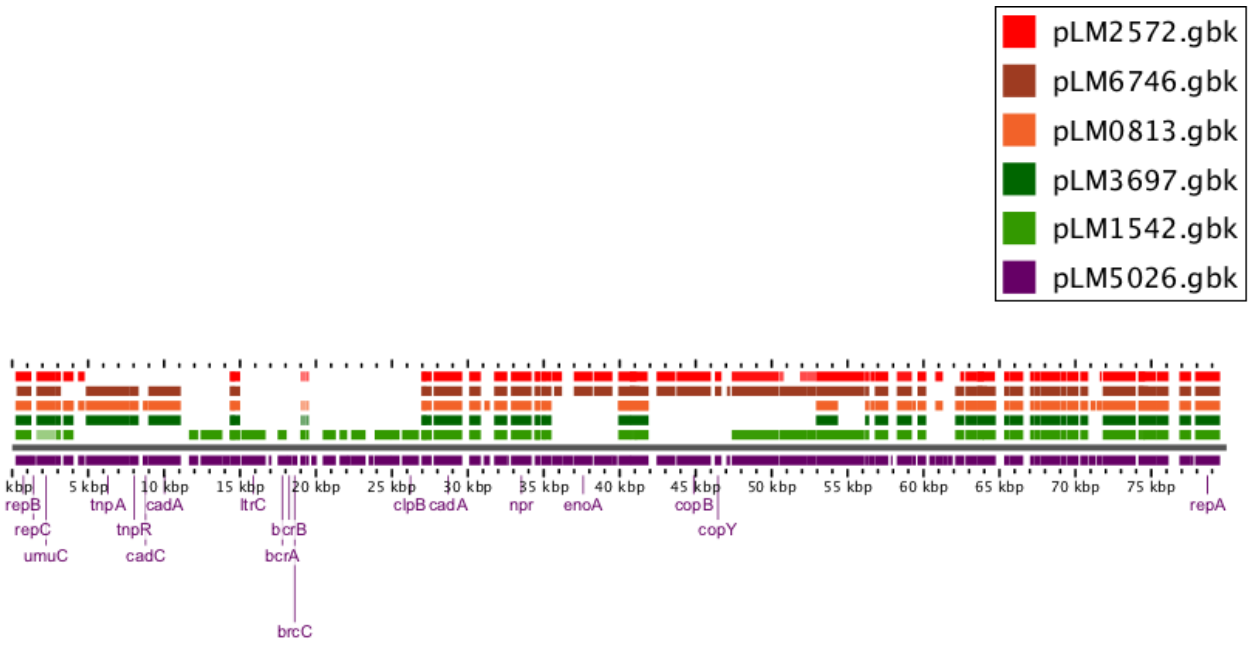

pLM2572.gbk
pLM6746.gbk
pLM0813.gbk
pLM3697.gbk
pLM1542.gbk
pLM5026.gbk
kbp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
50 kbp
55 kbp
60 kbp
65 kbp
70 kbp
75 kbp
repB
repC
umuC
tnpA
tnpR
cadC
cadA
ltrC
bcrB
bcrA
brcC
clpB
cadA
npr
enoA
copB
copY
repA

Figure 21: BLAST atlas of annotated plasmid DNA sequences from the clade containing pLM8809 in Figure 19, depicting putative genes shared between these plasmids. Figure was generated using the Gview server.

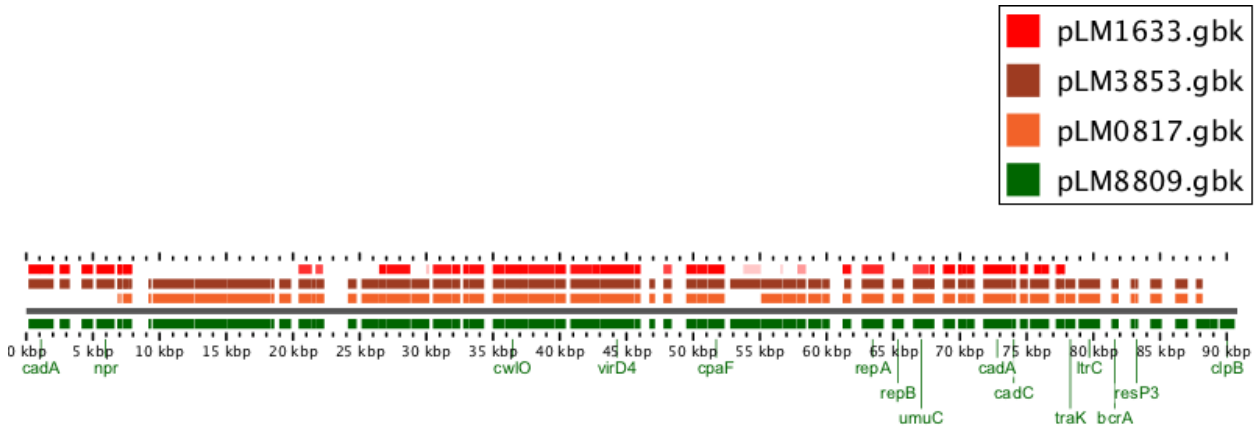
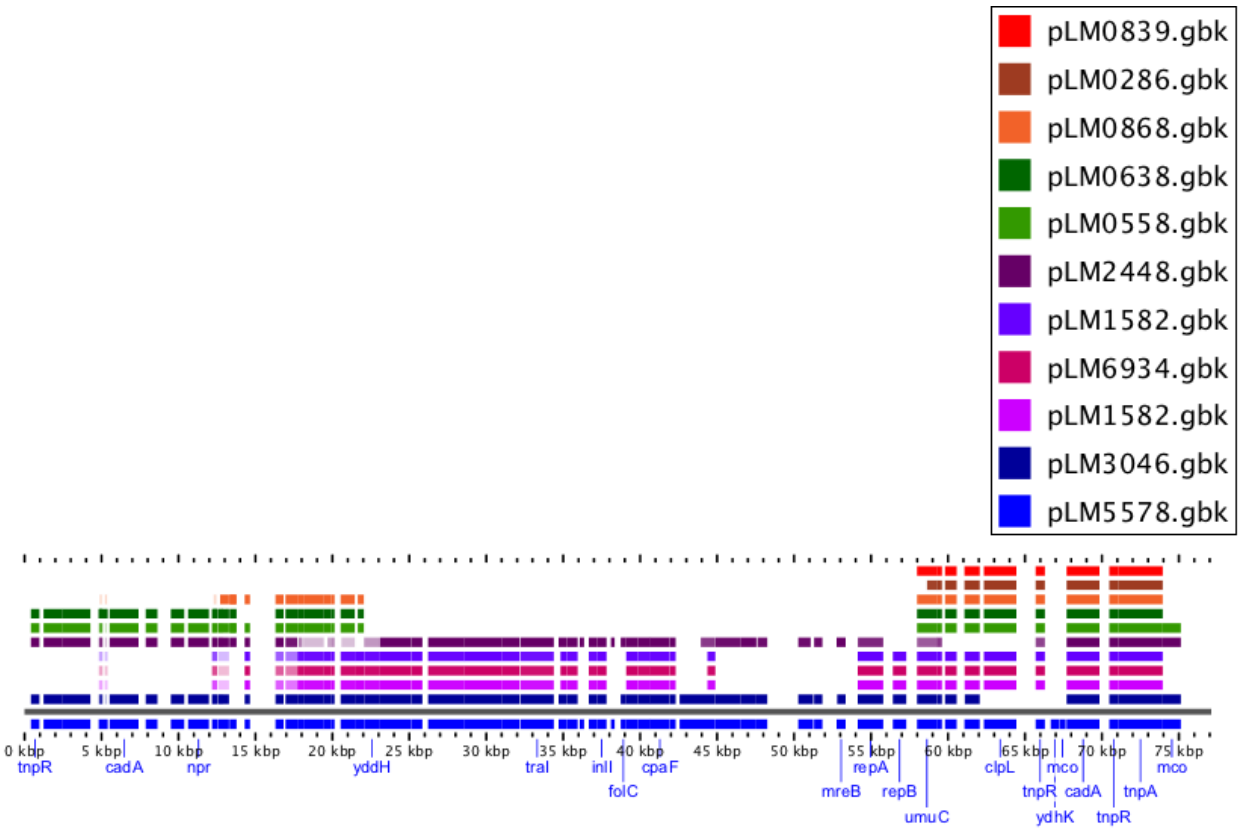

pLM1633.gbk
pLM3853.gbk
pLM0817.gbk
pLM8809.gbk
0 kbp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
50 kbp
55 kbp
60 kbp
65 kbp
70 kbp
75 kbp
80 kbp
85 kbp
90 kbp
cadA
npr
cwlO
virD4
cpaF
repA
repB
umuC
cadA
cadC
ltrC
traK
bcrA
resP3
clpB

Figure 22: BLAST atlas of annotated plasmid DNA sequences from the clade containing pLM5578 in Figure 19, depicting putative genes shared between these plasmids. Figure was generated using the Gview Server.

.


pLM0839.gbk
pLM0286.gbk
pLM0868.gbk
pLM0638.gbk
pLM0558.gbk
pLM2448.gbk
pLM1582.gbk
pLM6934.gbk
pLM1582.gbk
pLM3046.gbk
pLM5578.gbk
0 kbp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
45 kbp
50 kbp
55 kbp
60 kbp
65 kbp
70 kbp
75 kbp
tnpR
cadA
npr
yddH
traI
inlI
folC
cpaF
mreB
repA
repB
umuC
clpL
tnpR
mco
ydhK
cadA
tnpR
tnpA
mco

Chapter 4

Discussion and Conclusions

4. Discussion

4.1 The frequency of plasmid carriage is higher in food and environmental isolates, as well as in those belonging to *L. monocytogenes* lineage II

The role of *L. monocytogenes* as an important foodborne agent has only recently been recognized, and as a result there are considerable gaps in our knowledge of the pathogen. Over the past 30 years only a handful of studies have examined the prevalence of plasmids within *L. monocytogenes* and an even smaller subset have attempted to characterize any of the plasmids subsequently identified (Bertsch et al., 2013; Canchaya et al., 2010; den Bakker et al., 2012; Elhanafi et al., 2010; Gilmour et al., 2010; Hadorn et al., 1993; Kuenne et al., 2010; Lebrun et al., 1994; Margolles & de los Reyes-Gavilan, 1998; Poyart-Salmeron et al., 1990; Romanova et al., 2002).

Studies examining plasmid prevalence in *L. monocytogenes* have varied in their isolation of plasmids: a study of *Listeria* spp. from raw milk in Ontario, Canada, by Fistrovici and Collins-Thompson (1990) did not isolate plasmid DNA from only 19 *L. monocytogenes* isolates, whereas Kolstad et al. (1991) isolated plasmids from 77% of 307 Norwegian *L. monocytogenes* isolates from various clinical, environmental, and food sources. However, it should be noted that comparisons between studies are difficult as there is often considerable variation between the sources and serotypes of isolates examined for plasmid carriage. Other studies reported a range of results: McLauchlin et al. (1997) recovered plasmids from 111/322 (34%) of *L. monocytogenes* isolates from food and clinical sources from the United Kingdom. Peterkin et al. (1992) isolated plasmid DNA from 25/122 (20%) of Canadian clinical, food, environmental and veterinary isolates; Lebrun et al. (1992) observed plasmids in 48/173 (28%) of clinical,

food, and environmental isolates from France; Flamm et al. (1984) identified plasmids in 7/29 (24%) *L. monocytogenes* strains from the United States; Vaz-Velho et al. (2001) isolated extrachromosomal DNA in 20/56 (36%) strains from English and Portuguese fish production lines; and plasmids were found in 26/45 (58%) food-associated isolates from Northern Ireland (Harvey & Gilmour, 2001).

Overall, these studies had an overall average of approximately 35%, while our study found that 57% (75/147) of isolates harboured plasmids. A possible explanation for the higher prevalence of plasmids within our panel is the limited number of serotype 4b isolates in our study. These studies also observed that isolates belonging to serotype 4 carried plasmids less frequently than serotype 1 isolates, as well as that plasmids were overrepresented in food or environmental isolates than those isolated from clinical sources. In this thesis work, a higher incidence of plasmid carriage was observed in food/environmental isolates (70%) compared to clinical isolates (41%), as well as higher frequencies of plasmid carriage in lineage II isolates (55%) than in those belonging to lineage I (34%).

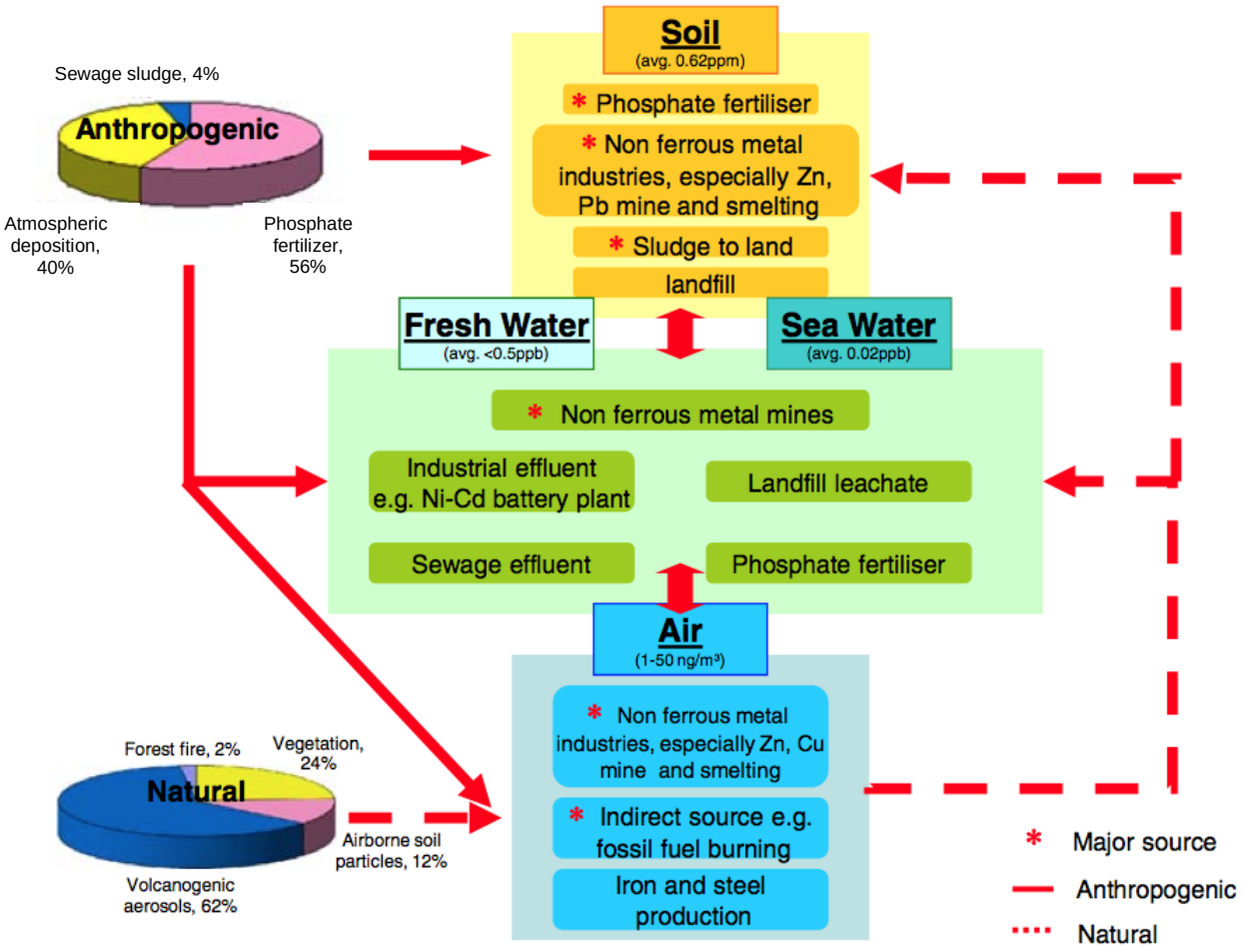
4.2 *L. monocytogenes* plasmids identified in this study contribute to increased resistance to the heavy metal cadmium

Awareness regarding the presence of the heavy metal cadmium within the environment first began in the 1960s in Japan. An outbreak of a painful osteoporosis-like bone disease, termed Itai-itai, was attributed to cadmium pollution of river water and rice fields, traced to waste water from an upstream zinc-lead mine (Murata et al., 1970). Today, it is known that chronic exposure to cadmium can result in osteoporosis, renal dysfunction, leukemia, and various cancers (Satarug et al., 2003). Cadmium

accumulates in the environment from a variety of geogenic (natural) and anthropogenic (industrial) sources (Figure 23). The heavy metal is found at naturally high levels in zinc and lead ores, as well as in phosphate fertilizers (McLaughlin & Singh, 1999). It is estimated that more than 90% of cadmium found in water and soil is due to anthropogenic sources, including rock phosphate fertilizer, waste from ore smelting, ash from fossil-fuel combustion, and sewage sludge (Pan et al., 2010). Of primary concern are the relatively high cadmium concentrations in agricultural soils, and the resulting bioaccumulation of the metal within plants and other organisms.

A number of other studies have examined the prevalence of cadmium resistance in *L. monocytogenes*, although not all attempted to determine if plasmids were involved in providing resistance. There have been considerable discrepancies between cutoffs denoting susceptible and resistant strains of *L. monocytogenes* throughout the literature, making comparisons of cadmium resistance considerably more difficult. Lebrun et al. (1992) found that 47/90 (52%) *L. monocytogenes* isolates were resistant to cadmium (>32 µg/mL); extrachromosomal DNA was detected in 41/47 (87%) of the resistant isolates. McLauchlin et al. (1997) investigation of clinical and food isolates from the UK found that 326/565 (58%) were resistant to cadmium (>75 µg/mL). A subset of these isolates was tested for the presence of plasmid DNA, and 66/210 (45%) of resistant isolates were found to carry plasmids. However, there was considerable discrepancies between serogroups 1/2 and 4, as 87/120 (72%) resistant isolates from serogroup 1/2 carried plasmids, whereas only 9/90 (10%) of serogroup 4 contained plasmid DNA. Vaz-Velho et al. (2001) tested plasmid-positive *L. monocytogenes*

Figure 23: The pathway of cadmium contamination from both natural and anthropogenic sources. Figure reproduced directly from (Pan et al., 2010)


Soil
(avg. 0.62ppm)
* Phosphate fertiliser
* Non ferrous metal industries, especially Zn, Pb mine and smelting
* Sludge to land
landfill
Sewage sludge, 4%
Anthropogenic
Atmospheric deposition, 40%
Phosphate fertilizer, 56%
Fresh Water
(avg. <0.5ppb)
Sea Water
(avg. 0.02ppb)
* Non ferrous metal mines
Industrial effluent e.g. Ni-Cd battery plant
Landfill leachate
Sewage effluent
Phosphate fertiliser
Air
(1-50 ng/m³)
* Non ferrous metal industries, especially Zn, Cu mine and smelting
* Indirect source e.g. fossil fuel burning
Iron and steel production
Forest fire, 2%
Vegetation, 24%
Natural
Airborne soil particles, 12%
Volcanogenic aerosols, 62%
* Major source
Anthropogenic
Natural

isolates from fish production facilities, and found that 19/20 plasmid-harbouring strains were cadmium resistant (>75 µg/mL). Finally, Harvey & Gilmour (2001) found that 25/45 (66%) *L. monocytogenes* isolates from milk and other food samples exhibited cadmium resistance to concentrations greater than 64 µg/mL. They further investigated isolates with MICs greater than 128 µg/mL, and found that all serogroup 1 strains that contained plasmid DNA were resistant to cadmium, whereas only 3 of 17 resistant strains did not contain plasmid DNA.

In this thesis work, 40/57 (70%) of wild-type isolates were resistant to cadmium with MICs over 64 µg/mL. However, comparisons between wild-type strains and their corresponding plasmid-negative strains revealed that plasmids conferred increased resitance to cadmium in 42/57 (74%) of strains. A further 9 isolates had reduced susceptibility to cadmium, but exhibited similar levels between wild-type and corresponding plasmid-cured strains, indicating that these strains carry resistance determinants that are encoded on the bacterial chromosome. Overall, it was determind that plasmid loss was associated with at least a partial loss of cadmium resistance in 15/19 (79%) of unique plasmids examined in this study, inferring that plasmids conferred the cadmium resistance phenotypes. Examination of plasmid coding sequences obtained through WGS revealed the presence of putative cadmium resistance genes, further supporting this inference.

Cadmium resistance in *L. monocytogenes* has been attributed to the presence of three cadmium resistance determinants: *cadA1*, which is encoded on the plasmid-borne transposon *Tn5422* (Lebrun et al., 1994); *cadA2*, harbored by large plasmids such as pLM80 (Nelson et al., 2004); and *cadA3* which is associated with an integrative

conjugative element on the chromosome of *L. monocytogenes* EGDe (Glaser et al., 2001). Annotation of plasmid sequences revealed the presence of both *cadA1* and *cadA2* on multiple plasmids identified from our panel. It is likely that chromosome-borne *cadA3* confers resistance to cadmium that cannot not be attributed to plasmid-carriage. As a saprophyte, *L. monocytogenes* is frequently isolated from soil and water sources, often in agricultural environments. Due to the frequent cadmium contamination in these environments, it is likely that *L. monocytogenes* encounters the heavy metal and this has necessitated the acquisition of mechanisms to prevent cellular death due to the fatal accumulation of cadmium.

4.3 Plasmid-encoded benzalkonium chloride resistance is attributed to the *bcrABC* cassette

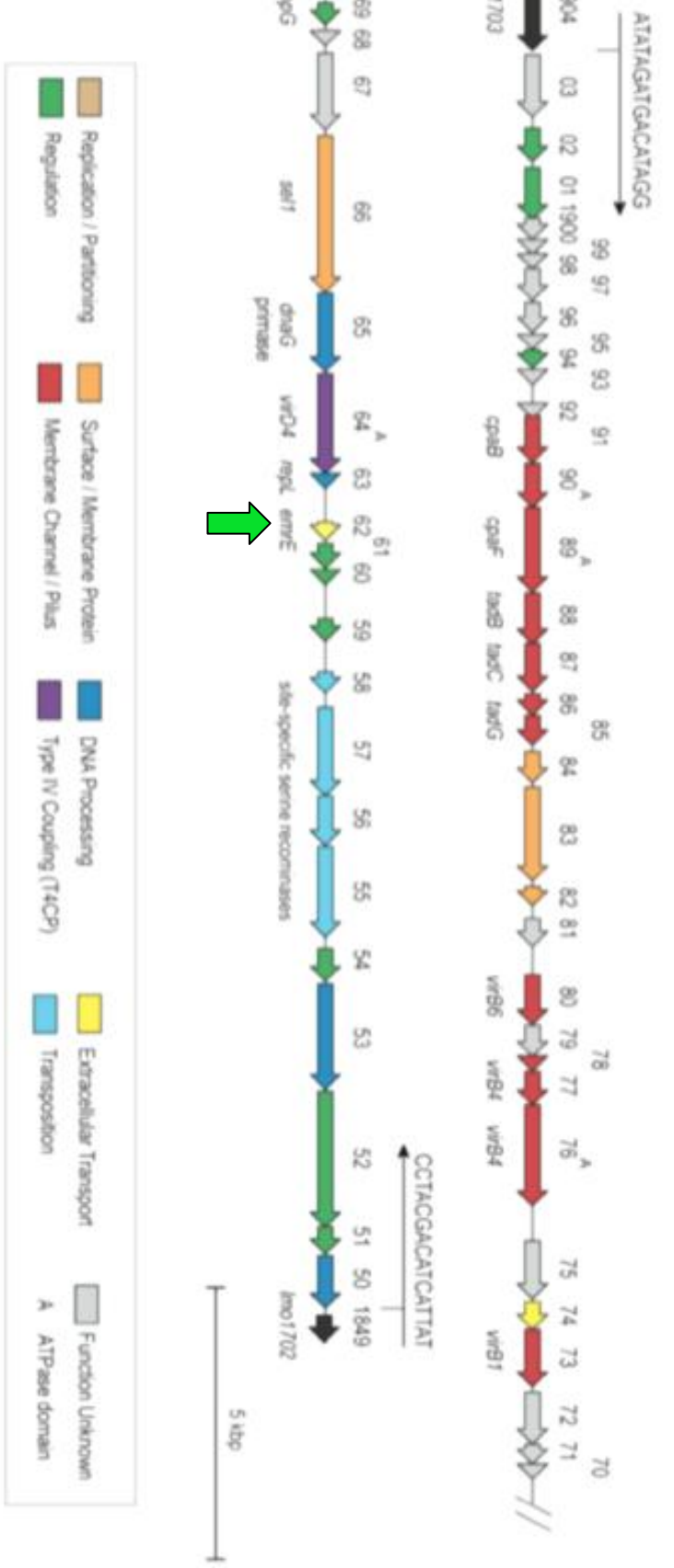
Resistance to chemical sanitizers commonly used in food-processing environments has been investigated for its role in the persistence of *L. monocytogenes*. QACs are often one of the primary components in these sanitizers, which are used extensively not only in food processing, but also in retail and household settings. BKC is the most common QAC in use today, and as a result is one of the most studied in terms of resistance to this class of sanitizers (Buffet-Bataillon et al., 2012). While there have been a number of studies that have focused on resistance to BKC in *L. monocytogenes*, only a few have explored the relationship between plasmids and BKC resistance. It should be noted that the terms “resistant” and “susceptible” are not well defined in regards to QACs, with *L. monocytogenes* breakpoints often arbitrarily defined by different research groups (Soumet et al., 2005).

BKC resistance in *L. monocytogenes* isolated from food and the food processing environment has been found to range from as low as 10% (Aase et al., 2000) to as high as 61% (Dutta et al., 2013). While BKC resistance in *L. monocytogenes* is not yet fully understood, resistance has been attributed to both chromosomal (Dutta et al., 2013; Earnshaw & Lawrence, 1998; Romanova et al., 2002; Romanova et al., 2006; Soumet et al., 2005; To et al., 2002) and plasmid-associated mechanisms (Elhanafi et al., 2010; Gilmour et al., 2010; Lemaître et al., 1998; Romanova et al., 2002; Romanova et al., 2006).

At least part of the resistance to BKC has been determined to be due to the presence of MDR efflux pumps, which are systems that often convey resistance to a range of structurally different dyes, surfactants, and antibiotics (Paulsen et al., 1996). Gilmour et al. (2010) discovered the presence of a 49.8 kbp genomic island, denoted LGI1 (Figure 24), that was present on the chromosome of the outbreak-associated serotype 1/2a *L. monocytogenes* isolate 08-5578. Investigation into LGI1 determined that presence of the island was associated with a 4-fold increased tolerance to the QAC compounds BKC and benzalthonium chloride (Ziegler, 2011). Annotation of the DNA sequence of LGI1 revealed the presence of the SMR gene *emrE* (Figure 24), which was upregulated in the presence of BKC along with 13 other LGI1 genes.

Elhanafi et al. (2010) recently elucidated a plasmid-associated BKC resistance mechanism involving MDR efflux pumps. This BKC resistance cassette, termed *bcrABC*, was identified on an *IS1216* transposon found on the *L. monocytogenes* plasmid pLM80 (Figure 25). Elhanafi et al. (2010) determined that the *bcrABC* cassette conferred BKC resistance (>20 μg/mL) when transferred to susceptible strains. This

Figure 24: The genetic and structural organization of the Listeria Genomic Island 1 and its predicted functions. The *emrE* gene is indicated by the green arrow. Figure adapted from (Gilmour et al., 2010).


ATATAGATGACATAGG
CCTACGACATCATTAT
5 kbp
Replication / Partitioning
Surface / Membrane Protein
DNA Processing
Extracellular Transport
Function Unknown
Regulation
Membrane Channel / Pilus
Type IV Coupling (T4CP)
Transposition
A ATPase domain

same gene cassette was found in all BKC-resistant isolates to which resistance was attributed to plasmid carriage. When examined along with WGS data, we determined that four newly identified plasmids, pLM5026, pLM8809, pLM1542, and pLM3083, carried the *bcrABC* resistance cassette.

4.4 Plasmids encode genes with a wide range of putative functions, including efflux and stress responses

Annotation and analyses of the 24 plasmids identified revealed considerable variation in gene content. Gene content analysis determined a total of 402 unique putative genes, for which none were on found every plasmid. Some plasmids shared a considerable number of genes with one another, such as pLM5578, pLM0366, pLM6934, and pLM3046, while others such as pLM3083, shared almost no genetic content with other plasmids. All plasmids contained a large number of genes related to transposition; 22/64 (34%) and 19/52 (37%) of the coding sequences on pLM5026 and pLM0813 were putatively identified as relating to transposition, respectively. Many of these transposons contain genes required for self-mobilization, essentially creating genetic elements capable of moving within DNA sequences (Toleman & Walsh, 2011). Many of these plasmid-borne transposons also carry additional genes, forming groups of identical genes that are found on multiple plasmids. These conserved groups of genes make up structural 'blocks' that make up the many different plasmids found within *L. monocytogenes*. Canchaya et al. (2010) found that the full sequence of pLM33, a 33 kb plasmid commonly isolated from *L. monocytogenes*, is contained within other larger plasmids. It is possible that *L. monocytogenes* plasmids are made up of mobilizable

units ranging from a few kb containing a handful of genes to large structural blocks that are made up of entire plasmids.

The prevailing hypothesis is that all species of *Listeria* evolved from a common ancestor that contained the main virulence genes found in *L. monocytogenes*. Divergence occurred primarily through evolutionary reduction, resulting in attenuation of some species through gene loss (den Bakker et al., 2010; Hain et al., 2006; Ragon et al., 2008; Schmid et al., 2005). The mobile nature of *L. monocytogenes* plasmids and their constituent transposon structural units make reconstruction of their phylogenetic history exceedingly difficult. It is possible that all *Listeria* plasmids may be traced to a common ancestor plasmid, and countless events of acquisition and loss of transposon units over time led to the wide range of plasmids seen today. Evolution of *L. monocytogenes* plasmids is further complicated by HGT through conjugation. Conjugation involving *L. monocytogenes* plasmids has been poorly characterized, and little is known regarding the mechanisms responsible for plasmid transfer between *Listeria*. Conjugative ability of plasmids from this study was not investigated, and annotation did not identify any coding sequences known to be associated with conjugation machinery. However, a number of studies have shown that *Listeria* plasmids are capable of transfer between *Listeria* spp. and other Gram-positive and Gram-negative bacteria (Bertsch et al., 2013; Poyart-Salmeron et al., 1990). Further complicating evolutionary studies was the distinct lack of SNPs between identical plasmids isolated from different isolates. For example, pLM5578 was identified in 25 different isolates that were isolated from years spanning 1995 to 2010, but no SNPs were detected in any of the pLM5578 plasmid sequences. Nearly all the plasmids that

we identified encoded a putative translesion repair polymerase V, an enzyme that is normally involved in ultra-violet light-induced DNA repair. If this putative enzyme is responsible for replication of these low-copy number plasmids, it would be expected that this pro-mutagenic enzyme would be likely to make a number of errors during replication (Kuenne et al., 2010).

Antibiotic resistance in *L. monocytogenes* is rarely observed, but some studies have observed limited plasmid-encoded antibiotic resistance (Bertsch et al., 2013; Poyart-Salmeron et al., 1990). The first known MDR *L. monocytogenes* strain was attributed to a plasmid harboured by an isolate from France in 1988. A 37 kb plasmid, pIP811, that encoded resistance to chloramphenicol, erythromycin, streptomycin, and tetracycline was isolated and was determined to be self-transferable to other *L. monocytogenes* strains as well as to *Streptococcus* spp. and *S. aureus* (Poyart-Salmeron et al., 1990). Recently, a 7.6 kb MDR plasmid was isolated from an *L. innocua* isolate, and was found to be capable of replication in both Gram-positive and Gram-negative bacteria (Bertsch et al., 2013).

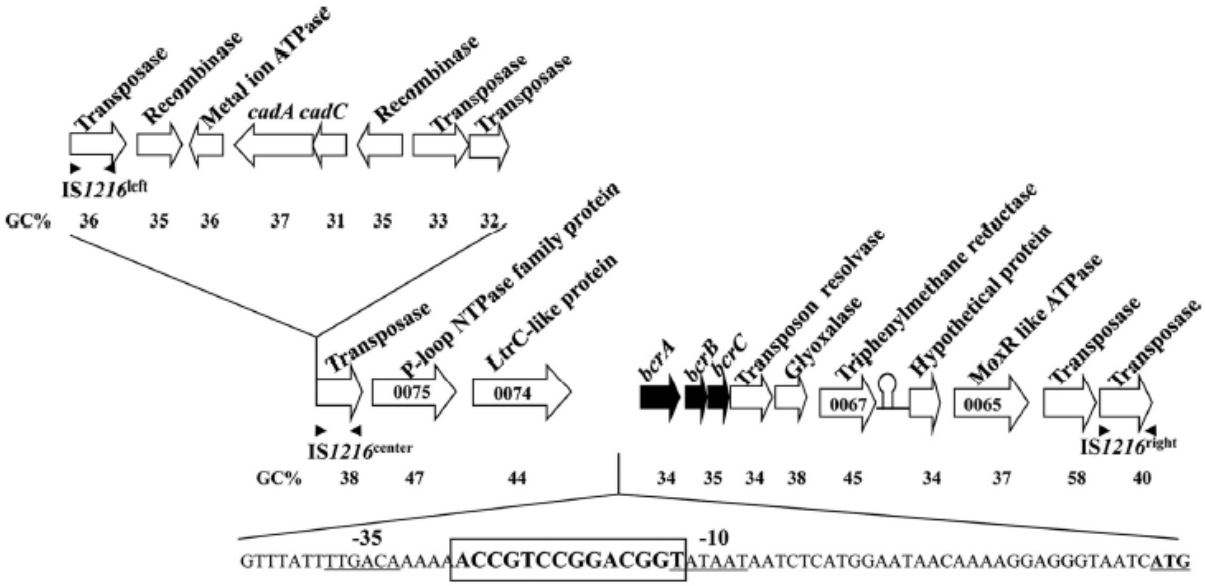
Another plasmid of considerable importance was recently identified in a lineage IV serotype 4a *L. monocytogenes* strain isolated from a goat (den Bakker et al., 2012). This plasmid, termed pLMIV, is unique in that while it does not carry genes related to resistance to antibiotics or heavy metals; instead it was found to carry genes belonging to the internalin family. Internalin genes are involved in cellular invasion in humans and other animal hosts, and pLMIV, which also contains putative conjugative genes, is the first potential virulence plasmid found in *L. monocytogenes* (den Bakker et al., 2012).

No antibiotic resistance determinants were found on any of the 24 plasmids characterized in this study, nor were any coding sequences directly related to virulence within *L. monocytogenes*. However, a number of genes putatively related to survival of various environmental stresses were encountered frequently during plasmid annotations. These included putative genes such as: pLM5026_35 (CDS location bp 31729 - 32598) an ABC transporter involved in surviving osmotic stress; *clpB*, a chaperone protein involved in heat-shock responses; *npr*, which encodes an NADH peroxidase related to surviving oxidative stress; and *ltrC*, encoding a protein that regulates growth at low temperatures. These genes were present on multiple plasmids identified in this study, and require further analysis to determine how these elements contribute to *L. monocytogenes's* ability to survive and persist in a wide range of environments.

4.5 Plasmid carriage in *L. monocytogenes* is associated with varied levels of biofilm production

L. monocytogenes is a pathogen that excels at survival in a wide range of environments. Due to the ubiquitous nature of the bacterium entry into food processing facilities is all but inevitable, and once established it is nearly impossible to eradicate (Tompkin, 2002). Studies have shown that *L. monocytogenes* is able to adhere to and form biofilms on materials that are commonly found in food industry facilities, including stainless steel, aluminum, rubber, and polyvinyl chloride (PVC) (Beresford et al., 2001). *L. monocytogenes* has also been isolated from a wide range of equipment and environments within these facilities, including knives, tables, drains, floors, walls, gaskets, conveyor belts, and various equipment (Destro et al., 1996; Holah & Gibson,

Figure 25: The benzalkonium chloride resistance cassettee *bcrABC* found on the 80 kb plasmid pLM80 in *L. monocytogenes*. Figure reproduced directly from (Elhanafi et al., 2010).


Transposase
Recombinase
Metal ion ATPase
cadA cadC
Recombinase
Transposase
Transposase
IS1216left
GC% 36 35 36 37 31 35 33 32
Transposase
P-loop NTPase family protein
LtrC-like protein
0075
0074
IS1216center
GC% 38 47 44
bcrA
bcrB
bcrC
Transposon resolvase
Glyoxalase
Triphenylmethane reductase
Hypothetical protein
MoxR like ATPase
0067
0065
Transposase
Transposase
IS1216right
34 35 34 38 45 34 37 58 40
-35
-10
GTTTATTTTGACAAAAAACCGTCCGGACGGTATAATAATCTCATGGAATAACAAAAGGAGGGTAATCATG

2000; Klausner & Donnelly, 1994; Nelson, 1990; Salvat et al., 1995; Wenger et al., 1990).

Persistence of *L. monocytogenes* within food processing environments increases the risk of contamination of food products. The ability to form biofilms is considered to be a key component of persistence in these environments for *L. monocytogenes*. Bacteria within biofilms are considerably more difficult to eliminate from surfaces compared to free-living planktonic cells. This has been attributed to the resistance of biofilms to physical cleaning methods (Holah & Gibson, 2000; Wirtanen et al., 2000) and increased resistance to antimicrobial compounds, due to decreased penetration of antibacterial agents and physical means through the biofilm matrix, and via altered growth and gene expression of listerial cells within the biofilm (Donlan, 2002; Gandhi & Chikindas, 2007; Robbins et al., 2005). Increased levels of resistance to common sanitizers, including BKC, as a result of *L. monocytogenes* residing in biofilms has been documented in a number of studies (Ibusquiza et al., 2011; Pan et al., 2006; Yang et al., 2009).

The exact mechanisms of biofilm formation by *L. monocytogenes* have not yet been fully elucidated, but existing evidence indicates that it is influenced by a considerable number of factors. These include the serotype and the genetic composition of the given strain, as well as environmental conditions such as pH, oxygen availability, availability of nutrients, hydrodynamics, osmolarity, and temperature (Goller & Romeo, 2008; Møretrø & Langsrud, 2004). A recent study found that temperature is one of the most important factors influencing biofilm production, with biofilm production decreasing with decreased temperature (Kadam et al., 2013). In the present study, similar results were observed with strongest biofilm production occurring at 30°C and

the weakest at 4°C. Various studies have reached contradictory conclusions regarding the relationships between serotypes and the nutrient content of growth medium on biofilm formation. Serotypes 1/2a, 1/2b, 1/2c, and 4b have exhibited considerable variation in biofilm production when grown in either nutrient rich or nutrient poor growth media (summarized by Kadam et al. 2013). Our laboratory has found that overall biofilm production was increased when MWB, a nutrient-poor minimal growth medium, was used for biofilm assays (Ziegler, 2011).

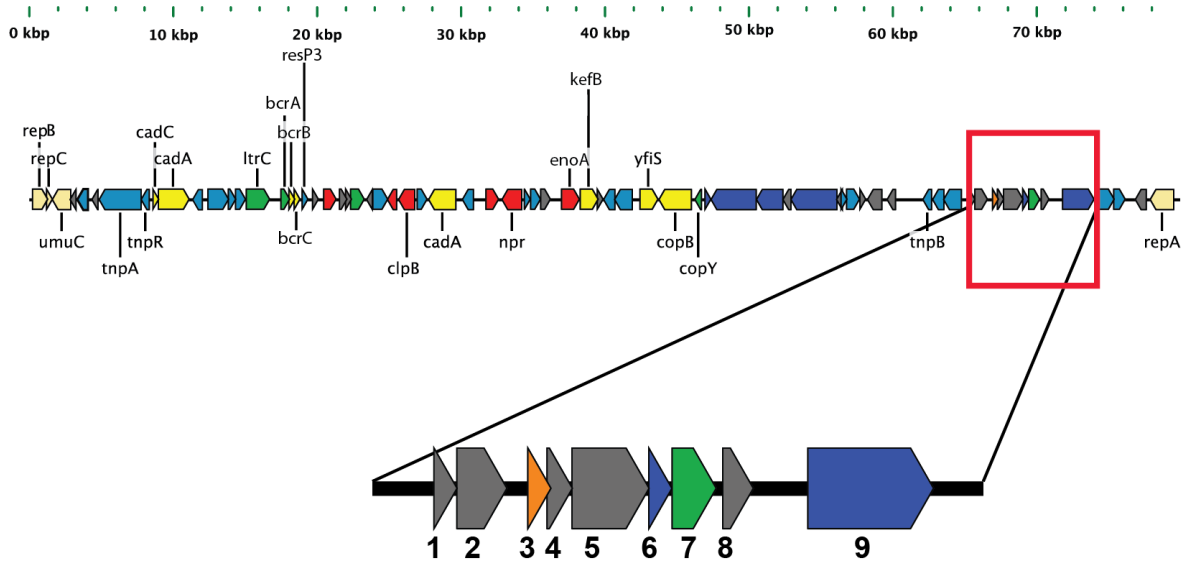
To date and to the best of our knowledge, there has been no exploration into how plasmids may be involved in biofilm formation in *L. monocytogenes*. Bacterial cells living within biofilms are in close proximity to one another, and as such biofilms are considered hotspots for the exchange of genetic materials, including plasmids (Ghigo, 2001). A number of studies in Gram-negative bacteria have shown that conjugative plasmids are capable of directly inducing biofilm formation through various mechanisms. The TOL plasmid has been shown to enhance biofilm formation through the increase of extracellular DNA content in *Pseudomonas putida* by increasing production of extracellular DNA (D'Alvise et al., 2010). In *E. coli*, the 42 kbp plasmid pMAS2027 was shown to promote biofilm formation through plasmid-encoded type 3 adhesion fimbriae (Ong et al., 2009). Finally, Ghigo observed that natural conjugative *E. coli* plasmids expressed pili capable of inducing planktonic cells to form biofilms (Ghigo, 2001).

This thesis work revealed that biofilm formation in *L. monocytogenes* was affected by the presence of wild-type plasmids. Plasmid-carriage was associated with greater biofilm formation in 61% of wild-type isolates. Examination of plasmid DNA sequences from these isolates revealed the presence of identical plasmids within numerous strains. Twenty-four strains belonging to serotype 1/2a were found to carry pLM5578, and of

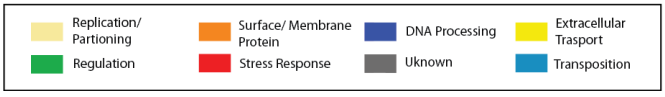
these 23 were associated with greater biofilm-forming capacity than their corresponding plasmid-cured strains. Nine additional unique plasmids were found to be associated with elevated biofilm production compared to their corresponding plasmid-cured strains, 4 of which were carried by serotype 1/2a isolates, 4 by serotype 1/2b, and one by 1/2c. Plasmid carriage by serotype 1/2a isolates was not found to be associated with decreased biofilm formation. Alternatively, plasmid carriage was associated with weaker biofilm formation in 21% of wild-type isolates. WGS revealed that these isolates contained 5 unique plasmids, 2 of which (pLM0558 and pLM5026) were found in multiple serotypes and were associated with varied effects on biofilm production.

To our knowledge, this is the first study in which plasmid-carriage has been found to affect biofilm formation in Gram-positive bacteria. Annotation of plasmid sequences did not reveal any coding sequences that have been shown to be involved in biofilms. However, there were numerous hypothetical proteins, as well as putative efflux pumps and regulatory proteins that have yet to be characterized. Plasmid alignments found an 8.5 kbp region (Figure 26) containing a number of hypothetical proteins, a putative DNA methylase, and a putative regulatory gene that is shared by all the plasmids for which carriage was associated with enhanced biofilm formation. However, this region was also shared by 3 of the 5 plasmids that were associated with diminished biofilm production. It may be that this region encodes a regulatory factor that affects chromosomally-encoded genes involved in biofilm formation, or some form of signaling molecule that has varied effects within different serotypes or strains. Studies have found that cell-to-cell signaling plays a role in biofilm formation in *L. monocytogenes* (Rieu et al., 2007; Sela et al., 2006), and genes related to quorum-sensing systems have previously been found on plasmids (Penalver et al., 2006; Piper et al., 1993).

Figure 26: The 8.5 kb region shared by numerous wild-type plasmids that affect biofilm formation in *L. monocytogenes*. The shared region is highlighted by the red box on pLM5026.



1 - Conserved hypothetical protein
2 - Conserved hypothetical protein
3 - Regulation; related to *fic* family
4 - Conserved hypothetical protein
5 - Conserved hypothetical protein
6 - Homology to DNA binding proteins
7 - Unknown transcriptional activator
8 - Conserved hypothetical protein
9 - Putative adenine-specific DNA methylase



Alternatively, it is possible that there are different genetic elements on different plasmids that each are involved in biofilm formation. Further investigation is required to determine how plasmid carriage is affecting biofilm formation in *L. monocytogenes*. The construction of plasmids with gene deletions would serve to determine if specific plasmid regions or genes were directly responsible. Furthermore, it is worth investigating if carriage of a single plasmid would yield similar results if carried by a strain of a different serotype. However, repeated attempts to insert various plasmids into strains of differing serotypes using a considerable number of electroporation protocols proved intractable. While the 6.9 kb shuttle vector plasmid, pKSV7, was successfully inserted using a number of electroporation protocols, the larger wild-type plasmids failed to be taken up by recipient cells. Electroporation experiments were further complicated by the lack of any antibiotic resistance determinants on the wild-type plasmids, necessitating the use of naturally occurring BKC or cadmium resistance for selection purposes.

5. Conclusions

Plasmids were found with high frequency within *L. monocytogenes*, and isolates from food and food-associated environments had higher levels of plasmid carriage than those from other sources. Twenty-four newly identified plasmids were detected within our panel of 147*L. monocytogenes* isolates. Determinants for resistance to cadmium were identified on 15 of these plasmids, while the BKC resistance cassette *bcrABC* was present on 4 plasmids. Plasmid carriage was also found to affect biofilm formation at 30ºC; 9 plasmids were associated with enhanced biofilm formation, while carriage of 5 plasmids was associated with diminished biofilm formation. No genes were identified within these plasmid sequences to which the biofilm phenotype could be attributed. However, there were a considerable number of putative genes for which the functions remain unknown. Annotation of the plasmid DNA sequences also revealed the presence of additional genes putatively involved in surviving environmental stresses. Further investigation is required, but the data from this study takes an important step to lay the groundwork in order to fully elucidate how plasmid-related biofilm formation, BKC resistance, and other uncharacterized coding sequences may contribute to persistence of *L. monocytogenes* within food processing environments, and subsequent contamination of foods leading to listeriosis outbreaks.

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