The Distribution, Diversity and Functional Characterization of the *Listeria* Genomic Island 1

by

Jennifer Ziegler

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Abstract

Listeria monocytogenes was the causative agent of the nationwide 2008 outbreak associated with contaminated ready-to-eat meat products. Within the whole genome DNA sequences of the outbreak isolates we previously identified a novel 50kb genomic island, designated as *Listeria* Genomic Island 1 (LGI1). LGI1 is predicted to contribute to *Listeria* pathogenesis and/or environmental persistence because it encodes genes related to known virulence factors and mobilization functions, including a putative type IV secretion system and a putative small multidrug resistance efflux pump. The distribution of LGI1 in Canadian *L. monocytogenes* isolates was determined by PCR screening for LGI1 within 126 isolates from 1987 to 2010 that represented different serotypes and pulsed-field gel electrophoresis (PFGE) patterns. To assess the evolutionary history and genetic diversity of this island, total LGI1 sequences from 15 whole-genome sequences were compared, and from the full study panel of isolates, PCR screening for the chromosomal insertion site and multiple LGI coding sequences were performed. LGI1 was detected almost exclusively in serotype 1/2a isolates, and within those, the isolates predominantly had the same PFGE patterns. These LGI1-encoding isolates also exclusively belonged to the multi-locus sequence typing (MLST) clonal complex 8. LGI1 was highly genetically conserved and it was inserted at the same location within the genome in 65 of the 67 isolates that harboured the island. To study the function and expression of LGI1, antimicrobial susceptibility assays, bioinformatic analyses and real-time reverse-transcription PCR were used. Isolates encoding LGI1 had an increased tolerance to quaternary ammonium compounds commonly used in sanitizing agents (benzalkonium chloride (BCl) and benzethonium chloride (BeCl)) compared to isolates lacking LGI1 (but still highly related by MLST and PFGE). LGI1 is also tightly regulated, with expression of 13 of 16 tested coding

sequences only being induced by the presence of sub-inhibitory concentrations of BCl, and one predicted regulator being expressed under all conditions. This study indicates that the vast majority LGI encoding CC8 isolates share a common progenitor *L. monocytogenes* ancestor that acquired LGI1 in a single evolutionary event. LGI1 has remained genetically conserved since that time, and the functions contributed by this island minimally include an increased tolerance to sanitizer agents.

Foreward

A portion of this research was included in the manuscript “Human listeriosis cases across Canada from 1988-2010 were caused by a novel epidemic clone of *Listeria monocytogenes”* that was co-authored by Stephen J. Knabel, Aleisha Reimer, Bindhu Verghese, Mei Lok, Jennifer Ziegler, Jeffrey Farber, Franco Pagotto, Morag Graham, Celine A. Nadon, the Canadian Public Health Laboratory Network and Matthew W. Gilmour. This manuscript was submitted to the Journal of Clinical Microbiology in October 2011.

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List of Abbreviations

AMP	Ampicillin
ATP	Adenosine triphosphate
BCl	Benzalkonium chloride
BeCl	Benzethonium chloride
BHI	Brain Heart Infusion
BHIA	Brain Heart Infusion agar
CBAB	Columbia blood agar base
CDC	Centers for Disease Control and Prevention, Atlanta
cDNA	Complementary DNA
CDS	Coding sequence
CFU	Colony forming unit
CIHR	Canadian Institutes of Health Research
ddH_2O	Double distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
INDEL	Insertion/deletion event
LB	Luria Bertani
LGI1	*Listeria* Genomic Island 1
LPI1	*Listeria* Pathogenicity Island 1
MH	Mueller-Hinton
MLST	Multi-locus sequence typing
mM	Millimolar
NESP	National Enterics Surveillance Program

NML	National Microbiology Laboratory, Public Health Agency of Canada
ORF	Open reading frame
PCR	Polymerase chain reaction
PHAC	Public Health Agency of Canada
PFGE	Pulsed-field gel electrophoresis
QAC	Quaternary ammonium compound
qRT-PCR	Quantitative real-time PCR
RPM	Revolution per minute
RNA	Ribonucleic acid
RT-PCR	Real-time PCR
SMR	Small multidrug resistance protein
T2SS	Type II secretion system
T4CP	Type IV coupling protein
T4SLS	Type IV secretion-like system
T4SS	Type IV secretion system
TE	Tris-EDTA
µL	Microlitre
µM	Micromolar

Chapter 1

Introduction

1. Introduction

1.1 Emerging infectious disease

Throughout history, infectious diseases have influenced population densities, the fate of wars and battles, population migration, colonization, scientific and medical discoveries, as well as the flow of genes from one generation to the next (Morens et al., 2004; Morse, 1995). One notable example of the influence of infectious disease on human kind is the Black Plague that devastated Europe between the 13th and 15th centuries, killing between 30-60% of Europe's population during this time (Zietz & Dunkelburg, 2004; Echenberg, 2002). However, medical and scientific advancements during the last twenty years of the 19th century revolutionized medical beliefs and our way of life (Drews, 2000; Hewitt, 1967). By the 1950s, the widespread use of antibiotics and the development of polio vaccines left many experts with the notion that infectious diseases would soon be eradicated (Andrews & Langmuir, 1963; Molina & Puffer, 1955).

Despite such optimism, nearly half a century later, over a quarter of the total annual deaths worldwide are directly attributable to infectious diseases (Lopez et al., 2001). Although we may have developed sufficient scientific knowledge and effective mechanisms to control the predominant infectious diseases of the past, infections that have newly appeared in a population, or that have existed previously but are rapidly increasing in incidence and/or geographic range continue to emerge (Morens et al., 2004). Additionally, infectious diseases that were once controlled by modern medicine are re-emerging due to evolutionary acquisitions such as antibiotic resistance or new genetic features that result in increased virulence, infection of new body sites or even allow broadening of the host species that can be infected. Many social and economic factors also contribute to the emergence of a disease and include the lack of access to health care, population growth and changes in demographics, changes in human

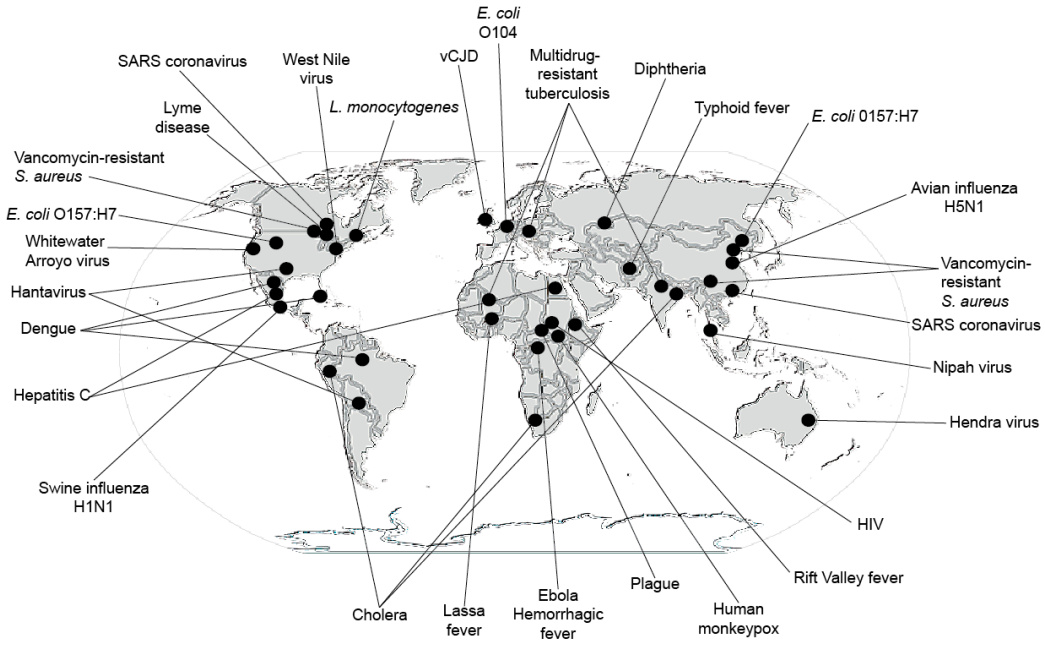
behaviors, misuse of antimicrobial drugs, microbial adaptation, urbanization, global trade and modern travel (Endy et al., 2011). Most importantly, changes in human behavior and population growth significantly impact the mechanisms by which diseases emerge. However the social and economic factors that lead to the emergence of the pathogens vary with each geographical region. Thus, emerging pathogens affect different regions of the world with varying degrees (Figure 1).

1.2 Foodborne diseases

1.2.1 The burden of foodborne diseases

Like other infectious diseases, foodborne diseases have been causing illness in humans for centuries. Since the Middle Ages, municipal authorities and medical professionals have recognized the relationship between food and health and regulators dealing with food products have enforced sanitation regulations in order to protect consumers (Nicoud, 2008). It is difficult to estimate the true burden of foodborne disease because most cases of diarrhea are self-limiting and most cases cannot definitively be linked to a contaminated food source therefore public health data is largely under-reported (Scallan et al., 2011). But, diarrheal diseases in general are one of the leading causes of morbidity and mortality in developing countries causing an estimated 2 billion cases and nearly 2 million mortalities globally every year (WHO, 2008). In the developed world, an estimated one in four individuals experiences a significant foodborne illness each year, however the mortality rates are much lower due to access to health care systems and treatment (Tauxe, 2002). In the United States alone, foodborne illness causes approximately 9.4 million illnesses, 56,000 hospitalizations, and 1,400 deaths each year (Scallan et al., 2011). In Canada, it is estimated that approximately 70% of the population suffers from acute gastroenteritis

Figure 1: **The global range and prevalence of current emerging infectious diseases (Adapted from Fauci, 2001)**


E. coli O104
SARS coronavirus
West Nile virus
vCJD
Multidrug-resistant tuberculosis
Diphtheria
Lyme disease
L. monocytogenes
Typhoid fever
E. coli 0157:H7
Vancomycin-resistant *S. aureus*
Avian influenza H5N1
E. coli O157:H7
Whitewater Arroyo virus
Vancomycin-resistant *S. aureus*
Hantavirus
SARS coronavirus
Dengue
Nipah virus
Hepatitis C
Hendra virus
Swine influenza H1N1
HIV
Rift Valley fever
Plague
Cholera
Lassa fever
Ebola Hemorrhagic fever
Human monkeypox

annually, costing the nation approximately $120 per capita per year (Henson et al., 2008; Ruzante et al., 2011; Sargeant et al., 2008; Thomas et al., 2006).

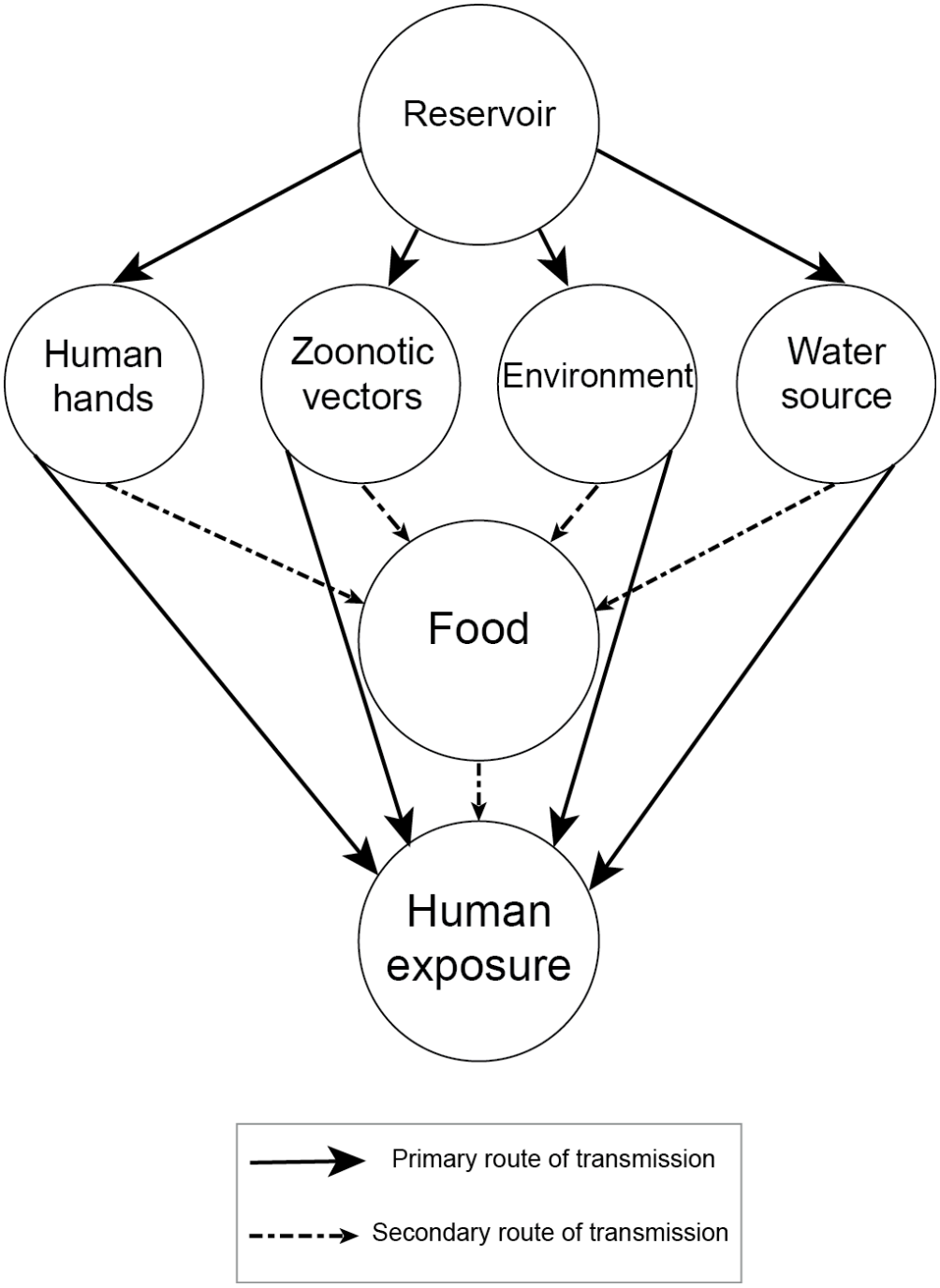
1.2.2 Etiology and transmission

The etiology of foodborne illness varies worldwide with common causes including infection by pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp or *Vibrio cholera*; viruses such as Rotavirus or Hepatitis A virus; or parasitic organisms such as cryptosporidium, cryptospora or trematodes. These pathogens are transmitted via the fecal-oral route, through consumption of contaminated food or water, via contact with contaminated fomites such as flies and cooking utensils, or they can be transmitted person-to-person or animal-to-person due to poor hygiene practices and contaminated hands (Curtis et al., 2000) (Figure 2). Some pathogens such as *Shigella* spp. require the human host as part of their life cycle, whereas other pathogens have primary reservoirs in other animals or the environment. The primary mechanisms of transmission are those in which the pathogen is consumed directly from contaminated water, fomites or hands. The secondary mechanisms of transmission occur when the pathogen progresses into the environment and is then transferred to objects such as food that subsequently becomes contaminated. Upon ingestion, the microbe invades the gastrointestinal system, eliciting the body to secrete large volumes of liquid. The host not only becomes highly susceptible to dehydration, but loss of nutrients can also occur, resulting in malnourishment and sometimes death (Curtis, Cairncross et al., 2000; Fewtrell et al., 2005).

1.2.3 Emerging foodborne pathogens

The spectrum of foodborne pathogens too, like other infectious diseases, has changed over time as a result of social, economic and behavioral factors. For

Figure 2: The primary and secondary routes of transmission of foodborne illness.


Reservoir
Human hands
Zoonotic vectors
Environment
Water source
Food
Human exposure
Primary route of transmission
Secondary route of transmission

pathogens that are well understood, eliminating or reducing the behaviors that cause a disease controls the spread of the pathogens, thereby reducing the prevalence of the pathogen. For example, trichinosis, which was once a common parasitic foodborne illness, was nearly eradicated in the 1970s when the custom of feeding pigs uncooked garbage was stopped because it was determined to be the source of the infection (Bailey & Schantz, 1990). New foodborne pathogens emerge either via mutation or by moving into a new niche within the food chain that enables infection (Käferstein et al., 1997; Tauxe, 2002). *Escherichia coli* O157:H7 is an example of an emerging foodborne pathogen that arose due to the acquisition of virulence factors, including a pathogenicity island that encodes factors for cellular adherence and a prophage that encodes the Shiga cytotoxins, via horizontal gene transfer (Perna et al., 2001). It was identified for the first time in 1979 and has subsequently caused illness and deaths due to its presence in ground beef, vegetables, and drinking-water in several countries, including the large waterborne outbreak in Walkerton, Ontario. *Salmonella Typhimurium* DT104, another emerging foodborne pathogen has developed resistance to five commonly prescribed antibiotics and is now a major concern in many countries (Briggs & Fratamico, 1999). More recently, a newly identified strain of enteroaggregative *E. coli* O104:H4 that acquired the Shiga toxin genes caused a large outbreak in Europe that included over 3,000 confirmed cases of illness (Mellmann et al., 2011). Consequently, an intricate balance exists between the ecologies within the food chain that support bacterial populations, cultural habits and technologies that help to limit the occurrence of particular pathogen within the foodborne illness transmission chain (Altekruse et al., 1997). Thus, as technologies and human behaviours change over time (including those related to food production and distribution), pathogens will continue to emerge. Currently, the most common foodborne pathogens in Canada include nontyphoidal

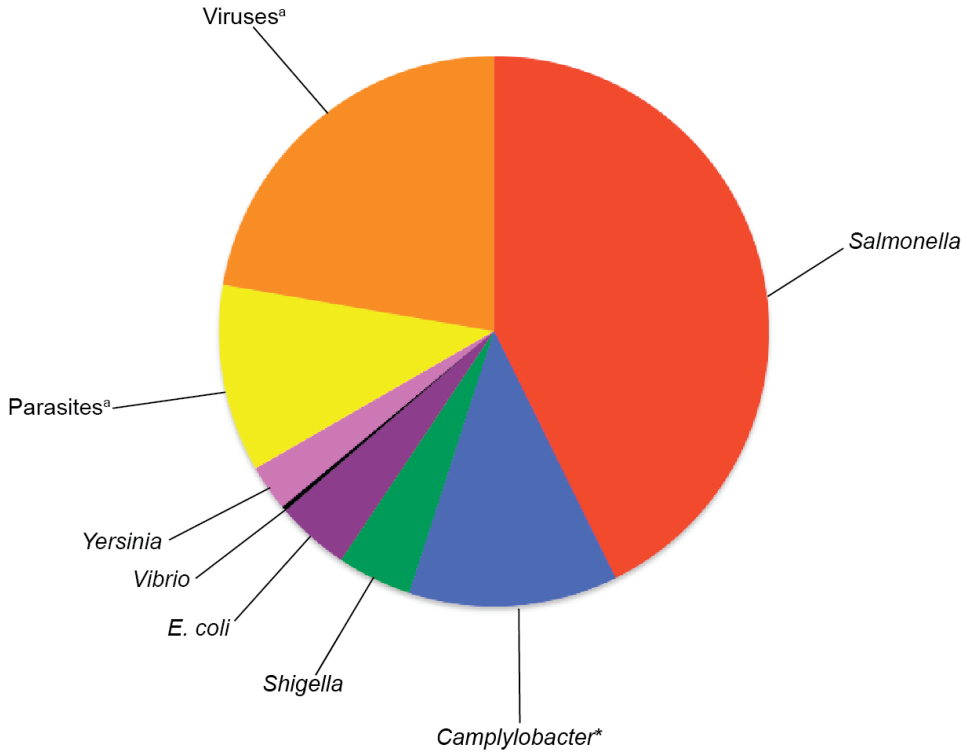
Salmonella subspecies I, *Campylobacter* species, and pathogenic viruses such as Rotavirus, Norovirus and Adenovirus (Figure 3) (NESP, 2009).

1.3 *Listeria* species

The genus *Listeria* was named after the pioneer of sterile surgery Joseph Lister, and compromises of a group of ubiquitous Gram-positive bacteria with a low GC-content that is closely related to the genera *Bacillus, Clostridium, Enterococcus* and *Staphylococcus* (Collins et al., 1991; Sallen et al., 1996). *Listeria* spp. are characterized as facultative anaerobic, non-sporulating and non-encapsulated rods. They are also motile at temperatures between 10°C and 25°C, with their motility being strongly suppressed at 37°C. *Listeria* have been isolated from a wide range of sources including soil, water, plants, feces, meat, seafood, dairy products, food processing facilities, symptomatic and asymptomatic domestic and wild animals as well as symptomatic and asymptomatic humans (Cox et al., 1989; Weis & Seeliger, 1975). However, the natural environment of *Listeria* spp. is soil and decaying plant matter. *Listeria* spp. can grow at a wide range of temperatures from less than 0°C up to 45°C, although optimal growth occurs between 30°C and 37°C. *Listeria* can also grow at salt concentrations up to 10% NaCl and in pH ranges of 5.2 to 9 (Cole et al., 1990; Sorrells & Enigl, 1990). *Listeria* spp. are susceptible to a wide range of antimicrobial agents, such as the tetracyclines, penicillins, macrolides, chloramphenicol and rifampin, but they are also intrinsically resistant to others such as nalidixic acid (Troxler et al., 2000). Current guidelines recommend penicillin combined with gentamycin for a synergistic effect as the drug of choice for treating *Listeria* infections (Irving et al., 2006).

There are currently eight recognized species within the genus and include *L. monocytogenes*, *L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi, L. marthii*

Figure 3: The frequency of the 6 most prevalent enteric pathogens from humans in Canada from 2007 to 2009 (NESP, 2009). At this time, NESP did not complete national laboratory surveillance of *L. monocytogenes*, but approximately 200 cases of invasive listeriosis infections are recorded each year in Canada.



* - Although *Campylobacter* is the most commonly reported enteric organism in Canada, isolates are not routinely forwarded to provincial or central reference laboratories and are therefore greatly under-represented in the NESP.

a - Parasitic (*Giardia*, *Cryptosporidium*, *Entamoeba Histolytica/Dispar* and *Cyclospora*) and viral infections (Norovirus, Rotavirus and Adenovirus) are not routinely reported to the provincial or central reference laboratories and are greatly under-represented in the NESP.

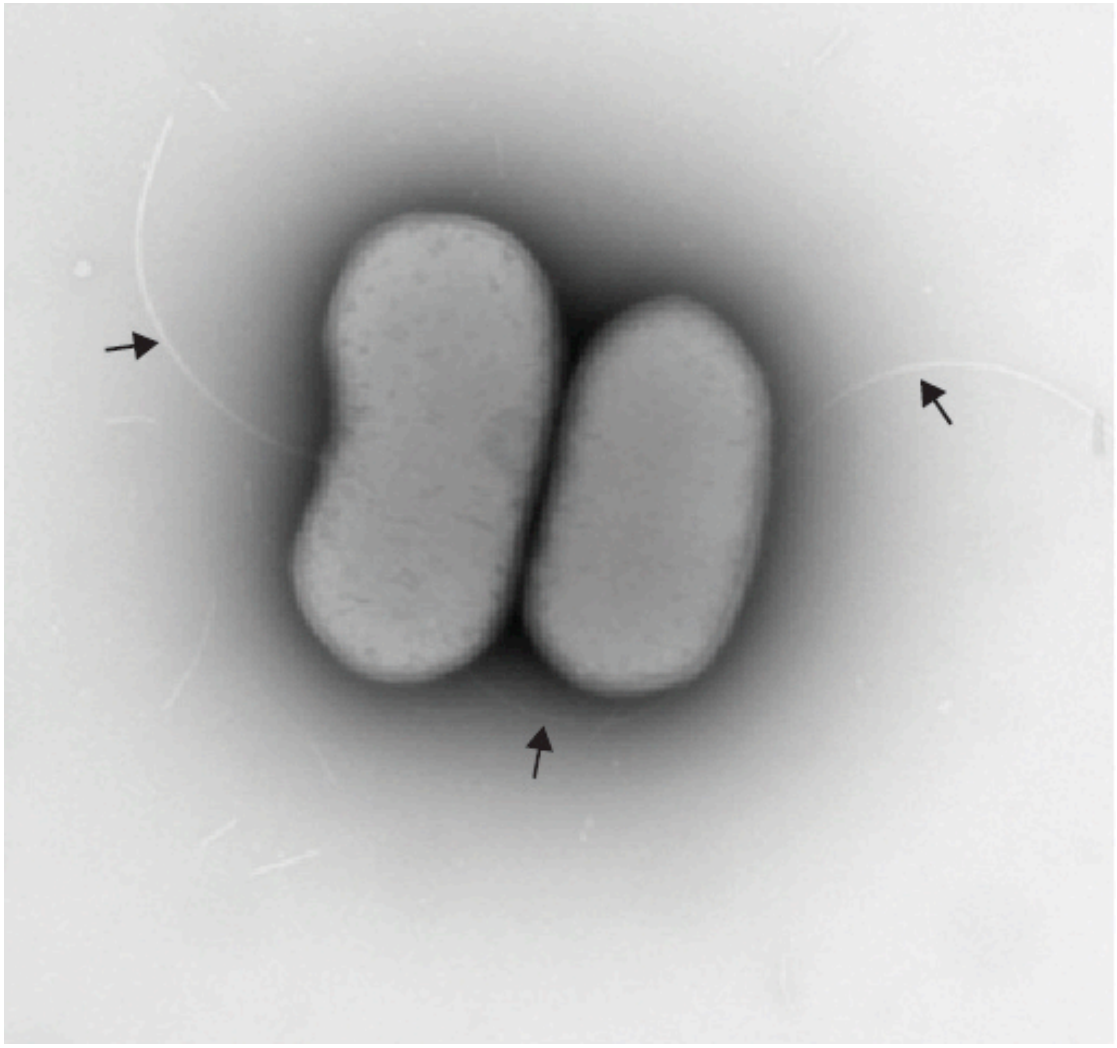
and *L. rocourtiae* (den Bakker et al., 2010). *L. monocytogenes* and *L. ivanovii* are the only pathogens of warm-blooded animals within this genus. *L. monocytogenes* causes a serious foodborne disease in humans and other warm-blooded animals, whereas *L. ivanovii* predominantly causes infections in ruminant animals with human infection being rare (Lessing, Curtis, & Bowler, 1994). Human infections caused by *L. seeligeri* have been rarely documented (Gilot & Content, 2002). *L. innocua* is physiologically similar to *L. monocytogenes* and is frequently isolated from food products, however this species lacks *L. monocytogenes* virulence genes, thus it can serve as an indicator for possible food contamination with the pathogenic *L. monocytogenes* (Buchrieser et al., 2003).

1.4 Listeria monocytogenes

1.4.1 *L. monocytogenes* infection

L. monocytogenes (Figure 4) is an important Gram-positive bacterial pathogen that was first described in 1926 by E.G.D Murray as the causative agent of monocytosis in laboratory rabbits (Murray, Webb, & Swann, 1926). However, the first definitive human cases of listeriosis were not documented until 1929 when *L. monocytogenes* was isolated from the blood cultures of patients with mononucleosis, and in 1936 when it was identified as a cause of sepsis in infants and meningitis in adults (Gray & Killinger, 1966). Despite its identification early in the 20th century, human listeriosis attracted limited attention and remained an infrequent cause of human disease. It was not until the 1980s that *L. monocytogenes* was recognized as being a foodborne pathogen when an outbreak of listeriosis in Halifax, Nova Scotia involving 41 cases and 18 mortalities, who were mostly pregnant women and neonates, and were linked to the consumption of contaminated cabbage that was treated with sheep manure (Schlech et al., 1983). During the next ten years, additional foodborne listeriosis outbreaks occurred in North America and Europe that were linked to contaminated milk,

Figure 4: A transmission electron microscope image of two *L. monocytogenes* 08-5578 cells after growth in BHI broth at 37°C with shaking. Flagella (marked with arrows) are observed on the sides of the bacterial cells. This image was taken during the course of this study.



soft cheese and pate (Fleming et al., 1985; Linnan et al., 1988; McLauchlin et al., 1991). Subsequently, there was a rise in the global incidence rate of listeriosis.

It is now recognized that listeriosis can cause a severe and sometimes life-threatening disease in humans that manifests as a mild self-limiting gastroenteritis to meningitis, encephalitis, septicemia and/or spontaneous abortion of the fetus in pregnant women. Subclinical infections can also occur, but these are rarely documented. Those at the most risk for listeriosis include the elderly, immunocompromised individuals, pregnant women and their fetuses. *L. monocytogenes* is primarily transmitted via the consumption of contaminated food products; however infection can also be transmitted directly from the environment, infected animals or by vertical transmission from the mother to the neonate. Infections can be readily treated with antibiotics such as the penicillins, yet recent estimates have ranked listeriosis as one of the top causes of death from foodborne diseases, with an average case mortality rate of 20-30% (Behravesh et al., 2011; Swaminathan & Gerner-Smidt, 2007). In comparison, the case-mortality rate is approximately 10% for *Vibrio*, 1% for Shiga toxin-producing *E.coli* O157, and less than 1% for *Salmonella*, *Campylobacter* and *Shigella* (Behravesh et al., 2011; Scallan et al., 2011).

Since the 1980s when *L. monocytogenes* was identified as a foodborne pathogen, the annual incidence rate of listeriosis has been increasing in developed countries. In the US, there was a reduction in listeriosis cases between 1996 and 2002, however the incidence of listeriosis has since been increasing (CDC, 2003; Gillespie et al., 2006). The member states of the European Union also reported the highest number of annual cases in 8 years in 2006, as well as an increasing and statistically significant trend for many of the member states during the recent years (Denny & McLauchlin,

2008). Similarly, in Canada, from 2000-2004, there was a steady increase from 2.3 cases per million in 2000 to 3.0 cases per million in 2004 (Clark et al., 2010). These increases in incidence rates may be attributable to behavioural and social changes in developed countries including the increasing prevalence of ready-to-eat foods that do not require further heating before consumption. Accordingly, certain countries including France actively reduce *L. monocytogenes* exposure in at-risk groups such as pregnant women and the elderly by providing specific guidelines on which high-risk foods to avoid.

However, despite our frequent encounters with this pathogen in the environment and food products, *L. monocytogenes* rarely causes human infection. In fact, many ready-to-eat food products in Canada have a safe allowable limit of *L. monocytogenes* that may be present in the food products, as with the ubiquitous nature of this organism, it would not be possible to recall all foods that contain even a trace amount of *L. monocytogenes*. Therefore, we likely consume *L. monocytogenes* on a semi-regular basis without contracting disease symptoms. Additionally, up to 5% of the population may be asymptomatic carriers of *L. monocytogenes,* and up to 25% of contacts of symptomatic patients may also be infected with the pathogen without exhibiting clinical symptoms (Bartt, 2000). This is because *L. monocytogenes*, unlike other foodborne pathogens such as *Vibrio cholera*, does not have an overt virulence factor such as a cytotoxin. Rather, a combination of strain-specific virulence factors, environmental persistence mechanisms and/or host considerations such as immunodeficiency may need to occur for *L. monocytogenes* to cause human disease. More specifically, factors such as the ability to reproduce at a wide range of temperatures and pH (0.4°C to 45°C, pH 4 to 9.6), the tolerance to sanitizers and the ability to form biofilms may allow this pathogen to persist in food or food-processing environments (Cole et al., 1990). This

provides the pathogen with an increased opportunity to be consumed by a susceptible host, wherein the additional virulence factors will further dictate the course of disease.

1.4.2 Survival mechanisms

1.4.2.1 Adverse environmental conditions

In order to cause infection, *L. monocytogenes* present on food products must overcome many adverse environmental conditions that are used to preserve the foods, such as refrigeration, high salinity and/or exposure to sanitizers. These stress responses are primarily regulated by the alternative sigma factor σ^b. Refrigeration is a commonly used mechanism to extend the shelf life of foods, and *L. monocytogenes* is able to survive and replicate at temperatures as low as 0.4°C. It does so by changing the cell membrane fatty acid composition to maintain membrane fluidity, by expressing cold shock proteins that may have a role in degrading abnormal or damaged polypeptides that occur at low temperatures and by synthesizing cryoprotectants such as glycine betaine (Angelidis & Smith, 2003; 1997; Bayles, Annous, & Wilkinson, 1996). Upon exposure to mild acidic conditions, the *L. monocytogenes* acid tolerance response enables the bacteria to translocate excess protons across its cell membrane and to turn on the glutamate decarboxylase system which also results in the expulsion of protons to the extracellular space (Cotter, Gahan, & Hill, 2001; Shabala et al., 2002) Lastly, osmoadaptation to products of high osmolarity is achieved via the synthesis of osmoprotectants such as glycine betaine that serve to increase the osmolarity of the bacterial cell, disabling water loss from the cell (Bayles & Wilkinson, 2000).

1.4.2.2 Biofilms

When present in environments with low nutrient availability, *L. monocytogenes* can transition from planktonic cells to highly organized microbial communities attached to a surface known as biofilms. A polysaccharide matrix that protects the community

from unfavourable environmental stresses such as sanitizers, disinfectants and other antimicrobial agents encompasses biofilms (Lewis, 2001; Mah & O'Toole, 2001; Pan, Breidt, & Kathariou, 2006; Robbins et al., 2005). *L. monocytogenes* is able to form biofilms in food processing environments in such areas as food handing surfaces, conveyer belts and stainless steel equipment. Elimination of mature biofilms can be challenging because of the unique niches in which they can form, and because they are more resistant to sanitization methods than planktonic cells (Midelet & Carpentier, 2002). Biofilms can persist for many years, and in fact, one clone of *L. monocytogenes* persisted in a single food processing plant for more than 12 years between 1988 and 2000 (Orsi et al., 2008). Biofilms are particularly concerning because they enable the bacteria to persist for long periods of time within the food processing industry where cells can be transferred to food products and subsequently to susceptible hosts.

1.4.2.3 Resistance to antimicrobial agents and sanitizers

Like the other members of the *Listeria genus*, *L. monocytogenes* is susceptible to a wide range of antibiotics such as the β-lactams, however the rate of antibiotic resistant isolates is slowly increasing, and up to 10% of isolates are now resistant to at least one antibiotic (Conter et al., 2009). Moreover, *L. monocytogenes* is also becoming increasingly tolerant to many sanitizers and disinfectants commonly used in the food processing industry, such as quaternary ammonium compounds (QACs). QACs such as benzalkonium chloride (BCl) are cationic biocides that function by disrupting the cell membrane, causing leakage of cytoplasmic material. QACs are effective against most bacteria, fungi, amoeba and enveloped viruses and they are commonly used in household products, in commercial sanitizers and in the medical and food processing environments. Sanitizer resistance in *L. monocytogenes* occurs principally via the efflux of the toxic compound from the cell by chromosome or plasmid encoded efflux protein

pumps (Kumar & Schweizer, 2005; Mullapudi et al., 2008; Piddock, 2006; Poole, 2005; Poyart-Salmeron et al., 1990). The stress response systems in *L. monocytogenes* also permit cellular changes, such as the membrane fatty acid composition, that allow the bacterial cell to adapt and survive under the presence of sanitizers (To et al., 2002).

1.4.3 Lifecycle

Upon consumption by a susceptible host, *L. monocytogenes* transitions from a saprophyte to an intracellular pathogen capable of surviving and replicating within host cells. Once ingested, *L. monocytogenes*-specific factors trigger internalization of the pathogen and the intracellular life cycle begins. The *Listeria* Pathogenicity Island 1 (LPI1) encodes three virulence transcriptional units: *hly* (listerolysin O), the *mpl*, *actA* and *plcB* operon, and the *plcA prfA* operon (Vázquez-Boland et al., 2001). The transcriptional regulatory protein PrfA mediates *L. monocytogenes* pathogenicity and its expression is regulated by the combination of host cell derived signals and an RNA thermosensor mechanism that activates expression at 37°C (Cheng & Portnoy, 2003; Mansfield et al., 2003). Internalization of the bacterium into nonphagocytic cells is triggered by internalin A and internalin B, which induce the formation of pseudopods that entrap the bacteria and enable contact of the bacteria with the host cell. Upon internalization, the pore-forming hemolysin listeriolysin (hly/listeriolysin O) and the phospholipase PlcB lyse the membrane-bound vacuole, allowing the bacterium to be released into the cytoplasm where replication can occur (Glomski, 2003). PlcA, a phosphatidylinositol-specific phospholipase C, also synergizes with Hly and PlcB to help destabilize the primary phagosome (Smith et al., 1995). Concurrently, the surface protein ActA is expressed facilitating the actin-mediated propulsion of *Listeria* to neighbouring cells. Flagella-mediated motility is supressed thus the actin tails allow the bacteria to spread though host tissues without triggering a humoral immune response or

ingestion by phagocytes. The listeriopods that push outwards to neighbouring cells are again lysed by Hly and the phospholipases, releasing the bacterium into the new host cell, further enabling spread of the infection (Cossart & Toledo-Arana, 2008; Freitag, Port, & Miner, 2009; Gandhi & Chikindas, 2007; Kasper et al., 2005).

1.5 Current *L. monocytogenes* subtyping methods

Due to the ubiquitous presence of *L. monocytogenes* in the environment and in food products, robust subtyping of clinical and food isolates is necessary to establish epidemiological links during outbreak investigations. Serotyping to examine antigenic variation of the somatic (O) and flagella (H) antigens was the first subtyping method that was developed and it remains widely used today (Seeliger & Höhne, 1979). However, of the 13 identified *L. monocytogenes* serotypes, 3 serotypes (1/2a, 1/2b and 4a) cause over 90% of listeriosis cases worldwide (Wiedmann, 2002). Therefore, additional subtyping methods must be used in conjunction with serotyping to further distinguish isolates for the purposes of epidemiologic investigations. Likewise, serotyping is a slow and labour intensive technique so more recently, a multiplex PCR assay was developed to rapidly identify the four major serotypes (1/2a, 1/2b, 1/2c, and 4b) that are isolated from food and patient samples (Doumith et al., 2004). Several additional phenotypic and genotypic approaches have also been developed to subtype *L. monocytogenes* including phage typing, ribotyping, plasmid profiling, pulsed-field gel electrophoresis (PFGE), multiple-locus sequence typing (MLST) and multiple-locus variable-number tandem repeat analysis (MLVA) (Graves & Swaminathan, 2001; Miya et al., 2008; Pagotto et al., 2006; Sperry et al., 2008; Wiedmann, 2002). PFGE, which entails the macrorestriction of the entire genome with a restriction enzyme, provides high discriminatory power and it is a highly reproducible *L. monocytogenes* subtyping method (Graves & Swaminathan, 2001). Globally, the PulseNet system, including PulseNet

Canada, routinely applies standardized PFGE protocols during epidemiological investigations. Large PFGE databases have therefore been compiled worldwide and these help facilitate future outbreak investigations and long-term transmission analyses. Using subtyping methods, *L. monocytogenes* can be classified into phylogenetic groups such as evolutionary lineages, clonal complexes and epidemic clones, and further into distinct subgroups, such as serotypes, PFGE pattern types and sequence types. This information enables investigators to track and regulate *L. monocytogenes* based on the evolutionary relationships and genetic characteristics of particular isolates.

The application of whole genome sequencing has further enabled the characterization and evolutionary analysis of *L. monocytogenes*. Together, genomic analyses and phylogenetic subtyping studies provide evidence that *L. monocytogenes* isolates can be segregated into 4 divergent lineages (Doumith et al., 2004; Nightingale, 2010; Orsi et al., 2011; Ragon et al., 2008; Wiedmann et al., 1997). *L. monocytogenes* serotypes 1/2b, 4b, 3b, and 3c consistently group into lineage I, while serotypes 1/2a, 1/2c, and 3a strains group into lineage II. Lineage III represents a distinct taxonomic group and includes *L. monocytogenes* serotype 4a and 4c isolates. More recently, whole genome sequencing has been applied to epidemiological investigations during outbreak crises to rapidly gain detailed real-time information about the causative strains (Gilmour et al., 2010). Comparative genomics reveal that *L. monocytogenes* harbour a pan-genome of 2.8 to 3.2Mbp with high gene synteny and organization within which limited gene loss or acquisition occurs (den Bakker et al., 2010). However, despite the clonality observed within *L. monocytogenes* genomes, several serotype and strain specific genes have been identified (Doumith et al., 2004; Gilmour et al., 2010; Nelson et al., 2004). Genomic differences have also been observed as a result of prophage

insertions, transposable elements, virulence gene loss or acquisition and islands encoding genes of unknown function.

1.6 Global distribution of *L. monocytogenes*

Globally, the majority of listeriosis outbreaks are associated with lineage I serotype 4b isolates, while fewer outbreaks but more sporadic clinical cases are associated with lineage I serotype 1/2b isolates and lineage II serotype 1/2a isolates (Orsi et al., 2011). Lineage II serotype 1/2a and 1/2c isolates are also more frequently isolated from food and food processing plants compared to other serotypes, despite causing fewer outbreaks and clinical cases worldwide (Gray et al., 2004; Jeffers et al., 2001). This has led to the speculation that lineage I isolates may be more pathogenic than lineage II isolates, while lineage II isolates may have an increased ability to persist. Accordingly, several studies suggest that that the two lineages are composed of various clonal complexes that have different virulence and persistence characteristics (Gray et al., 2004; Nightingale et al., 2006). For example, a large proportion of lineage II isolates encode premature stop codons within the *inlA* gene, a protein that is necessary for attachment to host cells, whereas lineage I isolates encode intact *inlA* genes (Gaillard et al., 1991; Poyart et al., 1996). These isolates with mutated *inlA* genes demonstrate attenuated invasion and virulence abilities in mice and thus could explain the differences observed in isolate distribution (Poyart et al., 1996). Conversely, lineage II isolates are generally stronger biofilm formers than lineage I isolates, which could contribute to the higher proportion of isolates belonging to this lineage found in foods and food processing industries (Borucki et al., 2003). Despite these lineage specific traits, serotype distribution varies by region. For example, in the US, lineage I isolates are the predominant cause of human listeriosis, whereas in Northern Europe, lineage II serotype 1/2a isolates cause the majority of human listeriosis cases (Jeffers et al., 2001;

Lukinmaa et al., 2003). Recently, lineage II isolates have also caused several outbreaks worldwide. Notably, in Canada in 2008, a large nationwide outbreak associated with ready-to-eat meat products was caused by lineage II serotype 1/2a isolates (Gilmour et al., 2010). In the US, a large outbreak associated with a meat processing facility in 2000 and an outbreak in 2010 associated with hog headcheese were both linked to lineage II isolates (Olsen et al., 2005; CDC, 2011). Additionally, an outbreak in Austria and Germany associated with acid curd cheese was also caused by lineage II isolates (Fretz et al., 2010). The reasons behind the apparent regional differences with regard to the prevalence of lineage II isolates and their apparent global emergence remains to be determined.

1.7 *L. monocytogenes* in Canada

In Canada over the past twenty years, *L. monocytogenes* lineage II serotype 1/2a isolates caused a high prevalence of the reported human clinical listeriosis cases (Knabel et al., unpublished). In particular, a specific subtype of serotype 1/2a isolates with *AscI*/*ApaI* macrorestriction patterns similar to the PulseNet Canada designations LMACI.0001 and LMAAI.0001 has been responsible for approximately 20% of clinical cases and 40% of Canadian outbreaks. According to the MLST scheme proposed by Ragon et al. (2008), these isolates belong to the previously described MLST clonal complex 8 (CC8). CC8 includes the MLST sequence types ST120, ST292, ST387 and ST8. These related sequence types individually vary by no more than one allele, and those alleles differ by no more than one single nucleotide polymorphism (SNP). One lineage II serotype 3a isolate with the LMACI.0001 and LMAAI.0001 PFGE pattern has also been determined to be part of CC8. The global distribution of CC8 is largely unknown, but in France CC8 clinical isolates have been identified by the Institut Pasteur, but this does not represent a significant clone there (M. Lecuit, personal

communication). This subgroup of *L. monocytogenes* has also been proposed to represent 'epidemic clone V', or ECV (Knabel et. al., unpublished).

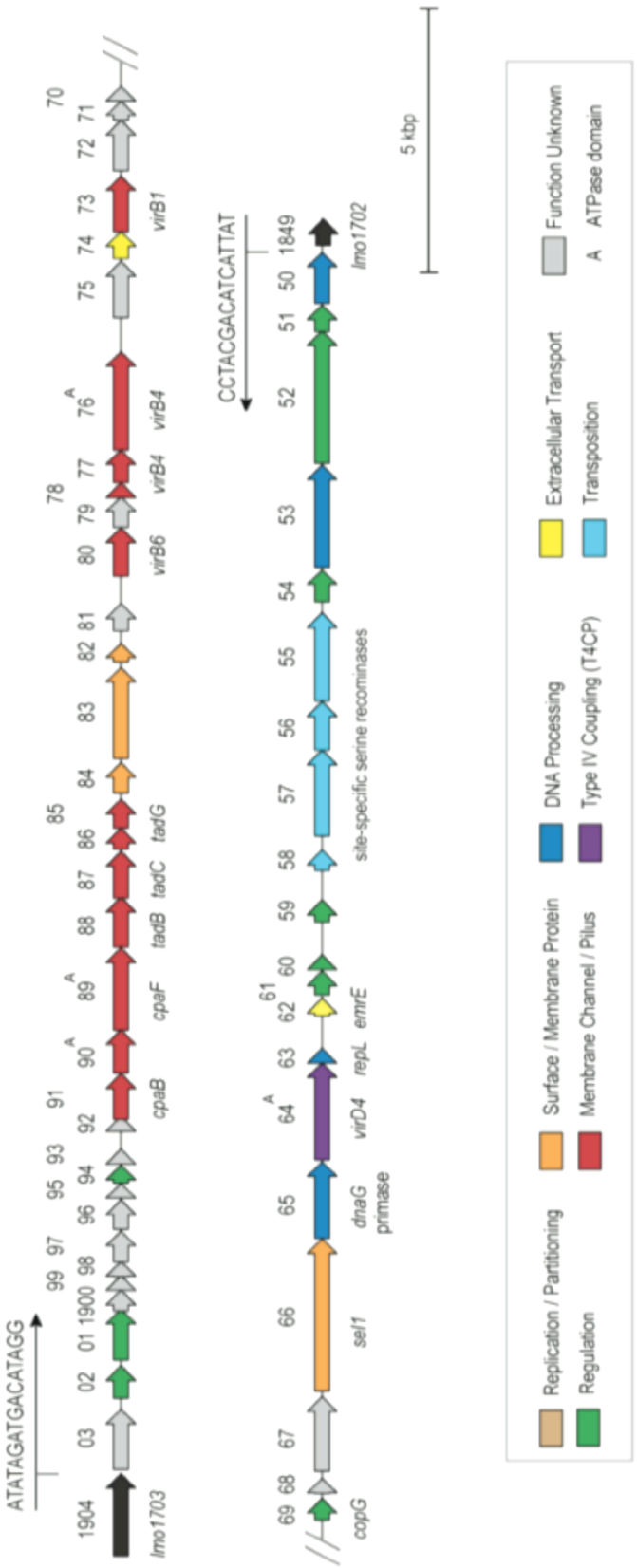
1.8 The 2008 Canadian listeriosis outbreak

In Canada in the fall of 2008, the CC8 subgroup of *L. monocytogenes* was the causative agent of a nationwide foodborne outbreak associated with ready-to-eat meat products that resulted in 57 laboratory confirmed human infections and 23 fatalities. In addition to the traditional subtyping methods, whole genome sequencing was performed on the primary outbreak strains (08-5578 and 08-5923) to gain further genetic information about these isolates. Whole genome sequencing allowed the NML to detect within these outbreaks 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). The isolate 08-5578 also encoded a 33kbp prophage (ΦLMC1) that accounted for the difference in the *AscI* PFGE patterns of the isolates (Gilmour et al., 2010). Additionally, a 77 kbp plasmid pLM5578 was identified within the genome sequences of isolate 08-5578 (Gilmour et al., 2010). 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; Figure 5) (Gilmour et al., 2010).

1.9 *Listeria* genomic island 1

LGI1 was present within the genomes of both 2008 outbreak isolates whose genomes were sequenced, however it was absent in all publicly available genomes (Figure 5). Coding sequences within LGI1 exhibited sequence homology within several environmental firmicutes, including *Clostridium kluyver*i, and *Desulfitobacterium*

Figure 5: The genetic and structural organization of the Listeria Genomic Island 1 and its predicted functions (Taken from Gilmour et al., 2010).


ATATAGATGACATAGG
CCTACGACATCATTAT
5 kbp
lmo1703
cpaB
cpaF
tadB
tadC
tadG
virB6
virB4
virB4
virB1
copG
sel1
dnaG primase
virD4
repL
emrE
site-specific serine recominases
lmo1702
Replication / Partitioning
Regulation
Surface / Membrane Protein
Membrane Channel / Pilus
DNA Processing
Type IV Coupling (T4CP)
Extracellular Transport
Transposition
Function Unknown
ATPase domain

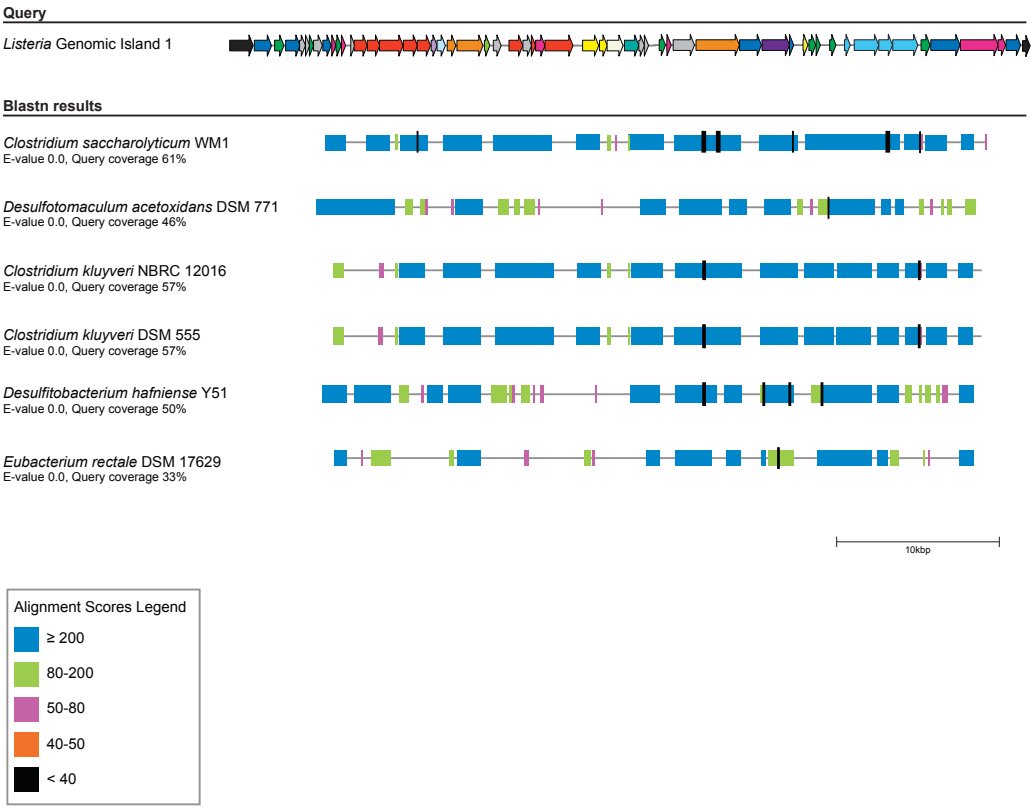
hafniense (Figure 6), although the entire LGI1 sequence was not present within these genomes. The bordering coding sequences LM5578_1849 and LM5578_1904 were present within the genome sequence of *L. monocytogenes* EGDe, a serotype 1/2a isolate used as a reference for the genome assembly. These two genes LM5578_1849 and LM5578_1904 were homologous to the adjacent EGDe coding sequences lmo1702 and lmo1703. This suggested that LGI1 represents a genomic insertion within the ancestral chromosome of these isolates.

Preliminary bioinformatic analyses show that LGI1 encodes several genes homologous to known virulence factors and mobilization functions, including components of known secretion systems, a multidrug efflux pump, and a putative histidine kinase two-component signal transduction system, which could be involved in environmental sensing and gene regulation. The multidrug efflux pump gene was adjacent to a putative MarR-family transcriptional regulator and a putative rpoE family DNA-directed RNA polymerase sigma-24 subunit, which is involved in the stress response regulation in other bacteria. LGI1 may therefore be tightly regulated, and it may function to translocate small molecules, DNA, proteins and/or nucleoprotein complexes. Furthermore, LGI1 encodes putative serine recombinases, and there are 16bp imperfect inverted repeats present at the borders in the intergenic regions between the loci LM5578_1849/50 and LM5578_1903/04 (Figure 5). This suggested that LGI1 may be horizontally acquired and mobile.

1.9.1 Secretion systems

The LGI1 loci LM5578_1864 and LM5578_1872 to LM5578_1891 encode genes homologous to secretion systems. Secretion systems are very versatile in bacteria and allow the bacteria to process and assemble multi-protein complexes that can secrete

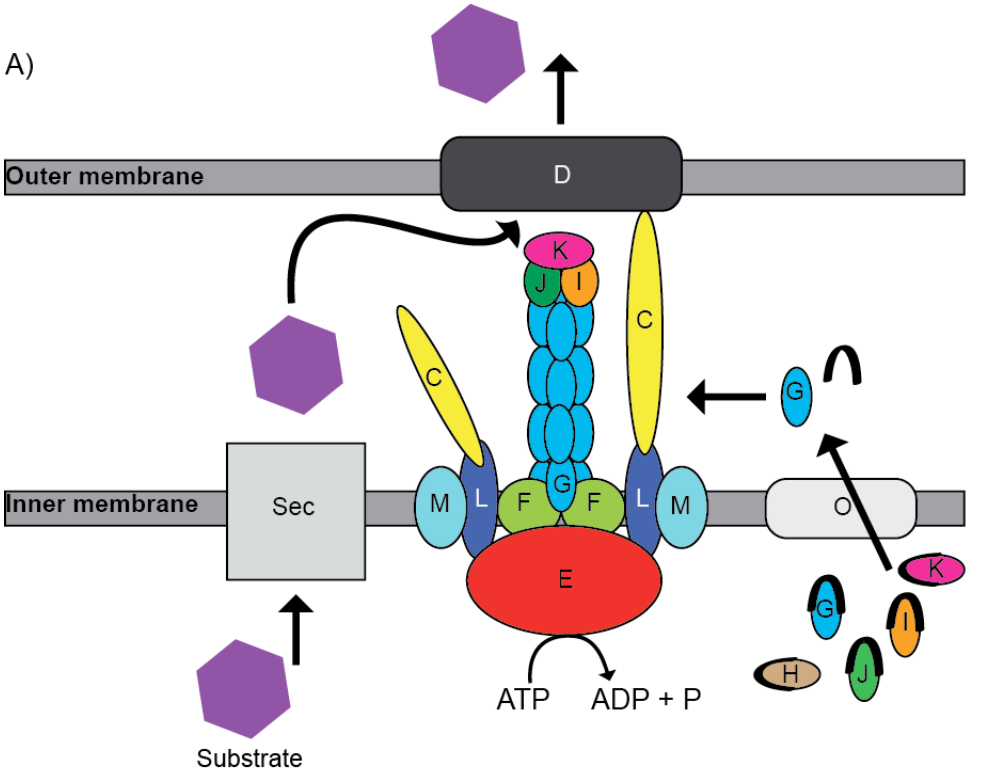
Figure 6: The blastn sequence homology of LGI1 to the other publically available genomes that shows the genetic origin of the LGI1 DNA sequence.

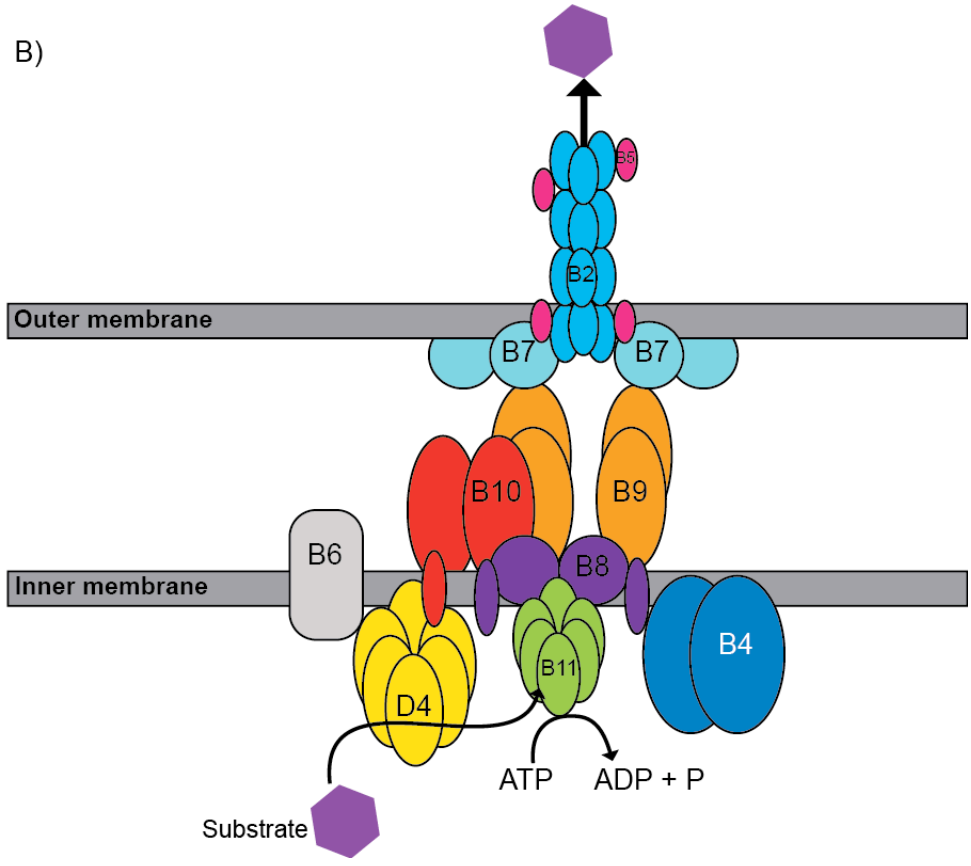

Query
Listeria Genomic Island 1
Blastn results
Clostridium saccharolyticum WM1
E-value 0.0, Query coverage 61%
Desulfotomaculum acetoxidans DSM 771
E-value 0.0, Query coverage 46%
Clostridium kluyveri NBRC 12016
E-value 0.0, Query coverage 57%
Clostridium kluyveri DSM 555
E-value 0.0, Query coverage 57%
Desulfitobacterium hafniense Y51
E-value 0.0, Query coverage 50%
Eubacterium rectale DSM 17629
E-value 0.0, Query coverage 33%
10kbp
Alignment Scores Legend
≥ 200
80-200
50-80
40-50
< 40

protein and DNA molecules to the extracellular environment or directly into any prokaryotic or eukaryotic target cell. These systems are widely used by bacteria for infection, invasion, conjugation and delivery of effector molecules such as cytotoxins. To date, up to 7 different types of secretion systems have been identified (Tseng et al., 2009). Preliminary bioinformatic analyses show that LGI1 encodes several proteins homologous to known components of putative type II and type IV secretion systems. These secretion systems are well studied in Gram-negative organisms, however current data does not elucidate complete mechanisms and structures within Gram-positive organisms.

Type II secretion systems (T2SS) are involved in protein translocation and are dependent on the secreton pathway. Protein secretion occurs in two steps where the proteins are first translocated across the inner membrane via the sec-pathway and then from the periplasm to the exterior of the cell by the T2SS (Filloux, 2004; Sandkvist, 2001). The T2SS consists of 12 core components that form a multi-protein complex, which spans the periplasmic compartment and is specifically required for translocation of the secreted proteins across the outer membrane. The core components of the T2SS are the outer membrane secretin (D), a cytoplasmic ATPase (E), an inner (trans)membrane protein (F), the major (G) and minor (H, I, J, K) pseudopilins, facilitators of the ATPase attachment to the inner membrane that appear, along with F, to form an inner membrane platform (L, M), the pre-pseudopilin peptidase/methyltransferase (O), and a protein that might be involved in substrate recognition and/or secretin interactions (C) (Figure 7) (Filloux, 2004). The T2SS is similar to the type IV secretion system (T4SS) and both have the ability to form a pilus-like structure that can be used to attach to surfaces or to secrete DNA or effector molecules.

Figure 7: The organization of the protein subunits in a Gram-negative type II secretion system (A) and type IV secretion system (B) (Adapted from Cianciotto, 2009; Shamaei-Tousi et al., 2004; Waksman, 2011).


A)
Outer membrane
D
K
J
I
C
C
G
Inner membrane
Sec
M
L
F
G
F
L
M
O
E
K
G
I
H
J
ATP
ADP + P
Substrate


B)
B5
B2
Outer membrane
B7
B7
B10
B9
B6
B8
Inner membrane
B4
B11
D4
ATP
ADP + P
Substrate

In Gram-negative bacteria, T4SS are categorized into one of 3 types: conjugation systems, effector translocation systems and DNA uptake and release systems. Conjugation is a mechanism of horizontal gene transfer that can allow the bacteria to exchange genetic information, and is thus important in the evolution of bacteria. Like T2SS, the T4SS components are evolutionarily related, and most of the T4SS genes are arranged in a single or a few operons (Lessl et al., 1992). The Gram-negative T4SS are named according to the *Agrobacterium tumefaciens* VirB/D scheme, which is composed of 12 main proteins named VirB1 to VirB11 and VirD4. Most T4SS encode an extracellular pilus composed of major (VirB2) and minor (VirB5) subunits. Three ATPases (VirB4, VirB11 and VirD4) provide energy for the system assembly and substrate secretion (Figure 7) (Fronzes et al., 2009). VirD4 is also referred to as a type 4 coupling protein (T4CP), and it functions to link the substrate to the secretion apparatus. The T4SS must allow the substrate to pass through both the inner and outer membrane of Gram-negative organisms. This is facilitated by an inner membrane channel, composed of the proteins VirB6, VirB8 and VirB10, but the outer membrane channel that would allow the substrate to reach the extracellular environment is unknown (Fronzes et al., 2009). However, recent studies speculate that VirB9 and VirB7 may form this channel (Bayliss et al., 2007). The functions of VirB1 and VirB3 are also unknown. Notably, the conjugativeT4SS must also encode a DNA transfer and replication proteins including a relaxase and an integrase to enable DNA mobilization (Alvarez-Martinez & Christie, 2009).

In Gram-positive organisms, conjugative type IV secretion-like systems (T4SLS) have been identified that are involved in both plasmid and chromosomal integrative conjugative element transfer. Like the Gram-negative counterparts, these Gram-positive systems encode proteins including relaxases involved in preparing the DNA for

translocation. T4CPs have also been identified that are similar to the Gram-negative proteins. However, less information is known about the type IV secretion channels in Gram-positive organisms. The T4SLS do encode a VirB4 like ATPase in addition to the T4CP, however none of the studied T4SLS encode a VirB11 ATPase homolog (Grohmann et al., 2003). Each T4SLS also encodes a putative murein hydrolase that is predicted to break down peptidoglycan linkages to allow for the channel formation across the cell membrane (Alvarez-Martinez & Christie, 2009). Lastly, these systems also encode VirB8 and/or VirB10-like scaffold proteins that may play a role in the apparatus assembly or membrane translocation (Alvarez-Martinez & Christie, 2009). Notably, a hydrolase and an adhesion protein are also encoded within LGI1, which could enable assembly of a secretion system through the dense peptidoglycan layer of the *Listeria* Gram-positive cell wall.

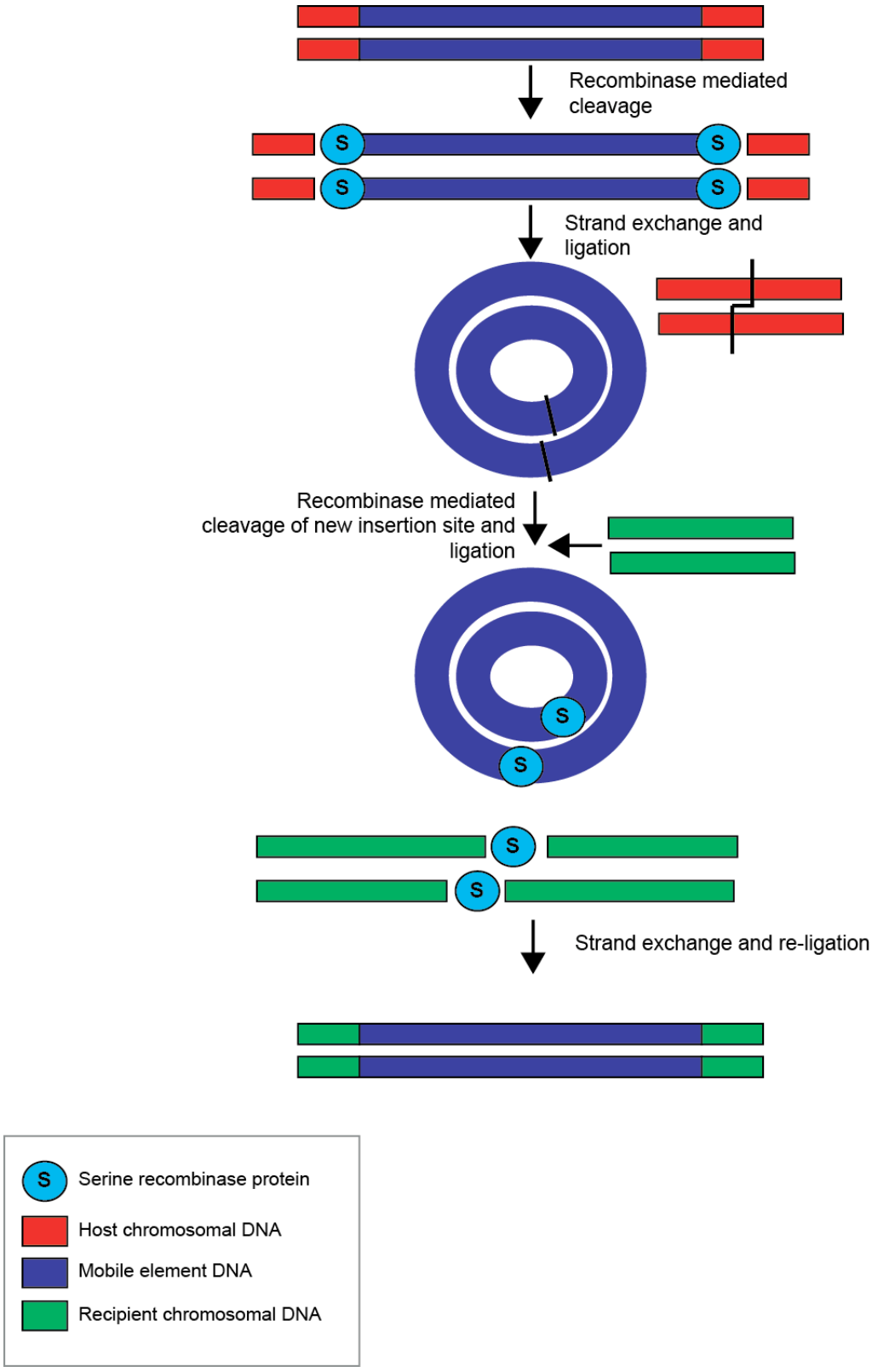
1.9.2 Small multidrug resistance proteins

The LGI1 locus LM5578_1862 encodes a putative small multidrug resistance protein (SMR). SMRs are integral inner membrane proteins ranging from 100 to 140 amino acids that span the bacterial membrane as 4 transmembrane α-helices (Paulsen et al., 1996). These proteins are very hydrophobic as a result of their short hydrophilic loops. Unlike other characterized multidrug transporter proteins, SMRs are only able to transport lipophilic compounds, including QACs, antibiotics such as tetracycline and chloramphenicol, antiseptics, dyes such as ethidium bromide and crystal violet, and detergents such as SDS. SMRs function via an electrochemical proton gradient that allows the substrate efflux against its concentration gradient (Grinius & Goldberg, 1994). SMRs are also widely distributed amongst bacteria as both Gram-positive and Gram-negative organisms encode them, and each species encodes an average of 2 SMRs (Bay, Rommens, & Turner, 2008).

1.9.3 Site-specific serine recombinases

The LGI1 loci LM5578_1855 to LM5578_1858 encode putative site-specific serine recombinases. Site-specific recombinases are proteins involved in site-specific DNA rearrangements. They are essential for bacterial genome replication, differentiation and pathogenesis, and have roles in the movement of mobile genetic elements such as plasmids, transposons, bacteriophages and integrons. Thus, site-specific recombination events have an important role in bacterial evolution and adaptation. The site-specific recombination event occurs in a series of steps mediated by the recombinase proteins. First, the recombinase interacts with the two short DNA target sequences at the ends of the DNA that will be transferred. The target sequences are usually between 30 and 200 nucleotides in length and have partial inverted-repeat symmetry. Next, the recombinase-bound sites are brought together in a synapse with the crossover sites juxtaposed (Grindley et al., 2006) (Figure 8). The recombinases then catalyze the DNA cleavage at the end sites to facilitate strand exchange. They then re-ligate the remaining DNA strands together, releasing the recombination DNA (Figure 8) (Grindley et al., 2006). This recombination reaction can result in the intregration, inversion and/or excision of DNA fragments and the outcome is tightly controlled by the recombinases. There are 2 main families of site-specific recombinases: the tyrosine and serine recombinases. These families differ with respect to the catalytic site, and more specifically the essential hydroxyl-bearing amino acid side chain that acts as a nucleophile to cleave a strand. LGI1 encodes putative site-specific serine recombinases as well as 16bp inverted repeats at the borders of the island, suggesting a putative horizontal mobilization mechanism.

Figure 8: The mechanism of site-specific serine-recombination.


Recombinase mediated cleavage
S
S
S
S
Strand exchange and ligation
Recombinase mediated cleavage of new insertion site and ligation
S
S
S
S
Strand exchange and re-ligation
S
Serine recombinase protein
Host chromosomal DNA
Mobile element DNA
Recipient chromosomal DNA

1.10 The current investigation

Due to the public health significance and national prevalence of the CC8 subtype of *L. monocytogenes* in Canada, it is plausible that the presence and function of LGI1 may contribute to its apparent virulence and persistence within Canada. LGI1 was identified within the genomes CC8 isolates, and it encodes coding sequences with homologies to known virulence and mobilization proteins. Furthermore, there is a high degree of synteny in gene sequence and organization amongst *L. monocytogenes* genomes, but LGI1 was unique to the 2008 outbreak isolates and it was inserted between two coding sequences that are adjacent in the reference strain, providing evidence that it is horizontally acquired. Thus, I hypothesize that LGI1 is a genetic trait that will consistently be found in clinical isolates across Canada because it contributes to bacterial persistence.

In order to test these hypotheses, the first objective of this study was to determine the distribution of LGI1 to provide an evolutionary model of its acquisition. A panel of isolates representing Canadian *L. monocytogenes* isolates from environmental, food and clinical sources was selected and screened using a real-time PCR assay to determine the distribution of LGI1. Bioinformatic analyses were then used to predict the functions of individual genes encoded by LGI1, to direct experiments on functions and phenotypes contributed by LGI1, and to determine the diversity amongst the LGI1 sequences of isolates whose whole genome sequences were available. Molecular techniques including gene mutagenesis, antimicrobial susceptibility testing, and quantitative real-time PCR were used to examine the cellular and virulence-related functions encoded by the genomic island.

Chapter 2

Materials and Methods

2. Methods

2.1 Bacterial isolates and growth conditions

A total of 126 *L. monocytogenes* isolates (Table 1) from human, food and environmental sources were included in this study. Isolates were collected by Canadian public health laboratories and the Canadian Food Inspection Agency from 1987 to 2010 and submitted to the Enteric Diseases Program at the National Microbiology Laboratory. Isolates were also obtained from the Bureau of Microbial Hazards (Health Canada, Ottawa, ON) and Cornell University, Department of Food Science (Ithaca, NY). This panel of isolates included epidemiologically related and unrelated isolates with the same serotype, and similar PFGE patterns to the 2008 outbreak strains that represented a wide range of year of isolation and source of infection. Isolates were also selected to represent a diverse range of serotypes and PFGE patterns unrelated to the 2008 outbreak strains isolated from a wide range of sources and years. The 2008 listeriosis outbreak isolate 08-5578, which has the complete genomic DNA sequence available (GenBank accession number NC_013766), was used as the reference for all 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. Serotyping was performed by slide agglutination with antisera prepared at the NML according to Seeliger and Höhne (1979). Pulsed-field gel electrophoresis (PFGE) was performed 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.

Table 1: The bacterial isolates included in this study. Isolates were selected to represent serotypes and PFGE patterns both related and unrelated to the 2008 outbreak isolates. The panel also represents diverse sources of infection, provinces of isolation, and years of isolation.

Isolate	Serotype	Source	Province[a]	PFGE pattern	
				AscI	*ApaI*
01-2129	1/2a	n/a	QC	LMACI.0001	LMAAI.0001
01-2417	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001
07-3417	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001
01-5080	1/2a	Human joint fluid	MB	LMACI.0001	LMAAI.0001
03-5360	1/2a	n/a	NF	LMACI.0001	LMAAI.0001
01-5379	1/2a	Human uterus swab	ON	LMACI.0001	LMAAI.0001
04-5457	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
07-5657	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
01-6771	1/2a	Human eye	ON	LMACI.0001	LMAAI.0001
06-6837	1/2a	Human blood	QC	LMACI.0001	LMAAI.0001
07-7193	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
01-7209	1/2a	Liverwurst sausage	BC	LMACI.0001	LMAAI.0001
08-7362	1/2a	Environmental	BC	LMACI.0001	LMAAI.0001
08-7376	1/2a	Food processing environment	ON	LMACI.0001	LMAAI.0001
08-7381	1/2a	Food processing environment	ON	LMACI.0001	LMAAI.0001
08-7382	1/2a	Food processing environment	ON	LMACI.0001	LMAAI.0001
08-7554	1/2a	Food processing environment	ON	LMACI.0001	LMAAI.0001
00-0338	1/2a	Human knee fluid	MB	LMACI.0001	LMAAI.0001
03-0402	1/2a	Human blood	AB	LMACI.0001	LMAAI.0001
08-5923*	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
08-7374*	1/2a	Food processing environment	ON	LMACI.0001	LMAAI.0001
08-7669*	1/2a	Human blood	SK	LMACI.0001	LMAAI.0001
10-0814*	1/2a	Food processing environment	ON	LMACI.0001	LMAAI.0001
10-1046*	1/2a	Human clinical	ON	LMACI.0001	LMAAI.0001
10-1047*	1/2a	Human clinical	ON	LMACI.0001	LMAAI.0001
10-1321*	1/2a	Human clinical	ON	LMACI.0001	LMAAI.0001
88-0478	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
88-1059	3a	Human blood	NF	LMACI.0001	LMAAI.0001
95-0093	1/2a	Human blood	AB	LMACI.0001	LMAAI.0001
96-0218	1/2a	n/a	n/a	LMACI.0001	LMAAI.0001
96-0247	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
97-0624	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001
97-1602	1/2a	Human urine	AB	LMACI.0001	LMAAI.0001
98-0290	1/2a	Human cerebral spinal fluid	ON	LMACI.0001	LMAAI.0001
98-1143	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001
98-1191	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001
98-2299	1/2a	n/a	SK	LMACI.0001	LMAAI.0001
99-6666	1/2a	Human cerebral spinal fluid	ON	LMACI.0001	LMAAI.0001
99-6871	1/2a	Human clinical	NF	LMACI.0001	LMAAI.0001
01-3506	1/2a	Human cerebral spinal fluid	ON	LMACI.0001	LMAAI.0003
95-0012	1/2a	Human blood	ON	LMACI.0001	LMAAI.0003
95-0151	1/2a	Human blood	ON	LMACI.0001	LMAAI.0003
97-1636	1/2a	Human blood	SK	LMACI.0001	LMAAI.0003
01-5373	1/2a	Human blood	ON	LMACI.0002	LMAAI.0001
99-3046	1/2a	Human blood	ON	LMACI.0002	LMAAI.0001
08-5375	1/2a	Human clinical	ON	LMACI.0002	LMAAI.0214
03-5833	1/2a	Human blood	AB	LMACI.0002	LMAAI.0214
06-4721	1/2a	Human blood	ON	LMACI.0003	LMAAI.0294
07-5577	1/2a	Human blood	BC	LMACI.0004	LMAAI.0013
07-5999	1/2a	Human blood	BC	LMACI.0004	LMAAI.0013
09-0290	1/2a	Food	AB	LMACI.0004	LMAAI.0013
07-3998	1/2a	Human cerebral spinal fluid	ON	LMACI.0005	LMAAI.0013

06-6833	1/2a	Human blood	QC	LMACI.0007	LMAAI.0068
10-1870	4b	Human clinical	NS	LMACI.0009	LMAAI.0234
08-8680	4b	Human blood	NB	LMACI.0009	LMAAI.0234
09-0498	1/2a	RTE Leeks	QC	LMACI.0011	LMAAI.0015
06-6880	1/2a	Human blood	QC	LMACI.0014	LMAAI.0183
09-2089	1/2a	Human clinical	BC	LMACI.0015	LMAAI.0024
09-0748	1/2a	Fish	QC	LMACI.0027	LMAAI.0677
06-6846	1/2a	Human blood	QC	LMACI.0031	LMAAI.0174
08-5587	1/2a	Human clinical	ON	LMACI.0035	LMAAI.0414
08-5596	1/2a	Smoked Salmon	ON	LMACI.0036	LMAAI.0433
07-1873	1/2c	Food	BC	LMACI.0036	LMAAI.0433
08-8749	1/2c	Pork Loin Chops	ON	LMACI.0036	LMAAI.0658
08-4803	4a	Human clinical	ON	LMACI.0038	LMAAI.0540
08-2593	1/2a	Human clinical	NF	LMACI.0040	LMAAI.0001
08-5243	1/2a	Human clinical	BC	LMACI.0040	LMAAI.0001
08-5319	1/2a	Human clinical	AB	LMACI.0040	LMAAI.0001
08-5828	1/2a	Human blood	ON	LMACI.0040	LMAAI.0001
08-6011	1/2a	Corned beef AU25	ON	LMACI.0040	LMAAI.0001
08-6040	1/2a	RTE meat	ON	LMACI.0040	LMAAI.0001
08-6055	1/2a	RTE meat	ON	LMACI.0040	LMAAI.0001
08-6061	1/2a	RTE meat	ON	LMACI.0040	LMAAI.0001
07-6082	1/2a	Human blood	NS	LMACI.0040	LMAAI.0001
08-6135	1/2a	Human cerebral spinal fluid	ON	LMACI.0040	LMAAI.0001
08-6421	1/2a	Human blood	ON	LMACI.0040	LMAAI.0001
08-6567	1/2a	Food processing environment	ON	LMACI.0040	LMAAI.0001
01-7210	1/2a	Liverwurst sausage	BC	LMACI.0040	LMAAI.0001
08-5578*	1/2a	Human blood	ON	LMACI.0040	LMAAI.0001
08-6569*	1/2a	Food processing environment	ON	LMACI.0040	LMAAI.0001
10-0815*	1/2a	Food processing environment	ON	LMACI.0040	LMAAI.0001
08-5871	1/2a	Human cerebral spinal fluid	BC	LMACI.0040	LMAAI.0003
08-6056*	1/2a	Turkey Meat	ON	LMACI.0040	LMAAI.0003
08-6997*	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003
98-2035*	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003
99-6370*	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003
06-7231	1/2a	Human blood	BC	LMACI.0041	LMAAI.0033
06-6891	1/2a	Human blood	QC	LMACI.0044	LMAAI.0074
08-5757	1/2a	Human clinical	ON	LMACI.0044	LMAAI.0193
08-8809	1/2b	Fromage Stilton Blue	QC	LMACI.0045	LMAAI.0317
06-3023	4b	Human blood	MB	LMACI.0060	LMAAI.0204
09-5042	1/2a	n/a	USA	LMACI.0083	LMAAI.0185
06-7047	1/2a	Human cerebral spinal fluid	ON	LMACI.0084	LMAAI.0175
08-6880	1/2a	Human clinical	MB	LMACI.0088	LMAAI.0448
01-1465	1/2a	Human blood	ON	LMACI.0096	LMAAI.0001
01-1468	1/2a	Human brain tissue	NF	LMACI.0098	LMAAI.0001
09-6935	4b	Human clinical	BC	LMACI.0107	LMAAI.0104
06-6902	1/2a	Human blood	QC	LMACI.0114	LMAAI.0182
06-4636	1/2a	Human blood	BC	LMACI.0116	LMAAI.0118
07-0129	1/2a	Human ascitic fluid	ON	LMACI.0118	LMAAI.0005
02-2448	1/2a	Human blood	ON	LMACI.0122	LMAAI.0003
08-7538	1/2c	n/a	CFIA-SHY	LMACI.0148	LMAAI.0442
08-6576	1/2a	Human cerebral spinal fluid	ON	LMACI.0149	LMAAI.0265
06-6878	1/2a	Human cerebral spinal fluid	QC	LMACI.0157	LMAAI.0126
10-1871	1/2a	Human clinical	NS	LMACI.0173	LMAAI.0834
05-7243	4b	Human blood	ON	LMACI.0207	LMAAI.0134
08-0291	1/2a	Human clinical	ON	LMACI.0222	LMAAI.0005
06-6865	3a	n/a	QC	LMACI.0226	LMAAI.1019

07-6342	4a	n/a	USA NY	LMACI.0306	LMAAI.0319
09-3419	4b	Human clinical	MB	LMACI.0308	LMAAI.0555
08-8753	1/2c	Human cerebral spinal fluid	BC	LMACI.0316	LMAAI.0559
07-6350	1/2a	n/a	USA NY	LMACI.0334	LMAAI.0320
08-6350	1/2a	Human blood	PEI	LMACI.0442	LMAAI.0551
09-3891	1/2a	Food	ON	LMACI.0590	LMAAI.0510
87-0192	1/2a	Human blood	ON	LMACI.0616	LMAAI.0818
87-0426	1/2a	Human cerebral spinal fluid	NS	LMACI.0531	LMAAI.0482
88-0286	1/2a	Cooked pork	ON	LMACI.0127	LMAAI.0013
88-0702	1/2a	Human cerebral spinal fluid	ON	LMACI.0351	LMAAI.0024
90-0558	1/2a	Human cerebral spinal fluid	AB	LMACI.0007	LMAAI.0068
91-0145	1/2a	Human blood	ON	LMACI.0373	LMAAI.0564
92-0366	1/2a	Human blood	n/a	LMACI.0002	LMAAI.0001
93-0024	1/2a	Human cerebral spinal fluid	QC	LMACI.0001	LMAAI.0001
93-0407	1/2a	Human blood	ON	LMACI.0034	LMAAI.0005
94-0096	1/2a	Human blood	BC	LMACI.0612	LMAAI.0217
94-0447	1/2a	Human blood	ON	LMACI.0034	LMAAI.0005
EGDe*	1/2a	Rabbit tissue		LMACI.0661	LMAAI.0944

a – Province: AB, Alberta; BC, British Columbia; MB, Manitoba; QC, Quebec; ON, Ontario; NS, Nova Scotia; PEI, Prince Edward Island; USA NY, United States of America state of New York; USA, United States of America; NF, Newfoundland; SK, Saskatchewan; NB, New Brunswick; CFIA-SHY, Canadian Food Inspection Agency St-Hyacinthe.
*- Whole genome sequence available

2.2 Bioinformatic analyses

Gene annotation analysis of the *L. monocytogenes* 08-5578 LGI1 sequence was performed using the GenDB version 2.2 annotation tool (Meyer et al., 2003). Similarity searches were performed by using BLASTN and BLAST2P (Altschul et al., 1990) against the nonredundant nucleotide and protein databases, respectively. A BLAST2P search was performed against the databases nr (Altschul et al., 2010; ftp://ftp.ncbi.nlm.nih.gov/blast/db), 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; Petersen 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 manipulations and additional annotations (Rutherford et al., 2000). DNA sequence alignments were performed to identify SNPs using ClustalX (Thompson et al., 2002). GView Server was used to compare the predicted LGI1 coding sequences and whole genome predicted coding sequences using the Blast Atlas analysis method created with the isolate 08-5578 serving as the reference strain (Petkau et al., 2010).

2.3 DNA template

DNA template used for PCR in this study was prepared according to the boiled cell method. Briefly, a loopful of bacteria 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. The 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

A single transformant colony from a selective plate was inoculated into 8 ml of BHI broth (Becton, Dickinson and Company) with the appropriate selective antibiotic if applicable to create a starter culture. Wild type plasmids were not selected for antibiotics because the resistance phenotypes were unknown. The starter culture was incubated overnight at 37ºC with rotation at 200 rpm. The starter culture was diluted 1/500 into fresh broth with the appropriate antibiotic if applicable and grown overnight at 37ºC with rotation at 200 rpm. Plasmids were isolated from 50 ml of overnight culture using a Qiagen plasmid midi kit (Qiagen, Mississauga, ON) according to the manufacturer's directions with the following modification. Plasmid DNA was resuspended in 5-100 µl 1xTE buffer (Sigma-Aldrich). The purified plasmid and a control plasmid of similar expected size was electrophoresed on a 1% TBE-agarose gel, stained with ethidium bromide (Sigma-Aldrich) and viewed under UV light.

2.5 LGI1 real-time PCR screening

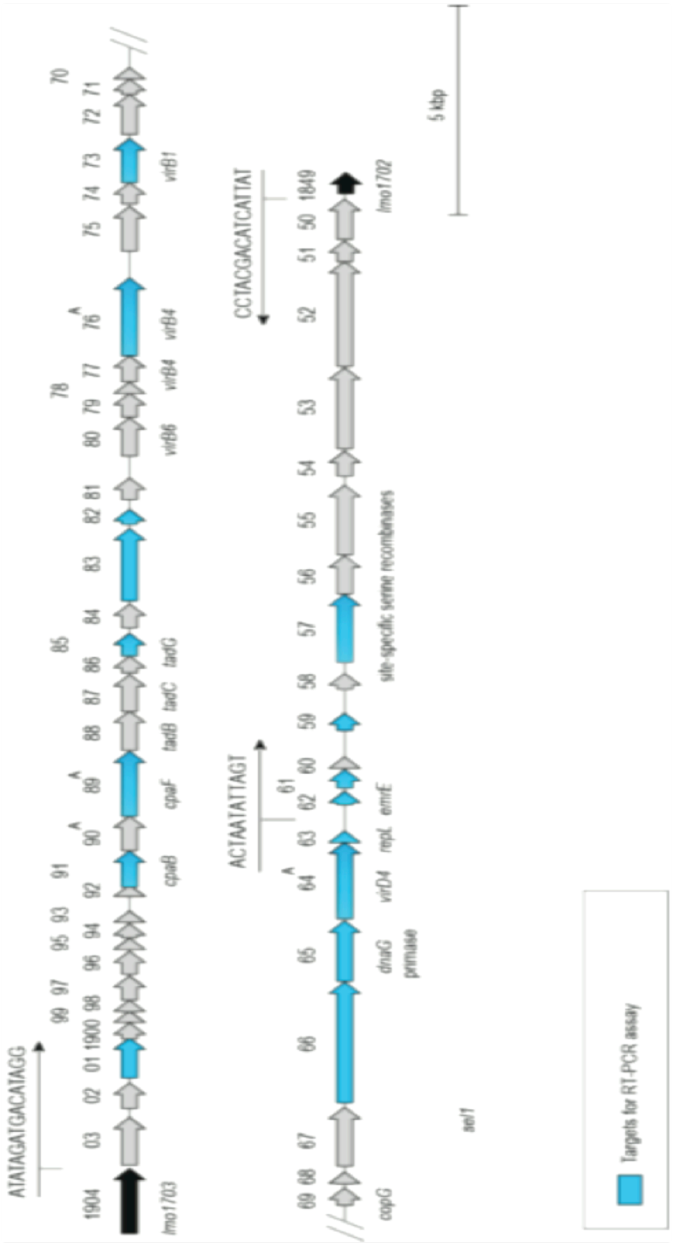
A TaqMan based real-time PCR (RT-PCR) assay with 16 LGI1 gene targets was designed (see Table 2 for primer and probe sequences). Gene targets were selected to represent the various functional units and operons within LGI1 (Figure 9). The assay was performed with ABI TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to manufacturer's instructions with 0.4 µM of each oligonucleotide primer, 0.2 µM of the TaqMan probe and 5 µL DNA template per 25 µL reaction. The thermocycling was performed using the Cepheid SmartCycler (Cepheid, Sunnyvale, CA) with the following conditions: 95°C for 8 minutes; and 35 cycles of 95°C for 10 seconds and 60°C for 30 seconds.

Table 2: The LGI1 TaqMan based real-time PCR screening assay primer and probe sequences. Product size indicates the approximate length of the amplicon generated.

08-5578 LGI1 Gene Target	Forward Primer (5'-3')	TaqMan Probe (5'-3')	Reverse Primer (5'-3')	Length	Reference
bglA[a]	AGCTGCTGCTG CAAACCAAT	CGAAGGCGCTTACAAC GTCGATGG	AGTAACATCTTGAACG GAAAGTCCTT	73	Tasara & Stephen, 2007
rRNA[a]	CTTCCGCAATG GACGAAAGT	TGACGGAGCAACGCCG CGT	TTACGATCCGAAAACC TTCTTCA	66	Tasara & Stephen, 2007
1859	AAGAGCGCGAA GCTGAAAGATA	AAGGAAGTGCATTCATC AATTTGAGCTTTCC	CCTCATCTTGGAATCG TTCCA	78	This study
1865	AGTTCCTATCCC TGCGGAAGA	CATCCACAGCTTATGCT TCGGGATGAA	TTGGGATACTTGCCC GCATA	70	This study
1866	CACGTCATTAGT CTTCGCAGAGA	ATGCTGCCCGCCTTGG ATATGACA	ATTAAATCCTGCCATT GCTTTCC	74	This study
1883	TTCACCTCACTA TGCTCCACTACT G	CTATGCGGTTGGTGAC GGGAATGTAGA	CATTCCACCACCGCC ATT	72	This study
1884	AGATGAAACACC AGCTCAGACTG A	AGCCGGAACAAAGCAT CCAACCG	TGGCACTGCCGGTTT AGTAAC	73	This study
1901	TGATCCGCCGT ATTACGAAAC	AGGGTCACTACGCTGT GGTGTTCCGA	AAGCCGTGCATGATC TTCCT	70	This study
1891	ACGGCAGGCAG TGGTTATG	TGCCAACACAGGTGAG CAGGAAGA	AAGGCAGTTCCTTTTC CTCAGA	67	This study
1889	GGCGGGAAAAC CGAGAAG	AACACTTTGACAGCCCA GAACACGCC	TGCGGCGTACCACAT TGA	70	This study
1885	CGGCGATGTGG TGGAATAC	TGTCGGGCTTGGAGGT AACAGTT	GAGCCAGCGGAGCAT TTC	65	This study
1882	CCGCTTTTGCAG CAGGAA	AGGCGATGTTGCCGGA GCGATT	GGGATGCAGTCGTCC ATGTC	66	This study
1876	CACTTACCGCTG CGTTTGG	CGCTCCGAGAATACCC GACTGCTACA	TGTCGAAGAATTGCCT GCTCTT	69	This study
1873	ACAGCATTCGTA TGAAAGCCATT	TTTACGCTTTGTTTTTCG GCACGGAA	TCGTCAGCGTTCATTT TGAGA	73	This study
1864	ACCGGGAGATG CTGACAATT	CTGATGAATACGGTGG CAAGCTGCC	CTGCGAAAATCATCAC ACGATT	69	This study
1863	TCTCCCGCAGTA CCGTGAA	CGTGCCATTGCTGACCT TGAA	CCTTGGAGAGATGGC CAGTCT	63	This study
1862	AAATGTCTGATG GACTTACAAAGC TATT	CCAAGTGCAGGGATGT TCATAGCATTTATCCTA	GCACTAACCGGTATTT TTTTTAATGC	109	This study
1861	GGAAATAGAAG CTTTCGCCAGTA T	CGCAGGAACAACGTAG GGCTATATCCG	GGAACTCCCGAGAAA AGAAAATG	79	This study
1857	CACTGCCGCCA AGAAAAAAA	CCAGCTTCTTGACAGTA ATGGTTTTGACCGA	GCAACTCTGTCCGAG CAATTT	77	This study

a - Housekeeping gene that is not encoded by LGI1

Figure 9: The LGI1 genes selected as target for the TaqMan based real-time PCR screening assay.


ATATAGATGACATAGG
ACTAATATTAGT
CCTACGACATCATTAT
5 kbp
lmo1703
cpaB
cpaF
tadB
tadC
tadG
virB6
virB4
virB1
copG
druG
primase
virD4
repL
emrE
site-specific serine recombinases
lmo1702
sel1
Targets for RT-PCR assay

2.6 Bridging PCR screening

A series of 3 PCR reactions were designed to determine if LGI1 was encoded at the same chromosomal location. The PCR reactions designed were to amplify from the gene LM5578_1904 that is upstream of LGI1 into LM5578_1903, the first gene of LGI1; to amplify from the gene LM5578_1849 downstream of LGI1 into LM5578_1850 at the end of LGI1; to amplify from LM5578_1904 to LM5578_1849, the 2 genes that flank either sides of LGI1. For the isolates 95-0093 and 95-0151, PCR reactions were designed were to amplify from the gene LM5578_1951 that is upstream of LGI1 into LM5578_1903, the first gene of LGI1; to amplify from the gene LM5578_1950 downstream of LGI1 into LM5578_1850 at the end of LGI1; to amplify from LM5578_1950 to LM5578_1951, the 2 genes that flank either sides of LGI1.

The bridging polymerase chain reactions were performed using Invitrogen Platinum Taq Polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and with 1.0μM of each oligonucleotide primer (see table 3 for oligonucleotide sequences). The thermocycling parameters used were: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 minute; followed by a final extension at 72°C for 5 minutes. Amplicons were visualized on an ethidium bromide (Sigma-Aldrich) stained 1% TBE-agarose gel after electrophoresis for 60 minutes at 110V.

2.7 SNP PCR screening

Oligonucleotide primers were designed to amplify a 373 bp region encompassing the detected SNP (see Table 3 for oligonucleotide sequences). SNP screening was then performed on the entire panel of PCR was performed using Invitrogen High Fidelity Platinum Taq Polymerase (Invitrogen) according to manufacturer's instructions, and with

Table 3: The oligonucleotide primers used in this study. Oligo refers to oligonucleotide. Product size indicates the approximate length of the amplicon generated.

Oligo[a]	Target	Sequence (5'- 3')	Product Size (bp)	Reference
SOE-A	LM5578_1864	ggaattctgatggggatattcattgctt	349bp	This study
SOE-B		tgtttagtgctgctccagcatccgctgcttg taattatgg		
SOE-C	LM5578_1864	tgctggagcagcactaaaca	372bp	This study
SOE-D		acgtcctgcggagaacatcgctctaga		
bridge1849-1904F	LM5578_1849 to LM5578_1904	tcgctatatgcaaccagtcg	219bp	This study
bridge1849-1904R		ttggaaagcgaaaaagtctga		
bridge1849-1850F	LM5578_1849 to LM5578_1850	ttctggcactgataacgaagtt	472	This study
bridge1849-1850R		ttggaaagcgaaaaagtctga		
bridge1903-1904F	LM5578_1903 to LM5578_1904	tcgctatatgcaaccagtcg	473	This study
bridge1903-1904R		attctccagctgggctgtta		
bridge1950-1850F	LM5578_1950 to LM5578_1850	tgaaaagtttgagtcagtggaaga	916	This study
bridge1950-1850R		tgggccatagctatcctgtt		
bridge1903-1951F	LM5578_1903 to LM5578_1951	acaagatgctcgtgcaaatg	808	This study
bridge1903-1951R		tgaatcaaatttttccggatct		
LGI1_SNP2Fa		cctcacgcacaccatgatac	373	This study
LGI1_SNP2R		tccaaacatttcgtggtcaa		

a - F; forward primer, R; reverse primer.

1.0 µM of each oligonucleotide primer. The thermocycling parameters used were: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 68°C for 1 minute; followed by a final extension at 68°C for 5 minutes. Amplicons were visualized on a 1% TBE-agarose gel after electrophoresis for 60 minutes at 110V. PCR products were purified using the Montage PCR Centrifugal Filter Device kit (Millipore, Billerica, MA) according to manufacturer's instructions and eluted in 30 µl DNA and Nuclease Free water (Invitrogen). Confirmation of SNP sites was achieved by Sanger sequencing of the amplicons using the oligonucleotide primers that were used for the PCR amplification at a concentration of 1 µM.

2.8 Sequencing

PCR and amplicon purification was conducted as previously described. 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 ABI3730 apparatus (Applied Biosystems) with the primers used to generate the template. Sequence data was analyzed using SeqMan II™ (DNAStar Inc. Madison, WI).

2.9 Multi locus sequence typing

MLST was performed as previously described (Ragon et al., 2008). Amplicons were sequenced at the NML with 3730xl DNA analyzers (Applied Biosystems). Allele, ST, and clonal complex designations were assigned by Dr. Sylvain Brisse and colleagues at the Institut Pasteur, and deposited in a publicly available database at http://www.pasteur.fr. The minimum spanning tree was created and visualized using BioNumerics v5.10 software (Applied-Maths).

2.10 LGI1 expression

2.10.1 Growth conditions

Single colonies of *L. monocytogenes* 08-5578 were obtained after incubation for 18–24 h at 37°C. These single colonies were used to inoculate BHI broth (Becton, Dickinson and Company). The cultures were grown for 18 h with shaking (250 rpm) at 37°C to reach the stationary phase. This was confirmed by following the growth curve of each strain by absorption measurements at 600 nm. To assess expression in the normal laboratory growth condition, the cells were then processed for total RNA isolation. To assess expression in the cold stress model, the stationary-phase cultures were pelleted by centrifugation, and resuspended in fresh BHI broth (Becton, Dickinson and Company). Each culture was further subdivided into two aliquots, followed by a 4-h incubation at 4 and 35°C, respectively. The samples were then processed for total RNA isolation. To assess expression in the heat stress model, the stationary-phase cultures were pelleted by centrifugation, and resuspended in fresh BHI broth (Becton, Dickinson and Company). Each culture was further subdivided into two aliquots, followed by a 4 h incubation at 52 and 35°C, respectively. The samples were then processed for total RNA isolation. To assess expression after DNA damage with UV light, the stationary-phase cultures were exposed to UV light for 0 seconds, 10 seconds, 1 minute, 5 minutes, 10 minutes, 15 minutes and 30 minutes. The cultures were then processed for total RNA isolation. To assess expression after exposure to quarternary ammonium compounds, bacterial cutlure were grown in BHI broth containing 0 µg/mL, 1 µg/mL, 5 µg/mL and 10 µg/mL benzalkonium chloride (BCl) (Sigma-Aldrich) at 37°C for 18 hours. The cultures were then processed for total RNA isolation. Samples were performed in duplicate to ensure reproducibility of the data.

2.10.2 Total RNA isolation

Total RNA was isolated from each sample using the RNeasy mini kit with the RNA protect reagent (Qiagen) according to the manufacturer's instruction. Briefly, *Listeria monocytogenes* cells were added to 2 volumes of the RNA protect reagent (Qiagen) and incubated for 5 minutes at room temperature. The cells were pelleted by centrifugation at 5,000xg for 10 minutes. The cells were resuspended in 15 mg/mL lysozyme (Roche Diagnostics) and 10 mg/mL proteinase K (Fisher Scientific, Fair Lawn, NJ), followed by vortexing to lyse the cells. The lysates were then passed over a DNA-binding column provided in the kit, followed by an on-column DNAse I digestion (Roche Diagnostics) of the samples bound to the RNA-binding column. The RNA templates were eluted in 30 µL of RNAse free water. To further eliminate genomic DNA contamination, the samples were treated with Ambion Turbo DNA-free DNAse (Ambion Inc, Austin, TX) according to the double digestion protocol provided by the manufacturer.

2.10.3 cDNA prodution

The total isolated RNA was used to produce cDNA. Briefly, 2.0 µL RNA was combined with 1.0 µL 2.5x random primers (Invitrogen), 1.0 µL 10x dNTPs (Invitrogen) and 16.0µL DNase RNase free water (Invitrogen). The samples were heated to 65°C for 5 minutes then placed on ice for 1 minute. Then, 4.0 µL 5x First Strand buffer (Invitogen), 1.0 µL 0.1 M DTT (Invitrogen), 1.0 µL RNase inhibitor (Invitrogen) and 200 units of Super Script III reverse transcriptase (Invitrogen) were added to the samples. The samples were incubated at room temperatures for 5 minutes, then at 50°C for 60 minutes. The reaction was inactivated by incubation at 70°C for 5 minutes. As controls, similar amounts of total RNA of each sample were also subjected to the cDNA synthesis reaction without the reverse transcriptase enzyme. These provided the minus RT control

samples used in the gene-specific real-time PCR assays to assess the potential residual DNA contamination of each sample.

2.10.4 Quantitative real-time PCR

The gene expression was determined using the LGI1 TaqMan based assay described above with the cDNA and minus RT control samples. Two housekeeping genes, rRNA and bglA, were used to ensure standardization of the cDNA samples based upon the crossing points (CP) values as computed by the SmartCycler software (Cepheid) (Tasara and Stephen, 2007). The cDNA was diluted if necessary to obtain standardized concentrations. LGI1 genes were determined to be expressed based on the CP values crossing the threshold value computed by the SmartCycler software (Cepheid).

2.11 LM5578ΔLM5578_1864 mutant creation

An internal deletion mutant of LM5578_1864 (*virD4*) was created using the *E. coli-L. monocytogenes* shuttle vector pKSV7 and allelic exchange in *L. monocytogenes* according to the protocol by Camilli et al. (1993). The *virD4* internal deletion allele was created using splicing by overlap extension (SOE) PCR (see Table 3 for oligonucleotide primer sequences). SOE oligonucleotide primers (SOE-A and SOE-B) were designed to amplify a 349bp DNA fragment at the 5'end of virD4. The primer SOE-A contained a 5' *Eco-R1* restriction endonuclease site and the primer SOE-B contained an overhang complementary to the primer SOE-C. SOE-C and SOE-D primers were designed to amplify a 372 bp DNA fragment at the 3'end of *virD4*. The primer SOE-D contained an *XbaI* restriction endonuclease site. The 5' and 3' target regions were amplified separately by PCR using High Fidelity Taq Polymerase (Invitrogen) according to the manufacturer's instructions with 1.0 μM of each oligonucleotide primer and *L.*

monocytogenes 08-5578 genomic DNA as a template. The thermocycling parameters used were: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 68°C for 1 minute; followed by a final extension at 68°C for 5 minutes. The PCR products were electrophoresed on a 1% TBE-agarose gel and viewed under UV light following staining with ethidium bromide (Sigma-Aldrich). The amplified fragments were purified using the Montage PCR Centrifugal Filter Device kit (Millipore) according to manufacturer's instructions. A subsequent round of PCR amplification was performed using the SOE-A and SOE-D primers and the purified PCR products from the first PCR reaction as template DNA. This PCR was also performed using High Fidelity Taq Polymerase (Invitrogen) according to manufacturer's instructions with 1.0 μM of each oligonucleotide primer and the PCR products from the previous reactions as the DNA template. The thermocycling parameters used were: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 48°C for 30 seconds and 68°C for 1 minute; followed by a final extension at 68°C for 5 minutes. The PCR products were electrophoresed on a 1% TBE-agarose gel and viewed under UV light following staining with ethidium bromide (Sigma-Aldrich). The 721bp band product was cut out from the agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen). This PCR fragment and the pKSV7 (Cornell University, Ithaca, NY) shuttle vector were subsequently digested with *XbaI* (Roche Diagnostics) and *EcoRI* (New England BioLabs) endonucleases. The digested PCR fragment was then cloned into pKSV7 using T4 ligase enzyme. The resulting plasmid pJZ01 was electroporated into *E. coli* DH5-α as described below and transformants were selected on LB agar (Becton, Dickinson and Company) plates containing 100 μg/mL ampicillin (Sigma-Aldrich). The plasmid pJZ01 was harvested from the transformants using the Qiagen Plasmid Mini Kit (Qiagen) and electroporated into electrocompetent *L. monocytogenes* 08-5578 as described below. Transformants

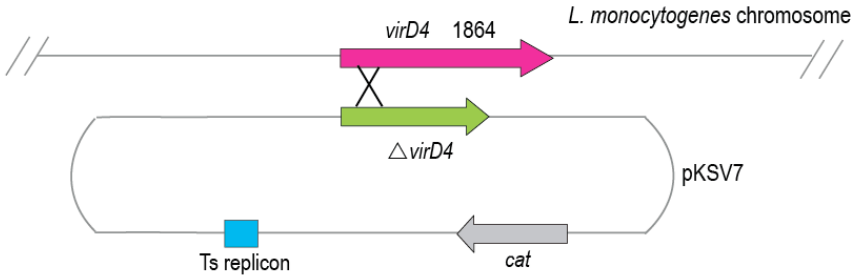
were selected on BHI agar plates containing 10µg/mL chloramphenicol (Sigma-Aldrich). A transformant was serially passaged at 42°C for 30 generations in BHI broth containing 10 µg/mL chloramphenicol to direct chromosomal integration of the plasmid by homologous recombination (Figure 10). The culture was diluted 1:100 in fresh BHI broth without antibiotics and incubated at 30°C for 50 generations to obtain an allelic exchange mutant. The culture was subsequently diluted 1:100 in frest BHI broth (Becton, Dickinson and Company) without antibiotics and incubated at 42°C for 30 generations to cure the excised plasmid. Single colonies were selected on BHI agar (Becton, Dickinson and Company) and allelic exchange mutagenesis was confirmed by PCR amplification using the SOE-A and SOE-D primers and Sanger sequencing.

2.11.1 Electrocompetent *L. monocytogenes* preparation

Electrocompetent *L. monocytogenes* cells were prepared using a modified version of the protocol developed by Park and Stewart (Park & Stewart, 1990). Briefly, a single colony of *L. monocytogenes* was subcultured into BHI broth (Becton, Dickinson and Company) and incubated overnight at 37°C with shaking. The culture was diluted 1:100 in BHI (Becton, Dickinson and Company) containing 0.5M sucrose (Sigma-Aldrich) and incubated at 37°C for 4-5 hours until OD_{600} was between 0.2 to 0.25. Penicillin G (Sigma-Aldrich) was added to a final concentration of 10 µg/mL and the culture was incubated for 2 more hours. The culture was added to a pre-chilled centrifuge bottle and centrifuged at 8000x g at 4°C for 10 minutes. The supernatant was decanted, the cells were washed twice with 5mL of ice cold 1 mM HEPES pH 7.0 (Sigma-Aldrich) containing 0.5 M sucrose (Sigma-Aldrich) diluted 1:2 with ice cold water. The supernatant was decanted and the cells were washed twice again with 2-5 mL ice cold 10% glycerol (Fisher Scientific) containing 0.5 M sucrose (Sigma-Aldrich). The supernatant was decanted and the cells were washed with 2-5 mL ice cold water. The

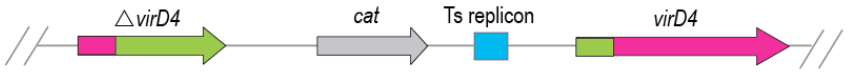
Figure 10: The molecular basis of homologous recombination of the plasmid vector pJZ01 and the *L. monocytogenes* 08-5578 chromosome during the creation of the LM5578_1864 gene deletion mutant.

A



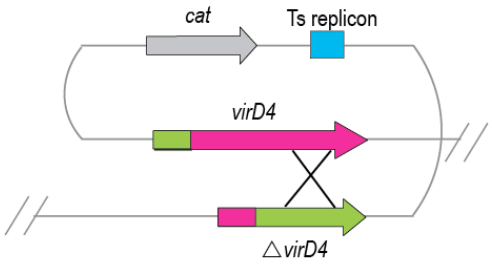
A single recombination event is induced by growth at the restrictive temperature of 42°C in the presence of chloramphenicol

B



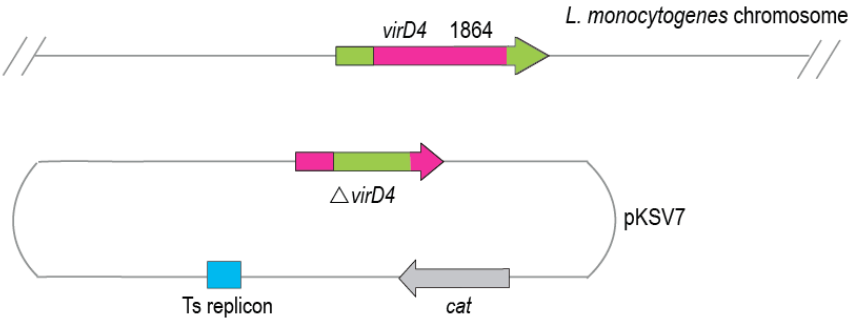
The plasmid is inserted into the *L. monocytogenes* chromosome

C



An additional single recombination event is induced by growth at the permissive temperature of 30°C in the absence of chloramphenicol

D



The excised plasmid is cured by sucessive rounds of replication in the absence of chloramphenicol

supernatant was decanted and the cells were resuspended in a minimal volume of 10% glyerol (Fisher Scientific) containing 0.5 M sucrose (Sigma-Aldrich). The cells were kept on ice and used in electroportations immediately following preparation.

2.11.2 Electroporations

Electroporation was carried out as described by Park and Stewart (1992). Briefly, 2 µl of the ligated plasmid was added to either 50 µl OneShot TOP10 Electrocompetent *E. coli* cells (Invitrogen) or 60 µL prepared electrocompetent *L. monocytogenes* 08-5578 and the mixture was transferred to a 0.1 cm chilled cuvette (Bio-Rad Laboratories, Hercules, CA). A BioRad Gene Pulser (Bio-Rad Laboratories) was used to apply a 1.1 kV pulse. Immediately following the pulse 1 ml of S.O.C medium (Invitrogen) was added to the cuvette containing *E. coli,* and 1 mL of BHI broth (Becton, Dickinson and Company) containing 0.5 M sucrose (Sigma-Aldrich) was added to the cuvette containing *L. monocytogenes*. The contents transferred to a sterile culture tube. Following incubation of the transformation culture at 35ºC for 60 min, 20 and 200 µl aliquots were plated onto the appropriate selective media and incubated for 24-72 hours at 35ºC.

2.12 Minimum inhibitory concentration assays

The MIC of several antimicrobial agents toward a subset panel of 7 isolates (Table 7) was determined by an agar dilution method according to the previously published method by Soumet et al. (2005). Each assay was performed in duplicate. Briefly, bacterial cultures were incubated overnight in BHI broth (Becton, Dickinson and Company) at 37°C with shaking. The cultures were then adjusted in Mueller-Hinton (MH) broth (Becton, Dickinson and Company) to an optical density value of 0.1 at 600 nm using a SmartSpec3000 instrument (Bio-Rad Laboratories). Serial dilutions in sterile

saline with subsequent plating on a BHIA plate were also performed to determine the cell densities and each culture contained approximately 10^8 bacteria per millilitre. A 10 µL sample of diluted culture was spread onto each plate containing the appropriate antimicrobial agent. For the crystal violet (Sigma-Aldrich) MIC assay, MH agar (Becton, Dickinson and Company) plates containing two-fold concentrations from 0.25 µg/mL to 4.0 µg/mL were made, inoculated with culture and incubated at 37°C for 24 and 48 hours. For ethidium bromide (Sigma-Aldrich), MH agar plates containing two-fold concentrations from 0.25 µg/mL to 40.0 µg/mL were made, inoculated with culture and incubated at 37°C for 24 and 48 hours. The plates were subsequently inspected for fluorescence under UV light to determine if the ethidium bromide had accumulated inside the cells (red fluorescence) or if it was effluxed out of the cells (white colonies with red fluorescent border). For BCl and BeCl (Sigma-Aldrich), MH plates (Becton, Dickinson and Company) supplemented with 2% sheep blood containing two-fold concentrations of each respective compound were made, inoculated with culture and incubated at 25°C and 37°C for 24 and 48 hours. For the BCl assay, plates were also incubated at 4°C for up to 4 weeks.

2.13 Antibiotic susceptibility test

The antibiotic susceptibility of the subset panel of 7 isolates was determined using Sensititre STP5 MIC Susceptibility 96-well plates (TREK Diagnostic Systems Inc., Clevland, OH) according to manufacturer instructions. The isolate *S. pneumoniae* ATCC 49619 was used as a control organism, as specified by manufacturer's instructions. Briefly, bacteria were emulsified in isotonic sterile saline to a turbidity equal to the 0.5 McFarland standard and 10-100 µL of this culture was added to the supplied tube of cation-adjusted MH broth containing lysed horse blood. Subsequently, 100 µL of this solution was added to each well of the Sensititre STP5 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. 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 Columbia Blood Agar (CBAB; Becton, Dickinson and Company) 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.

2.14 Motility

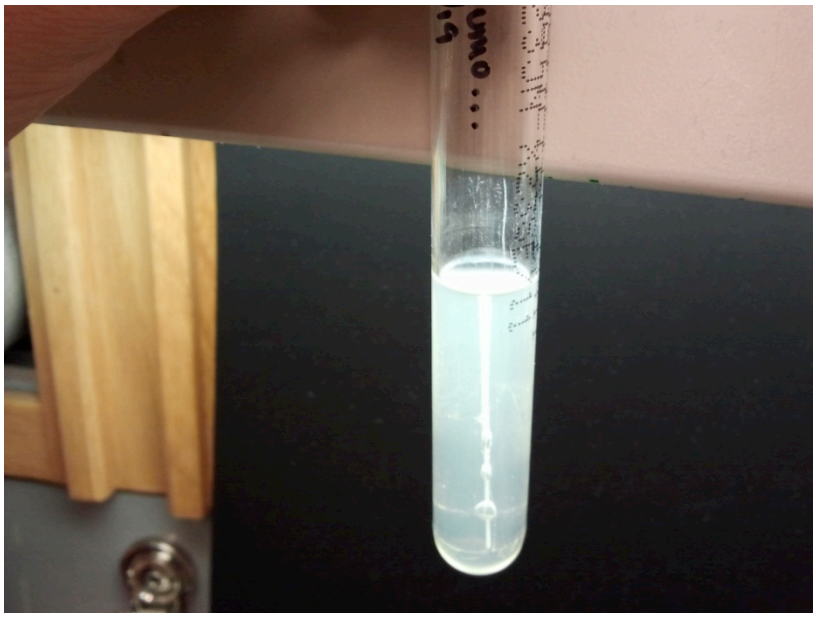
The motility of the subset panel of 7 isolates was determined using semi-solid agar slants. The isolates were grown overnight in BHI broth and in BHI broth (Becton, Dickinson and Company) containing 1.0 µg/mL BCl (Sigma-Aldrich) to induce LGI1 expression. A loopful of bacterial culture was stabbed through the middle of the slant. The slants were incubated for 48 hours at 25°C and 37°C. *L. monocytogenes* are usually motile at 25°C but motility is suppressed at temperatures above 35°C. Motility was observed based on the dispersion of the growth through the media (Figure 11).

2.15 Biofilms

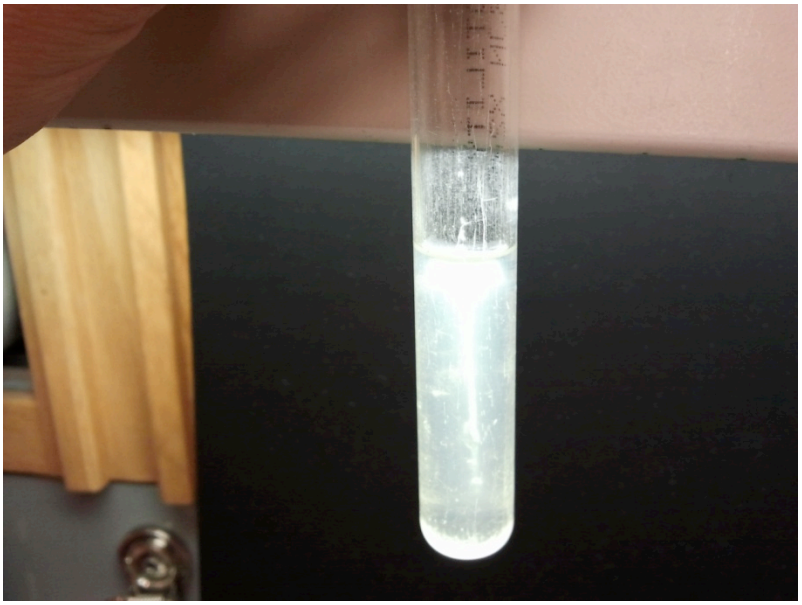
The biofilm forming ability of the subset panel of 7 isolates was determined according to a method developed by combination of the Calgary Biofilm Device protocol (Ceri et al., 1999) and the microtitre plate assay protocol (Djordjevic et al., 2002). Isolates were grown overnight on BHI agar (Becton, Dickinson and Company) at 35°C. A single colony was used to inoculate BHI broth (Becton, Dickinson and Company), and the culture was incubated overnight at 37°C with shaking at 200 rpm. The culture was diluted 1:40 in Modified Welshimer's broth (MWB) and 150 µL of culture was added to

Figure 11: The appearance of non-motile (A) and motile (B) *L. monocytogenes* in semi-solid agar.

A)

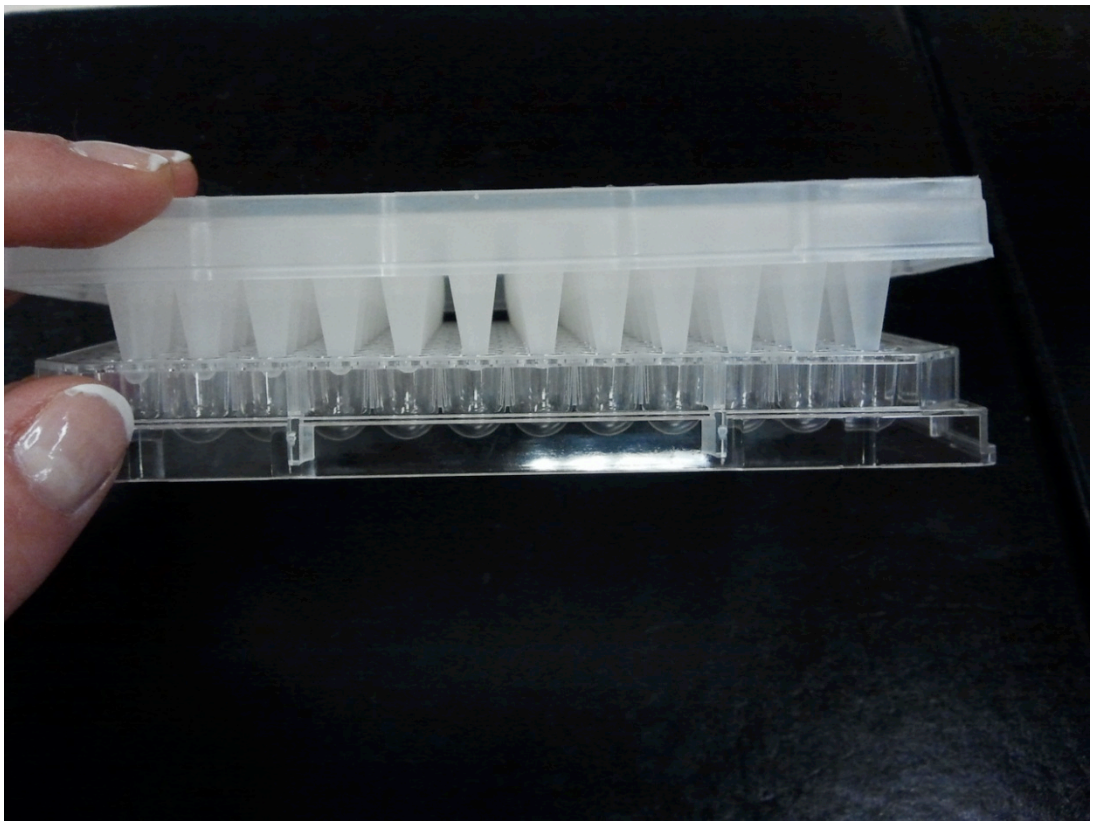


B)



each well of a sterile round bottom 96-well tissue culture plate (Thermo Scientific). A row of 8 wells was filled with 150 µL MWB to serve as a negative control. A sterile 96-well PCR plate (Applied Biosystems) 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 12). The cultures were incubated at 25°C for 48 hours because previous studies have determined that biofilm formation is inhibited above this temperature since motility is suppressed. [Note: Motility is a factor in biofilm formation and allows the bacteria to travel to the biofim 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). All isolates used in this study were confirmed to be motile at 25°C according to the protocol outlined in section 2.14]. The PCR plate was then removed from the tissue culture plate and the wells were inspected for turbidity in the sample wells and no turbidity in the negative control wells. The PCR plate was washed in a series of 4 water baths with shaking for 30 seconds each to remove the loosely adhering cells. The PCR plate was dried inverted 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 immunoassay plate (Thermo Scientific). Crystal violet binds to the bacterial cells and it therefore binds to the biofilm formed on 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 each to remove unbound crystal violet. The plate was dried inverted for 30 minutes and visual results were recorded. To destain 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

Figure 12: 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 in 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.



optical density at 580 nm (the absorbance wavelength of crystal violet) was then determined. The optical density corresponded to the concentration of crystal violet in each well, therefore a higher optical density compared to a greater concentration of biofilm.

2.16 Conjugation

L. monocytogenes 08-5578 strains were used as LGI1 donor strains. Recipient *L. monocytogenes* EGDe was serially passaged against rifampicin (Sigma-Aldrich) until resistance to 750 µg/ml was achieved. Rifampicin (500 µg/ml) (Sigma-Aldrich) was used as a selective agent for the recipient strains, and BHI broth (Becton, Dickinson and Company) containing BCl (1.0 µg/mL) (Sigma-Aldrich) was used to induce LGI1 in isolate 05-5578 prior to mating experiments. The LGI1 RT-PCR assay described above was used to indicate the presence or absence of LGI1 following mating according to the PCR protocol described above.

2.16.1 Cross-hatch mating

Single colonies of donor and recipient strains were inoculated into BHI broth (Becton, Dickinson and Company) containing BCl (Sigma-Aldrich) and rifampicin (Sigma-Aldrich) respectively and grown for 5 hours at 35ºC with shaking at 200 rpm. Recipient and donor strains were streaked out onto BHI agar (BD Diagnositics, Sparks, MD) in a crosshatch pattern and incubated overnight at 35ºC. Colonies at right angle intersections were resuspended in 1 ml of 1xTE buffer and LGI1 transconjugants were selected by plating onto BHI agar (Becton, Dickinson and Company) containing 500 µg/mL rifampicin (Sigma-Aldrich), followed by overnight incubation at 35°C. DNA template was made from the transconjugant colonies according to the boiled cell method and the colonies were screened for the presence of LGI1.

2.16.2 Filter mating

Single colonies of donor and recipient strains were inoculated into BHI broth (Becton, Dickinson and Company) containing BCI (Sigma-Aldrich) and rifampicin (Sigma-Aldrich) respectively and grown for 5 hours at 35ºC with shaking at 200 rpm. Donor and recipient cultures were mixed in a ratio of 1:10 on a 0.22 µm 150 mL bottle top filter (Corning Incorporated, Corning, NY) and vacuum filtered. The filter paper was removed from the filter device, placed onto a BHI agar (Becton, Dickinson and Company) plate containing 500 µg/mL rifampicin (Sigma-Aldrich), and incubated overnight at 35°C. Single colonies were selected, DNA template was made according to the boiled cell method, and the colonies were screened for the presence of LGI1.

2.16.3 Liquid mating

Single colonies of donor and recipient strains were inoculated into BHI broth (Becton, Dickinson and Company) containing BCI (Sigma-Aldrich) and rifampicin (Sigma-Aldrich) respectively and grown overnight at 35ºC with shaking at 200 rpm. The donor and recipient overnight cultures were combined, diluted 1:50 in BHI broth (Becton, Dickinson and Company), and incubated for 48 hours at 35ºC with shaking at 200 rpm. The culture was then diluted 1:50 in BHI broth (Becton, Dickinson and Company) containing 500 µg/mL rifampicin (Sigma-Aldrich). The culture was serially passaged twice more in BHI broth (Becton, Dickinson and Company) containing 500µg/mL rifampicin (Sigma-Aldrich) to select for transconjugants. The cells were then pelleted by centrifugation at 10,000 x g for 10 minutes. The cells were washed in phosphate buffered saline three times to remove excess rifampicin (Sigma-Aldrich). After the final wash, cells were resuspended in 1xTE buffer and DNA template was made according to the boiled cell method. The cell pellet was subsequently screened for the presence of LGI1.

Chapter 3

Results

3. Results

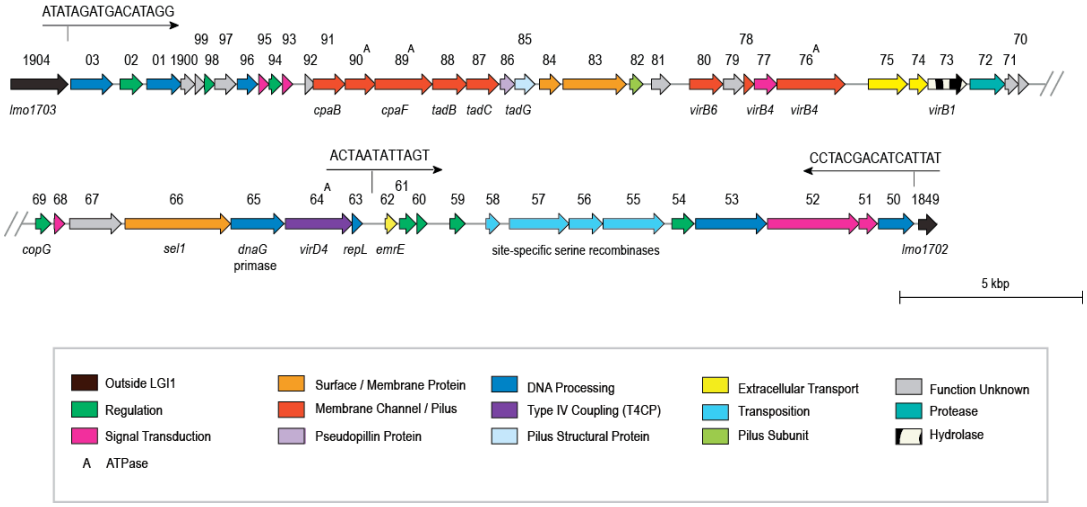
3.1 LGI1 encodes genes homologous to known secretion systems, virulence factors and mobilization proteins

During the 2008 listeriosis outbreak, genome sequencing was completed for 2 primary outbreak isolates, which had different but related *ApaI* PFGE patterns. Within the genomes of both isolates, the novel LGI1 was identified and a rapid bioinformatic analysis was performed to determine a putative function for this island. This preliminary analysis found that the LGI1 encoded genes related to T2SS and T4SS, regulatory proteins, a small multi-drug resistance protein, site-specific serine recombinases, as well as several genes of unknown function. Additionally, many of the predicted functions were determined by very distant homologies, therefore the function of LGI1 was largely speculative. Thus, one objective of this study was to build upon the preliminary data observed during the genome sequencing study to more definitively assign a function to the LGI1. In order to do so, a more thorough, in-depth bioinformatic analysis of each gene encoded by the LGI1 was necessary to facilitate and develop future experiments.

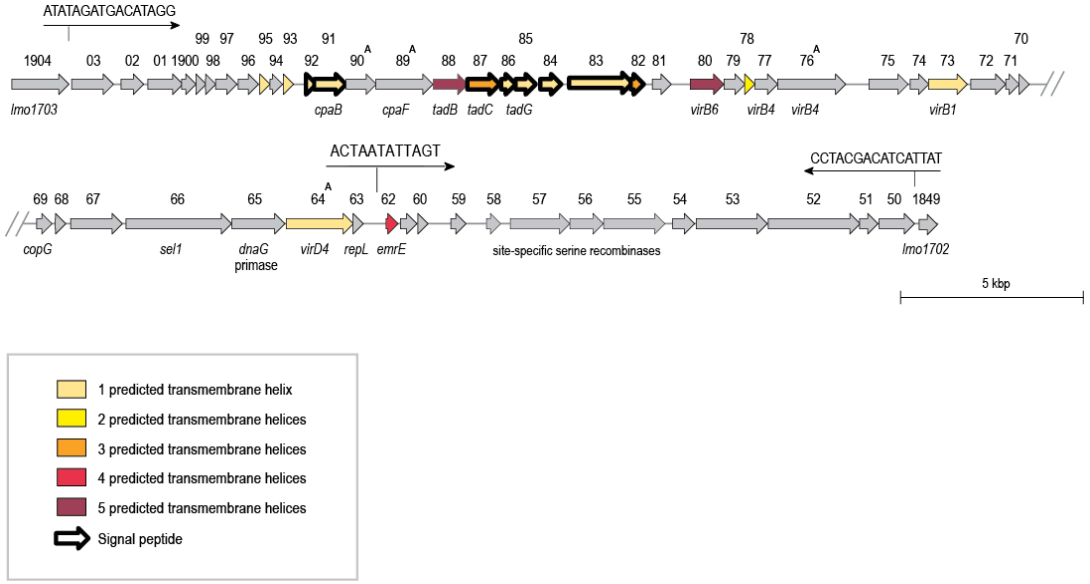
Using the GenDB genome annotation software package, bioinformatic analyses were performed on each of the genes of LGI1 to determine their putative individual function, and the function of LGI1 in its entirety (Figure 13A). Within the GenDB software package the protein family databases Pfam and TIGRFAM were used as the principal indicators of gene function if a known protein family spanned most of the query gene. The majority of the genes had the same predicted function as the results of the preliminary analysis, however, the function of several genes previously determined to be of unknown function was predicted and the annotations of several genes was refined. The results confirm that LGI1 encodes genes related to secretion systems, including a putative T4CP, pilus subunit, structural and pseudopillin protein homologs, several

Figure 13: The predicted function of the LGI genes (A) and the LGI1 genes with predicted signal peptide sequences and transmembrane helices (B).

A)


ATATAGATGACATAGG
1904 03 02 01 1900 99 98 97 96 95 94 93 92 91 90A 89A 88 87 86 85 84 83 82 81 80 79 78 77 76A 75 74 73 72 71 70
lmo1703 cpaB cpaF tadB tadC tadG virB6 virB4 virB4 virB1
ACTAATATTAGT
CCTACGACATCATTAT
69 68 67 66 65 64A 63 62 61 60 59 58 57 56 55 54 53 52 51 50 1849
copG sel1 dnaG primase virD4 repL emrE site-specific serine recombinases lmo1702
5 kbp
Outside LGI1
Regulation
Signal Transduction
A ATPase
Surface / Membrane Protein
Membrane Channel / Pilus
Pseudopillin Protein
DNA Processing
Type IV Coupling (T4CP)
Pilus Structural Protein
Extracellular Transport
Transposition
Pilus Subunit
Function Unknown
Protease
Hydrolase

B)


ATATAGATGACATAGG
1904 03 02 01 1900 99 98 97 96 95 94 93 92 91 90A 89A 88 87 86 85 84 83 82 81 80 79 78 77 76A 75 74 73 72 71 70
lmo1703 cpaB cpaF tadB tadC tadG virB6 virB4 virB4 virB1
ACTAATATTAGT
CCTACGACATCATTAT
69 68 67 66 65 64A 63 62 60 59 58 57 56 55 54 53 52 51 50 1849
copG sel1 dnaG primase virD4 repL emrE site-specific serine recombinases lmo1702
5 kbp
1 predicted transmembrane helix
2 predicted transmembrane helices
3 predicted transmembrane helices
4 predicted transmembrane helices
5 predicted transmembrane helices
Signal peptide

membrane channel homologs with ATPase domains, proteins involved in extracellular transport, as well as a putative hydrolase and protease that could function in breaking down the peptidoglycan layer of the Gram-positive bacterial cell wall (Table 4). Additionally, several genes with predicted DNA processing and signal transduction functions were observed, further suggesting a role for LGI1 in secretion.

The in-depth bioinfomatic analysis also enabled the detection of a predicted operon. The gene LM5578_1862, a putative SMR efflux pump, is predicted to be regulated by the gene LM5578_1861, a gene homologous to the repressor MarR and with a winged helix-turn-helix domain that is characteristic of regulatory proteins. This domain allows the protein to clamp onto DNA to prevent transcription. Between the -10 and -35 promoter elements of LM5578_1862, a palindrome sequence that is similar to known repressor binding sites was also detected (Alekshun & Levy, 1997; Evans et al., 2001; Wilkinson & Grove, 2006). This further suggests that LM5578_1861 may act as a repressor for LM5578_1862.

The GenDB software was also used to determine if a predicted signal peptide and/or transmembrane helix existed to better characterize the putative functions of each gene (Figure 13B). The gene LM5578_1862, a predicted SMR efflux pump, encodes 4 putative transmembrane helices, which is characteristic of SMR proteins. Therefore, further evidence is provided for this gene's function as an SMR protein. Additionally, several of the genes predicted to be involved in a secretion system also encode putative transmembrane helices.

3.2 LGI1 was only detected in isolates belonging to the MLST CC8

When the genome sequences of the 2 primary outbreak isolates were compared to all the publicly available bacterial genomes, LGI1 was only encoded by the outbreak

Table 4: The bioinformatics analysis observations and predicted function of each gene encoded by LGI1.

Locus identification	Gene name	Functional Annotation	Putative function
LM5578_1903		Superfamily II DNA/RNA helicases; related to transcription factors involved in chromatin remodeling	DNA/RNA helicase
LM5578_1902		Distant homologies to transferases, signal transduction domains, molecular chaperone, transcription initiation factor	Regulatory, signal transduction or transcription regulation
LM5578_1901		DNA methylase, DNA methyltransferase and DNA adenine methylase motifs	DNA methylase
LM5578_1900		Distant homologies to dynamin-like protein involved in membrane fission, fusion, and restructuring events	Membrane restructuring
LM5578_1899		Distant homologies to protein with ubiquitin fusion degradation motif, HSP20 like motif of unknown function	Unknown
LM5578_1898		Cell division MraZ protein; transcriptional regulator AbrB family; DNA binding transcription factors	Regulation
LM5578_1897		Phospholipase D/transphosphatidylase; ABC transporter	Phospholipid hydrolysis or substrate transport
LM5578_1896		Ubiquitin specific peptidase; DNA primase	DNA processing
LM5578_1895		Distant homologies to permease YjgP/YjgQ family	Permease or signal transduction
LM5578_1894		Septation protein SpoVG essential for protulation; uncharacterized protein involved in regulation of septum location	Regulation
LM5578_1893		Related to predicted symporter, predicted signal transduction protein with cAMP binding and CBS domains	Symporter involved in signal transduction
LM5578_1892		Poor results	Unknown
LM5578_1891	*cpaB*	Flp pilus assembly protein CpaB; related to CpaE, CpaC ATPase, TadB, TadC	Pilus assembly
LM5578_1890		ATPase involved in chromosome partitioning; related to flagella GTP-binding protein; related to flagella motor switch protein	Flagella GTP binding protein/motor switch
LM5578_1889	*cpaF*	CpaF ATPase pilus assembly protein; Type II/IV secretion system protein; T4SS VirB11 components and related to ATPases involved in archaic flagella biosynthesis	Pilus assembly
LM5578_1888	*tadB*	Flp pilus assembly protein TadB	Pilus assembly
LM5578_1887	*tadC*	Flp pilus assembly protein TadC	Pilus assembly
LM5578_1886		TadE pseudopilin protein	Pseudopilin protein
LM5578_1885	*tadG*	TadG structural subunit of Flp pilli that may be involved in anchoring pilus to cell	Pilus structural protein/anchoring protein
LM5578_1884		Serine-aspartate repeat containing protein C; related to cell surface protein in *S. aureus* which may be involved in interaction with extracellular matrix of eukaryotes	Cell surface protein
LM5578_1883		Tryptophan rich region motif; orthologous to putative outer membrane adhesion like protein	Adhesion
LM5578_1882		TrbC/VirB2 family motif (pilin like protein)	Pilus subunit
LM5578_1881		Poor results	Unknown
LM5578_1880	*virB6*	VirB6 structural component of T4SS transport apparatus	Stuctural protein of T4SS apparatus
LM5578_1879		Poor results	Unknown
LM5578_1878		ABC-2 type transporter, transmembrane transporter protein; DNA methylase, DNA methyltransferase	Transport or DNA methylase
LM5578_1877	*virB4*	Histidine kinase; translocon associated protein gamma subunit	Signal transduction
LM5578_1876	*virB4*	VirB4 ATPase component of T4SS that provides energy for translocation of virulence	ATPase translocation of effectors

		proteins	
LM5578_1875		Amidoligase; ABC-type bacteriocin antibiotic exporter	Extracellular transport
LM5578_1874		AIG-2 like family protein that may bind small ligands; family also includes bacteria tellurite resistance proteins.	Extracellular transport
LM5578_1873	*virB1*	Cell wall associated hydrolase invasion associated protein	Hydrolase
LM5578_1872		Thiol (cystine) proteases histidine; aminopeptidase	Protease
LM5578_1871		Distant homologies to methyl-accepting chemotaxis protein, glycosy hydrolase family protein, oligonucleotide/oligosaccharide-binding fold, helicase, peptidase	Unknown
LM5578_1870		Poor results	Unknown
LM5578_1869	*copG*	Ribbon-helix-helix protein; CopG transcriptional regulator	Transcriptional regulator
LM5578_1868		Membrane fusion protein; bacterial checkpoint controller; regulatory subunit of type II PKA R-subunit	Signal transduction or regulation
LM5578_1867		Poor results	Unknown
LM5578_1866	*sel1*	Sel1 repeat motif involved in mediating important protein-protein interaction	Mediates protein-protein interactions
LM5578_1865	*dnaG* primase	DnaG primase with Toprim domain	DNA processing
LM5578_1864	*virD4*	VirD4 T4SS coupling protein	T4SS coupling protein
LM5578_1863	*repL*	Repressor with helix-turn-helix motif that binds nucleic acid; transcription regulatory protein domains and motifs	Repressor
LM5578_1862	*emrE*	Small multidrug resistance protein	Multidrug resistance
LM5578_1861		Transcriptional regulator MarR family; regulation of genes involved in degradation of aromatic compounds, helix-turn-helix motif	Transcriptional regulator
LM5578_1860		Distant homology to GntR family transcriptional regulator	Transcriptional regulator
LM5578_1859		Sigma-70 region 4 motif; RNA polymerase sigma-24 subunit	RNA pol sigma subunit
LM5578_1858	Site-specific serine recombinases	Site specific serine recombinases/resolvases	Recombinases
LM5578_1857			
LM5578_1856			
LM5578_1855			
LM5578_1854		transcriptional regulator; conjugative transposon	Regulation
LM5578_1853		Adenine specific methyltransferase; Eco57I restriction endonuclease	DNA methylase
LM5578_1852		Histidine kinase; DNA mismatch repair protein	Signal transduction or DNA mismatch repair
LM5578_1851		Response regulator consisting of a CheY-like receiver domain and a helix-turn-helix domain	Signal transduction
LM5578_1850		Restriction endonuclease	Restriction endonuclease

isolates. However, regions of homology to LGI1 were identified within the genomes of other environmental firmicutes, and LGI1 encodes genes homologous to site-specific serine recombinases, suggesting that LGI1 may be horizontally acquired and broadly distributed amongst clinical, food and environmental isolates. Therefore, another objective of this study was to identify the distribution of LGI1 amongst *L. monocytogenes* isolates to indicate whether LGI1 was unique to the 2008 listeriosis outbreak isolates or if it was found in other *L. monocytogenes* isolates as well. Additionally, the LGI1 genomic regions have a skewed G/C content relative to neighboring sequences (Gilmour et al., 2010). A panel of 126 *L. monocytogenes* isolates from clinical, food and environmental sources (Table 1) were screened with the real-time PCR assay that included 16 LGI1 gene targets (Figure 9). This panel of isolates included strains genetically related to the 2008 outbreak strains, but from different years and geographical locations of isolation. The panel also included strains with a diverse range of serotypes and PFGE patterns unrelated to the 2008 outbreak strains that were isolated from a wide range of sources, time periods and geographical locations. LGI1 was detected in 66 serotype 1/2a isolates and 1 serotype 3a isolate from Canada, and all 67 of these isolates encoded all 16 LGI1 screening targets (Table 5). The earliest isolate included in this panel that encoded LGI1 was from 1988. Notably, all isolates that encode LGI1 also belong to the MLST CC8 group (Figure 14).

Four isolates belonging to the MLST CC8 did not encode LGI1, despite being highly genetically related to the isolates that did encode LGI1 (Table 6). These strains were isolated between the years of 1999 to 2008 from human clinical samples in the provinces of Alberta and Ontario. These isolates also all had the *AscI* PFGE pattern LMACI.0002 and the *ApaI* PFGE pattern of either LMAAI.0001 or LMAAI.0214 (Figure 15). In comparison, CC8 isolates encoding LGI1 had the *AscI* PFGE patterns

Figure 14: The distribution of the MLST sequence types of the isolates in Canada. The MLST sequence types were assessed using the protocol by Ragon et al. (2008). The numbers within each circle represents the sequence type number. The grey numbers between each circle represents the number of SNPs between each sequence type. The size of each circle is proportional to the number of isolates with that sequence type. The sequence types circled with a grey box indicates the isolates that belong to the MLST CC8. The CC8 isolates encode LGI1 (Reimer, Ziegler et al., unpublished).

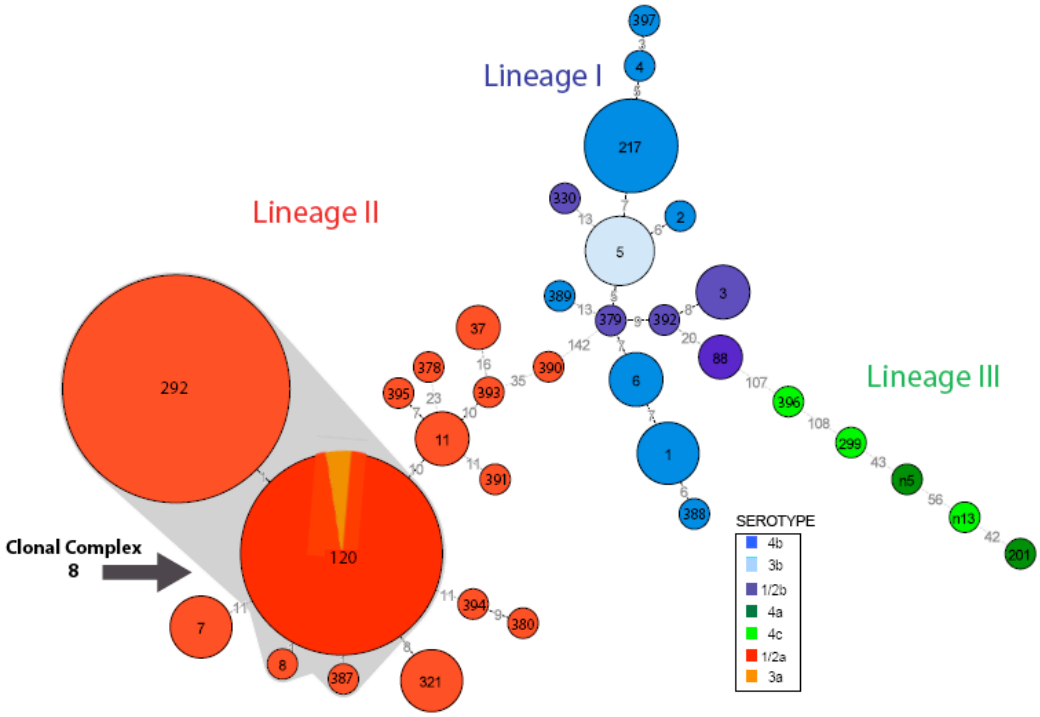

Lineage I
Lineage II
Lineage III
Clonal Complex
8
SEROTYPE
4b
3b
1/2b
4a
4c
1/2a
3a
397
4
217
330
2
5
3
389
379
392
88
37
378
390
6
395
393
11
1
391
388
396
299
n5
n13
201
292
120
7
8
387
321
394
380

Table 5: The results of the LGI1 RT-PCR screening assay and the bridging PCR that assessed the location of the genome where LGI1 was located. The complete table of results including individual gene data is included as appendix A.

Number of isolates	CC8[a]	LGI-1 (16 genes)	LM5578_1903 to LM5578_1904 PCR	LM5578_1849 to LM5578_1850 PCR	LM5578_1849 to LM5578_1904 PCR
65	+	+	+	+	-
2	+	+	-	-	+
4	+	-	-	-	-
44	-	-	-	-	+
11	-	-	-	-	-

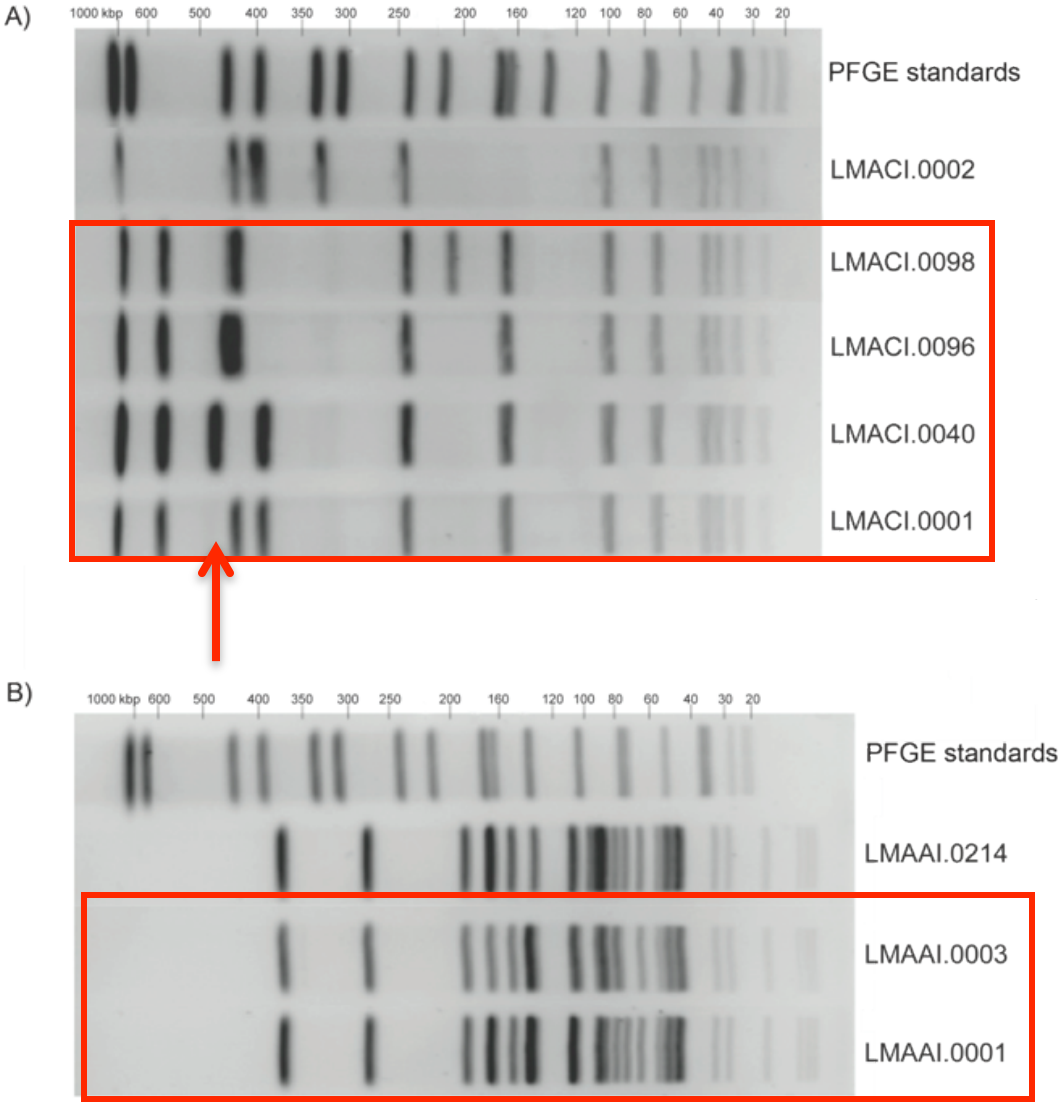
a - CC8 includes serotype 1/2a and 3a isolates

Table 6: The isolates belonging to the MLST CC8 that do not encode LGI1.

Isolate	Serotype	Source	Province	AscI	ApaI	MLST[a]	LGI1
01-5373	1/2a	Human clinical	ON	LMACI.0002	LMAAI.0001	ST120	-
99-3046	1/2a	Human clinical	ON	LMACI.0002	LMAAI.0001	ST120	-
08-5375	1/2a	Human clinical	ON	LMACI.0002	LMAAI.0214	ST120	-
03-5833	1/2a	Human clinical	AB	LMACI.0002	LMAAI.0214	ST120	-

a – MLST ST120 belongs to the CC8 group

Figure 15: The PFGE patterns of *L. monocytogenes* isolates that encode LGI1 (marked by the red boxes) and of highly related isolates belonging to the MLST CC8 that do not encode LGI1. The image A represents the *AscI* pattern combinations and the image B represents the *ApaI* pattern combinations. The *ApaI* pattern LMAAI.0001 is associated with both isolates that encode and that do not encode LGI1. The band on the LMACI.0040 image indicated with an arrow is shifted compared to the LMACI.0001 image due to the insertion of the prophage LMC1.


A)
1000 kbp 600 500 400 350 300 250 200 160 120 100 80 60 40 30 20
PFGE standards
LMACI.0002
LMACI.0098
LMACI.0096
LMACI.0040
LMACI.0001
B)
1000 kbp 600 500 400 350 300 250 200 160 120 100 80 60 40 30 20
PFGE standards
LMAAI.0214
LMAAI.0003
LMAAI.0001

LMACI.0098, LMACI.0096, LMACI.0040 and LMACI.0001; and the *ApaI* PFGE patterns LMAAI.0003 and LMAAI.0001. These PFGE pattern combinations were determined to be more than 90% similar. LGI1 was also present within the highest molecular weight band on the restriction digest gels therefore the absence of LGI1, a 50kbp fragment, was not visible within the gel images.

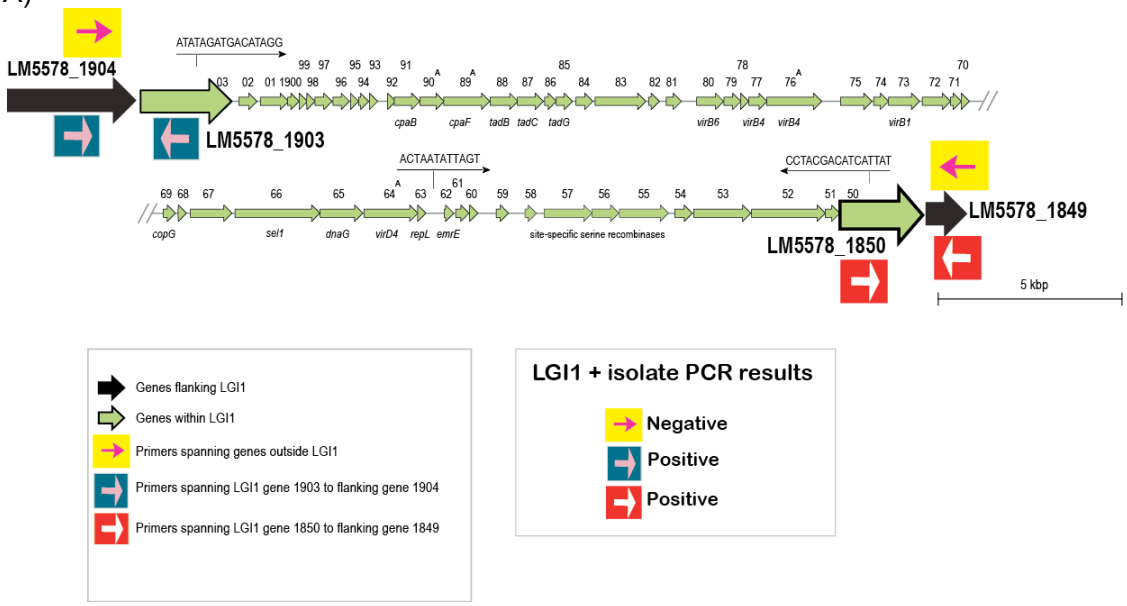
3.3 65 of 67 isolates encoded LGI1 at the same location within the genome

The series of 3 bridging PCR reactions was performed to assess if LGI1 was inserted within the same location in the chromosome for all isolates that encoded LGI1. For an isolate that lacks LGI1, the genes LM5578_1904 and LM5578_1849 are predicted to be adjacent and thus a product would be expected for only this PCR, whereas the other 2 PCR reactions would be negative (Figure 16A). However, for an isolate that does encode LGI1, it is predicted that LGI1 is inserted between these two genes, therefore a PCR spanning LM5578_1904 and LM5578_1849 would be negative, whereas a PCR spanning LM5578_1904 and the first gene of LGI1 and a PCR spanning LM5578_1849 and the last gene of LGI1 would both be positive (Figure 16A). Based on these PCR results, LGI1 was inserted at the same location within the genome in 65 of the 67 LGI1 positive isolates. Within these isolates, LGI1 was inserted between the genes LM5578_1904 and LM5578_1849.

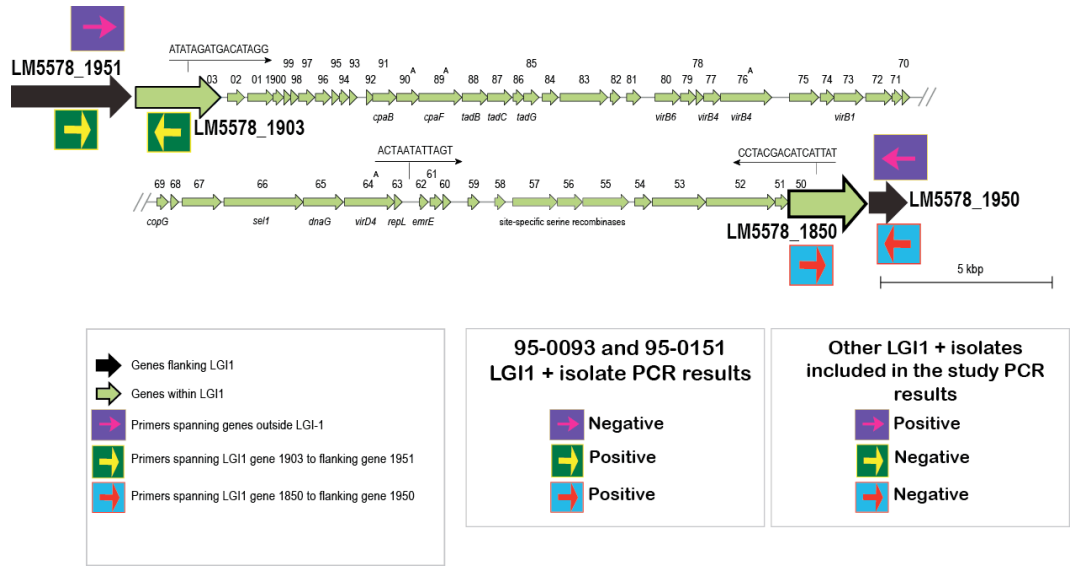
For two isolates (95-0093 and 95-0151), the bridging PCR results were characteristic of an isolate that does not encode LGI1 (Figure 16B). These isolates, 95-0093 and 95-0151, were both isolated from human clinical samples in 1995, however the isolates are from different geographical locations and they have different *ApaI* PFGE patterns. The isolate 95-0093 was isolated in Alberta and it has PFGE pattern LMACI.0001

Figure 16: The location of the bridging PCR primers and the expected PCR products for isolates encoding and lacking LGI1. A) The bridging PCR reactions for all LGI1 positive isolates included in this study except for isolates 95-0093 and 95-0151. B) The location of the bridging PCR primers and the expected PCR products for the isolates 95-0151 and 95-0093 that have LGI1 inserted at a different location within the genome.

A)


LM5578_1904
ATATAGATGACATAGG
LM5578_1903
cpaB cpaF tadB tadC tadG virB6 virB4 virB4 virB1
ACTAATATTAGT
CCTACGACATCATTAT
LM5578_1849
LM5578_1850
copG sel1 dnaG virD4 repL emrE site-specific serine recombinases
5 kbp
Genes flanking LGI1
Genes within LGI1
Primers spanning genes outside LGI1
Primers spanning LGI1 gene 1903 to flanking gene 1904
Primers spanning LGI1 gene 1850 to flanking gene 1849
LGI1 + isolate PCR results
Negative
Positive
Positive

B)

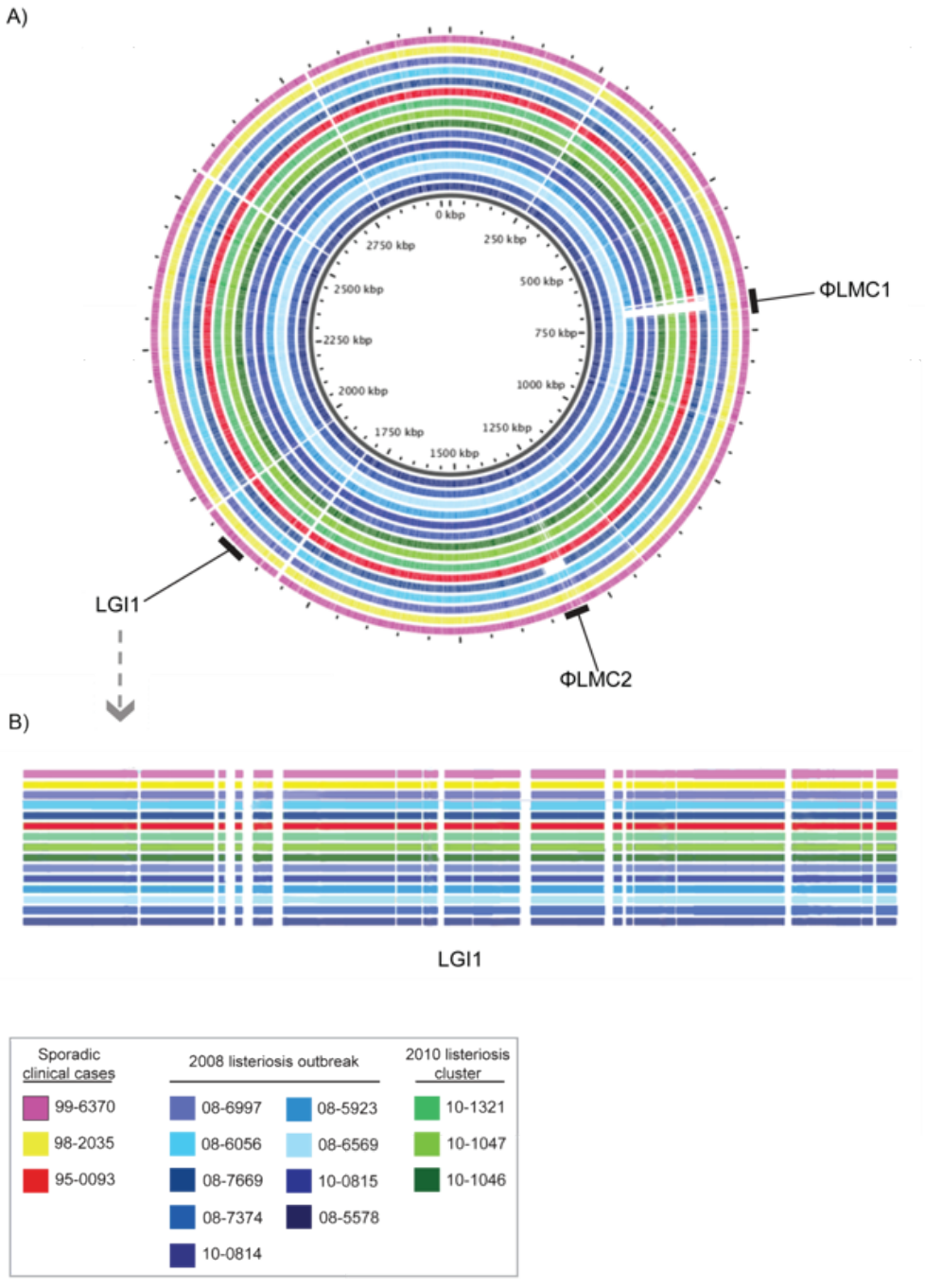

LM5578_1951
ATATAGATGACATAGG
LM5578_1903
cpaB cpaF tadB tadC tadG virB6 virB4 virB4 virB1
ACTAATATTAGT
CCTACGACATCATTAT
LM5578_1950
LM5578_1850
copG sel1 dnaG virD4 repL emrE site-specific serine recombinases
5 kbp
Genes flanking LGI1
Genes within LGI1
Primers spanning genes outside LGI-1
Primers spanning LGI1 gene 1903 to flanking gene 1951
Primers spanning LGI1 gene 1850 to flanking gene 1950
95-0093 and 95-0151 LGI1 + isolate PCR results
Negative
Positive
Positive
Other LGI1 + isolates included in the study PCR results
Positive
Negative
Negative

LMAAI.0001, whereas the isolate 95-0151 was isolated from Ontario and it has the PFGE pattern LMACI.0001 LMAAI.0003. Whole genome sequencing was previously performed on the isolate 95-0093, therefore the genome sequencing data was analyzed to determine where LGI1 was inserted. Corresponding PCR oligonucleotide primers similar in design principals to the other bridging reaction primers were used to screen isolate 95-0151. In these 2 isolates, LGI1 was inserted between the genes LM5578_1951 and LM5578_1950, approximately 100 genes downstream of the insertion site in the other isolates. For these 2 isolates that encode LGI1 at a different location within the genome, the PCR for the genes LM5578_1950 and LM5578_1951 was negative because LGI1 is suspected to be inserted between these two genes, whereas a product was observed for the other 2 PCR reactions (Figure 16B). The genes LM5578_1951 and LM5578_1950 are adjacent in the other 65 isolates that encode LGI1 therefore a second insertion site for LGI1 was identified.

3.4 LGI1 was highly genetically conserved

The complete LGI1 sequences of 15 isolates with whole genome sequence data available were compared to assess the variability amongst the LGI1 sequences. These isolates were isolated between the years of 1995 and 2010 from food, environmental and clinical sources. The entire genomes of these isolates exhibited a low degree of genomic variability (Figure 17A). Unrelated to LGI1, but as expected due to previous findings (Gilmour et al., 2010), the isolates with the *AscI* PFGE pattern LMACI.0040 encoded the phage ΦLMC1, whereas the isolates with the *AscI* PFGE pattern LMACI.0001 did not encoded this phage (Figure 17B). The isolate 08-7669 also lacked the phage ΦLMC2, although it was not detectable by a change in PFGE pattern. Accordingly, when the LGI1 sequences were compared, the majority of sequences were identical and only 1 SNP was identified that was distributed to 3 of the isolates. This

Figure 17: Blast atlas of predicted protein homologies mapped against the closed genome of the 2008 *L. monocytogenes* outbreak isolate 08-5578. Full color saturation represents 100% sequence homology while gaps indicate regions of divergence. Gaps in the innermost (dark blue) circle for reference isolate 08-5578 represent gaps between coding sequences, not genetic diversity. A, Entire chromosomes; B, Zoomed in view of the LGI1 sequences. From center: isolates with the PFGE patterns LMACI.0040 LMAAI.0001 08-5578, 10-0815 and 08-6569; isolates with the PFGE patterns LMACI.0001 LMAAI.0001 08-5923, 10-0814, 08-5374, 10-1046, 10-1047, 10-1321, 95-0093 and 08-7669; isolates with the PFGE patterns LMACI.0040 LMAAI.0003 08-6056, 08-6997, 98-2035 and 99-6370. Isolates with the *AscI* PFGE pattern LMACI.0040 encode a prophage ΦLMC1 that is absent in the genomes of isolates with the *AscI* PFGE pattern LMACI.0001. The isolate 08-7669 lacks the prophage ΦLMC1. The blue sequences are from isolates associated with the 2008 nationwide listeriosis outbreak, the green sequences are from isolates associated with a 2010 listeriosis cluster and the yellow, pink and red sequences are from isolates from sporadic clinical cases. The LGI1 sequences were genetically identical except for a single SNP encoded by the 3 isolates from the 2010 cluster. White spaces in figure B represent gaps between the predicted coding sequences, not genetic diversity.


A)
0 kbp
250 kbp
500 kbp
750 kbp
1000 kbp
1250 kbp
1500 kbp
1750 kbp
2000 kbp
2250 kbp
2500 kbp
2750 kbp
ΦLMC1
LGI1
ΦLMC2
B)
LGI1
Sporadic clinical cases
99-6370
98-2035
95-0093
2008 listeriosis outbreak
08-6997
08-5923
08-6056
08-6569
08-7669
10-0815
08-7374
08-5578
10-0814
2010 listeriosis cluster
10-1321
10-1047
10-1046

synonymous SNP was located within locus LM5578_1886, a predicted pseudopillin protein of the putative secretion system, and was only encoded by 3 isolates from a 2010 cluster linked to prosciutto ham. Furthermore, all 67 isolates that encoded LGI1 were positive for all 16 of the LGI1 screening targets, indicating conservation of gene content across all instances of LGI1.

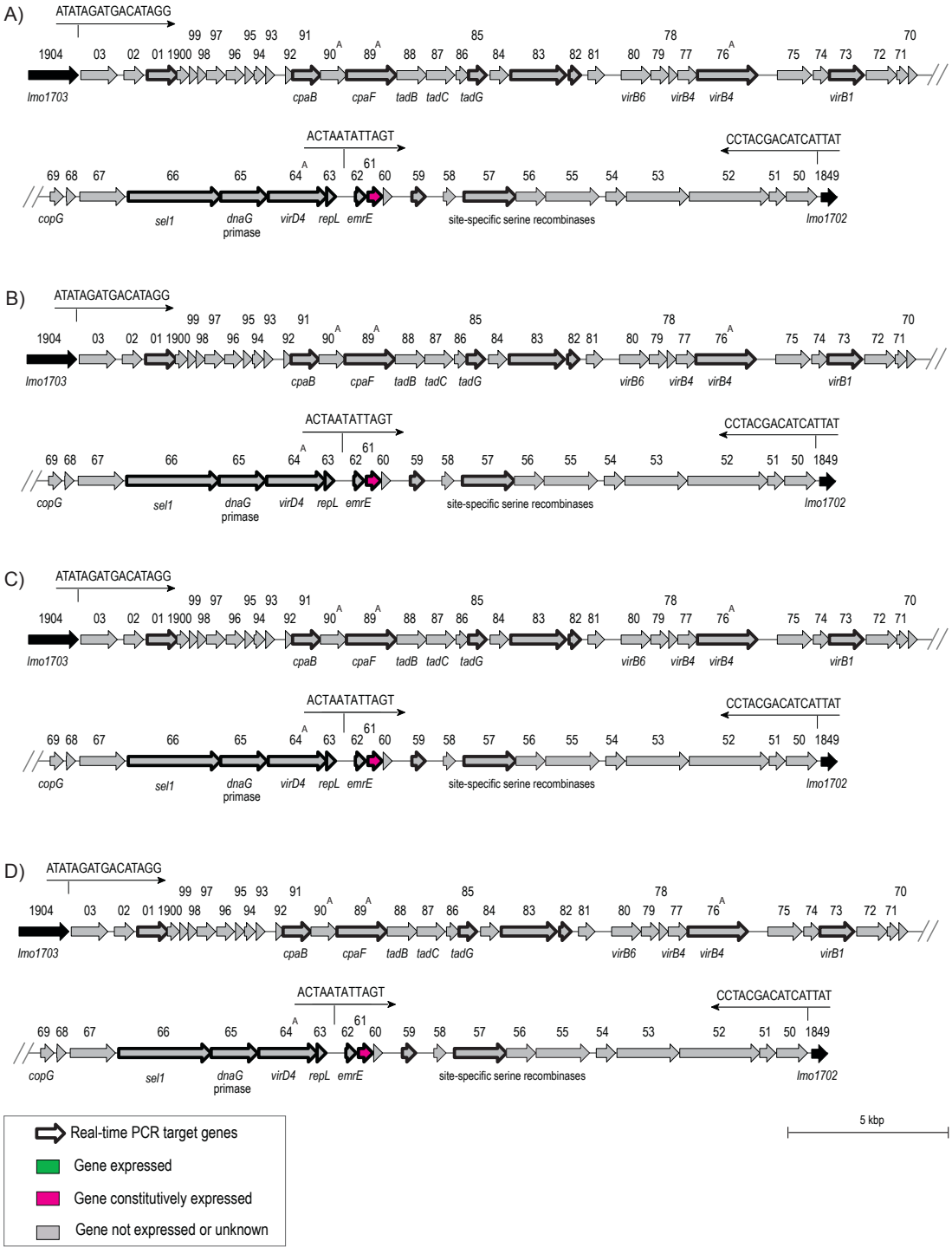
3.5 LGI1 expression was induced by the presence of BCl

The expression of LGI1 was analyzed under various growth conditions using qRT-PCR with 16 LGI1 gene targets to identify its possible functional roles. Under normal laboratory growth conditions when the cells were incubated in BHI broth at 37°C, only one gene target, LM5578_1862, a putative MarR family repressor, was expressed (Figure 18A). Similar results were obtained when the cells were subjected to heat shock, cold shock, when treated with UV light, and when the cells were grown in a mixed culture (Figure 18B-E). Alternatively, when the cells were grown at 37°C in BHI broth supplemented with 5μg/mL BCl, expression of 14 of the 16 LGI1 gene targets was detected (Figure 18F). This included the gene LM5578_1862, a putative MarR family repressor. The two genes for which expression was not detected were LM5578_1883, a putative surface/membrane protein, and LM5578_1863, a putative protein involved in DNA processing.

3.6 Isolates encoding LGI1 had an increased tolerance to BCl and BeCl compared to other isolates belonging to CC8 but not encoding LGI1.

To assess the putative function of LGI1, antimicrobial susceptibility assays were performed on the subset panel of 7 serotype 1/2a isolates (Table 7). This subset panel of isolates include the 2 primary isolates from the 2008 listeriosis outbreak which belong to the MLST CC8, and one of which (08-5578) encodes the plasmid pLM5578 and one

Figure 18: The expression profile of selected LGI1 genes under the different growth conditions. A, the expression profile when *L. monocytogenes* is cultured at 37°C with shaking in BHI broth; B, when heat shocked; C, when cold shocked; D, when treated with UV light; E, when grown in a mixed culture; E, when grown in BHI broth supplemented with 5µg/mL BCl.


A)
ATATAGATGACATAGG
1904 03 02 01 1900 98 96 94 92 90A 89A 88 87 86 84 83 82 81 80 79 77 76A 75 74 73 72 71
99 97 95 93 91 85 78 70
lmo1703 cpaB cpaF tadB tadC tadG virB6 virB4 virB4 virB1
ACTAATATTAGT
CCTACGACATCATTAT
69 68 67 66 65 64A 63 62 61 60 59 58 57 56 55 54 53 52 51 50 1849
copG sel1 dnaG primase virD4 repL emrE site-specific serine recombinases lmo1702
B)
ATATAGATGACATAGG
1904 03 02 01 1900 98 96 94 92 90A 89A 88 87 86 84 83 82 81 80 79 77 76A 75 74 73 72 71
99 97 95 93 91 85 78 70
lmo1703 cpaB cpaF tadB tadC tadG virB6 virB4 virB4 virB1
ACTAATATTAGT
CCTACGACATCATTAT
69 68 67 66 65 64A 63 62 61 60 59 58 57 56 55 54 53 52 51 50 1849
copG sel1 dnaG primase virD4 repL emrE site-specific serine recombinases lmo1702
C)
ATATAGATGACATAGG
1904 03 02 01 1900 98 96 94 92 90A 89A 88 87 86 84 83 82 81 80 79 77 76A 75 74 73 72 71
99 97 95 93 91 85 78 70
lmo1703 cpaB cpaF tadB tadC tadG virB6 virB4 virB4 virB1
ACTAATATTAGT
CCTACGACATCATTAT
69 68 67 66 65 64A 63 62 61 60 59 58 57 56 55 54 53 52 51 50 1849
copG sel1 dnaG primase virD4 repL emrE site-specific serine recombinases lmo1702
D)
ATATAGATGACATAGG
1904 03 02 01 1900 98 96 94 92 90A 89A 88 87 86 84 83 82 81 80 79 77 76A 75 74 73 72 71
99 97 95 93 91 85 78 70
lmo1703 cpaB cpaF tadB tadC tadG virB6 virB4 virB4 virB1
ACTAATATTAGT
CCTACGACATCATTAT
69 68 67 66 65 64A 63 62 61 60 59 58 57 56 55 54 53 52 51 50 1849
copG sel1 dnaG primase virD4 repL emrE site-specific serine recombinases lmo1702
Real-time PCR target genes
Gene expressed
Gene constitutively expressed
Gene not expressed or unknown
5 kbp

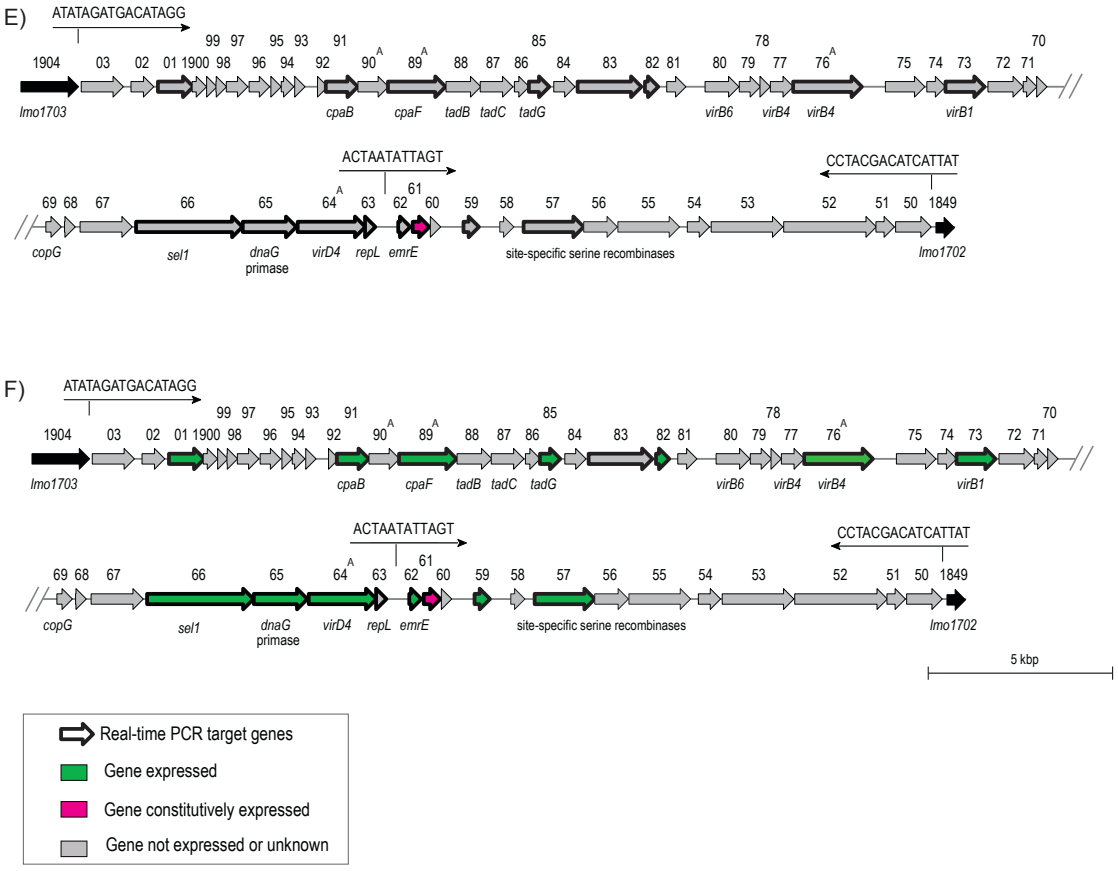

E)
ATATAGATGACATAGG
1904 03 02 01 1900 99 98 97 96 95 94 93 92 91 90A 89A 88 87 86 85 84 83 82 81 80 79 78 77 76A 75 74 73 72 71 70
lmo1703
cpaB
cpaF
tadB
tadC
tadG
virB6
virB4
virB4
virB1
ACTAATATTAGT
CCTACGACATCATTAT
69 68 67 66 65 64A 63 62 61 60 59 58 57 56 55 54 53 52 51 50 1849
copG
sel1
dnaG primase
virD4
repL
emrE
site-specific serine recombinases
lmo1702
F)
ATATAGATGACATAGG
1904 03 02 01 1900 99 98 97 96 95 94 93 92 91 90A 89A 88 87 86 85 84 83 82 81 80 79 78 77 76A 75 74 73 72 71 70
lmo1703
cpaB
cpaF
tadB
tadC
tadG
virB6
virB4
virB4
virB1
ACTAATATTAGT
CCTACGACATCATTAT
69 68 67 66 65 64A 63 62 61 60 59 58 57 56 55 54 53 52 51 50 1849
copG
sel1
dnaG primase
virD4
repL
emrE
site-specific serine recombinases
lmo1702
5 kbp
Real-time PCR target genes
Gene expressed
Gene constitutively expressed
Gene not expressed or unknown

Table 7: The subset panel of 7 isolates used in the MIC assays, the antimicrobial susceptibility assays and the biofilm assays.

Isolate	Serotype	Source	MLST[a]	CC8	LGI1	pLM5578
08-5578*	1/2a	Human clinical	ST292	+	+	+
08-5578Δ LM5578_1864	1/2a	Human clinical	ST292	+	+	+
08-5923*	1/2a	Human clinical	ST120	+	+	-
01-5373	1/2a	Human clinical	ST120	+	-	-
03-5833	1/2a	Human clinical	ST120	+	-	-
09-0290	1/2a	Food	ST321	-	-	-
EGDe*	1/2a	Rabbit tissue	ST35	-	-	-

a – MLST ST292 and ST120 belong to the CC8 group
*- Whole genome sequence available

of which does not encode the plasmid pLM5578 (08-5923); the LM5578_1864 gene deletion mutant (08-5578Δ LM5578_1864) which also encodes the plasmid LM5578; 2 older CC8 isolates epidemiologically unrelated to the 2008 outbreak isolates that do not encode LGI1 or the pLM5578 (01-5373 and 03-5833); a food isolate epidemiologically unrelated to the 2008 listeriosis outbreak not encoding LGI1 or pLM5578 with a MLST sequence type unrelated to the CC8 (09-0290); and the reference isolate *L. monocytogenes* EGDe, which is epidemiologically unrelated to the 2008 listeriosis outbreak, which is not related to the MLST CC8, and which does not encode LGI1 or pLM5578. Thus this panel could provide us with putative resistance characteristics common to CC8 isolates and/or LGI1.

The MIC values of the 7 isolates toward a panel of 20 antimicrobial compounds commonly used to treat clinical bacterial infections were assessed and no correlation was identified amongst the isolates encoding LGI1 and not encoding LGI1. The MIC values were similar for all 7 isolates except the isolates 08-5923, 03-5833 and EGDe, which had an increased susceptibility to daptomycin (>2.0 μg/mL compared to 2.0 μg/mL for the other isolates) and the food isolate 09-0290, which had higher MIC values for azithromycin and erythromycin, but lower MIC values for chloramphenicol (Table 8).

The MIC values of the 7 isolates against known targets of SMR efflux pumps were also assessed since LGI1 encodes a putative SMR protein (Table 8). The compounds tested were crystal violet, ethidium bromide, BCl and BeCl. Several antibiotics included in the previously described MIC assay are also known targets of SMR proteins, including chloramphenicol and tetracycline. The MIC of crystal violet and ethidium bromide for the 7 isolates was equitable, and all the isolates appeared red when viewed under UV light

Table 8: The motility and minimum inhibitory concentrations of the subset panel of 7 isolates to antimicrobials and known targets of SMR efflux proteins.

	08-5578	08-5578Δ LM5578_1864	08-5923	01-5373	03-5833	09-0290	EGDe
Serotype	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a
Source	Human clinical	Human clinical	Human clinical	Human clinical	Human clinical	Food	Rabbit tissue
MLST[a]	ST292	ST292	ST120	ST120	ST120	ST321	ST35
LGI1	+	+	+	-	-	-	-
pLM5578	+	+	-	-	-	-	-
Crystal Violet (μg/mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
EtBr (μg/mL)/color	10.0/red	10.0/red	10.0/red	10.0/red	10.0/red	10.0/red	10.0/red
Motility 25°C (+/- BCl)	+	+	+	+	+	+	+
Motility 37°C (+/- BCl)	slight	slight	slight	slight	slight	slight	slight
BCl - 25°C, 37°C (μg/mL)	25.0	25.0	25.0	5.0	5.0	20	5.0
BCl - 4°C (μg/mL)	20.0	20.0	20.0	5.0	5.0	15.0	5.0
BeCl - 25°C, 37°C (μg/mL)	20.0	20.0	20.0	5.0	5.0	15.0	5.0
Moxifloxacin (μg/mL)	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0
Penicillin (μg/mL)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Levofloxacin (μg/mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Meropenem(μg/mL)	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Azithromycin(μg/mL)	0.5	0.5	0.5	0.5	0.5	>2.0	0.5
Tetracycline(μg/mL)	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0
Ertapenem (μg/mL)	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Erythromycin (μg/mL)	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	>2.0	≤0.25
Cefuroxime (μg/mL)	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
Amoxicillin/clavulanic acid 2:1 ratio (μg/mL)	≤2/1	≤2/1	≤2/1	≤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	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5
Ceftriaxone (μg/mL)	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
Linezolid (μg/mL)	1.0	1.0	2.0	2.0	2.0	1.0	1.0
Vancomycin (μg/mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cefotaxime (μg/mL)	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
Clindamycin (μg/mL)	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
Daptomycin(μg/mL)	2.0	2.0	>2.0	2.0	>2.0	2.0	>2.0
Cefepime (μg/mL)	>8.0	>8.0	>8.0	>8.0	>8.0	>8.0	>8.0
Chloramphenicol (μg/mL)	8.0	8.0	8.0	8.0	8.0	4.0	8.0
Tigecyline (μg/mL)	0.06	0.06	0.06	0.06	0.06	0.06	0.06

a – MLST ST292 and ST120 belong to the CC8 group

after being grown with ethidium bromide. Isolates that efflux out ethidium bromide have a red halo surrounding the white colored colonies.

The MICs of the quaternary ammonium compounds BCl and BeCl were also assessed. The MIC for BCl was assessed at room temperature, 37°C and 4°C, and the MIC values for BeCl were only assessed at room temperature and 37°C. The MIC values obtained with BCl and BeCl did vary amongst the panel of 7 isolates, but no variation was observed among the different temperatures. Isolates belonging to CC8 and encoding LGI1 had a 4-fold increased tolerance to BCl and BeCl compared to those CC8 isolates not encoding LGI1. The isolate 09-0290 also had similar MIC values to those isolates encoding LGI1 even though it does not encode LGI1 and it is not part of CC8. There was no variability in MIC values amongst isolates with and without the plasmid pLM5578.

An additional panel of 7 isolates belonging to the MLST CC8 was selected to further confirm the results of the BCl assay (Table 9). This panel consisted of an environmental isolate encoding LGI1 and pLM5578; 3 clinical isolates encoding LGI1 but not pLM5578; a food isolate encoding pLM5578 but not LGI1; and 2 clinical isolates encoding pLM5578 but not LGI1. Again, the CC8 isolates encoding LGI1 had an increased tolerance to BCl (20 µg/mL) compared those that did not encode LGI1 (5.0µg/mL) (Table 9). There was however one isolate, 08-5375, that did not encode LGI1, that had a high tolerance to BCl (15 µg/mL).

3.7 Variations were observed in the biofilm forming ability of CC8 isolates

Since LGI1 putatively encodes a T4LSS, it is thus plausible that LGI1 may be involved in the secretion of proteins and/or DNA that could contribute to biofilm formation. During biofilm formation, *L. monocytogenes* cells must secrete a

Table 9: The tolerance to BCI of the additional CC8 isolates used in the BCI MIC assays including the serotype, the source of bacteria, the MLST sequence type, the presence or absence of LGI1 and the presence or absence of pLM5578.

	08-6569	98-2035	99-6370	10-1046	01-5991	01-7107	08-5375
Serotype	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a
Source	Environmental	Human clinical	Human clinical	Human clinical	Food	Human clinical	Human clinical
MLST[a]	ST292	ST292	ST120	ST120	ST120	ST321	ST35
LGI1	+	+	+	+	-	-	-
pLM5578	+	-	-	-	+	+	+
BCI tolerance (µg/mL)	20	20	20	20	5.0	5.0	15

a – MLST ST292 and ST120 belong to the CC8 group

* - Whole genome sequence available

polysaccharide matrix that protects the community from unfavourable environmental stresses such as sanitizers, disinfectants and other antimicrobial agents. Thus, the ability of the subset panel of 7 isolates to form biofilms was assessed using the microtitre plate method. Two additional serotype 4b isolates (81-0592 and 81-0861) were included in the biofilm experiment because serotype 4b isolates are weaker biofilm formers than serotype 1/2a isolates, therefore the inclusion of these 2 isolates would help validate the results obtained.

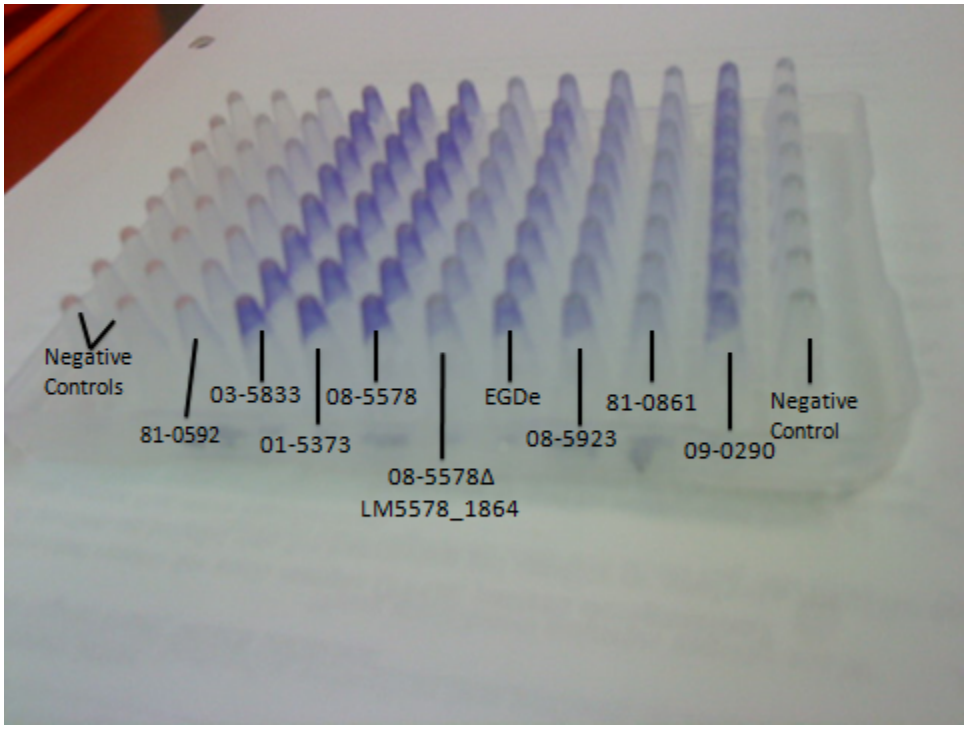
The isolates 01-5373 (CC8, LGI1 negative, pLM5578 negative), 03-5833 (CC8, LGI1 negative, pLM5578 negative), 08-5578 (CC8, LGI1 positive, pLM5578 positive) and 09-0290 (not CC8, LGI1 negative, pLM5578 negative) were classified as strong biofilm formers. The isolates 08-5578ΔLM5578_1864 (CC8, LGI1 positive, pLM5578 positive), EGDe (not CC8, LGI1 negative, pLM5578 negative) and 08-5923 (CC8, LGI1 positive, pLM5578 negative) were intermediate biofilm formers, and the 2 serotype 4b isolates were weak biofilm formers (Figure 19). Notably, there was a difference in biofilm forming ability between 08-5578 and 08-5923, the two isolates from the 2008 listeriosis outbreak whose genomes were sequenced. The genetic differences amongst these isolates are the presence of pLM5578 in 08-5578, 28 SNPs and the presence of a prophage within the genome of 08-5578. There was also a difference in biofilm forming ability between 08-5578 and the LM5578_1864 gene deletion mutant.

3.8 LGI1 was not transferred by conjugation

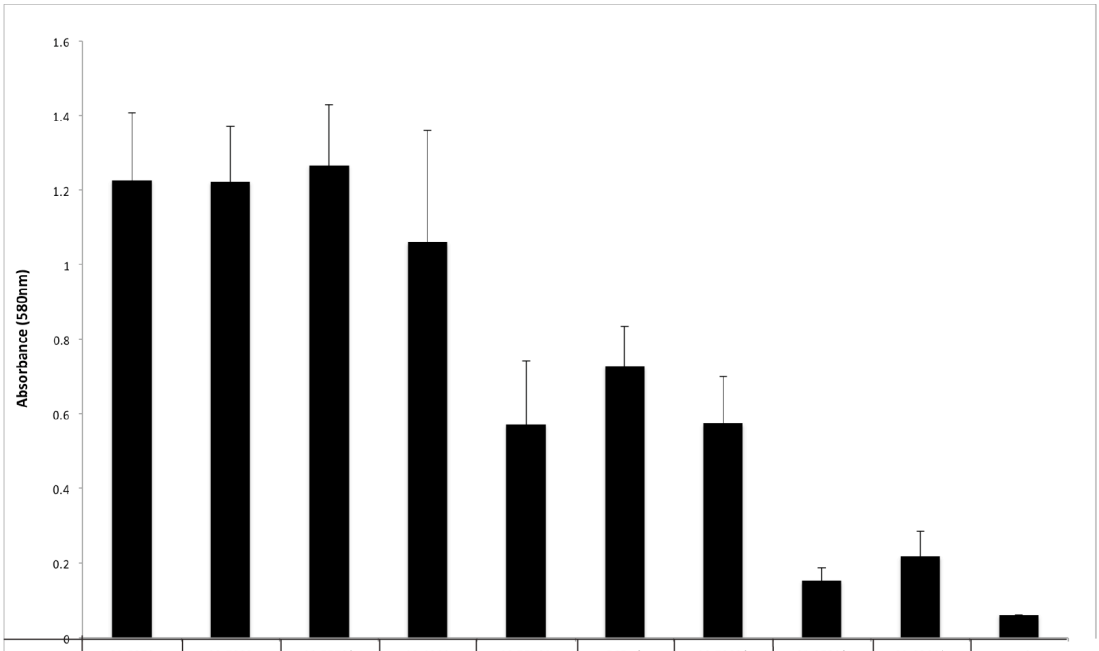
Conjugation studies were attempted to determine whether LGI1 could be transferred by this method since it encodes a putative T4LSS that are involved in transfer of nucleoprotein complexes, as well as encoding putative site-specific serine recombinase proteins that are involved in the excision/integration of genetic elements.

Figure 19: The PCR plate with the stained biofilms (A) and the absorbance readings at 580nm of the destained biofilm plates (B) of the subset panel of 7 isolates and the 2 additional serotype 4b isolates.

A)



B)



Isolate	01-5373	03-5833	08-5578*	09-0290	08-5578Δ LM5578_1864*	EGDe*	08-5923*	81-0592*	81-0861*	negative
Serotype	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	4b	4b	
CC8	+	+	+	-	+	-	+	-	-	
LGI1	-	-	+	-	+	-	+	-	-	
pLM5578	-	-	+	-	+	-	-	-	-	
ΦLMC1	-	-	+	+	+	-	-	-	-	

The start and end coding sequences of LGI1 are also bordered by imperfect inverted repeats, which are characteristic of the binding site of recombinases, therefore mobility of LGI1 is possible. Conjugation was attempted to determine if *L. monocytogenes* EGDe could serve as a recipient for LGI1. Multiple attempts to transfer the LGI1 from the donor *L. monocytogenes* 08-5578 to the recipient *L. monocytogenes* EGDe using various conjugation methods and inducing agents were unsuccessful. However, the conditions necessary to induce LGI1 transfer are unknown therefore the conditions used in the conjugation experiments may not be sufficient to induce transfer.

Chapter 4

Discussion and Conclusion

4. Discussion

4.1 LGI1 is exclusive to and is widely distributed within CC8 of *L. monocytogenes*

The contribution of LGI1 to the 2008 nationwide outbreak was unknown, but the discovery of this large genetic island that encoded multiple traits related to transfer, secretion and mobilization suggested the role of LGI could be significant. These contributions could include persistence within food production environments, survival and growth on contaminated foods, and/or virulence during infection. As LGI1 was a newly discovered element, and few *L. monocytogenes* whole-genome DNA sequences were available, it was also unknown if it was widely distributed beyond the 2008 outbreak strain.

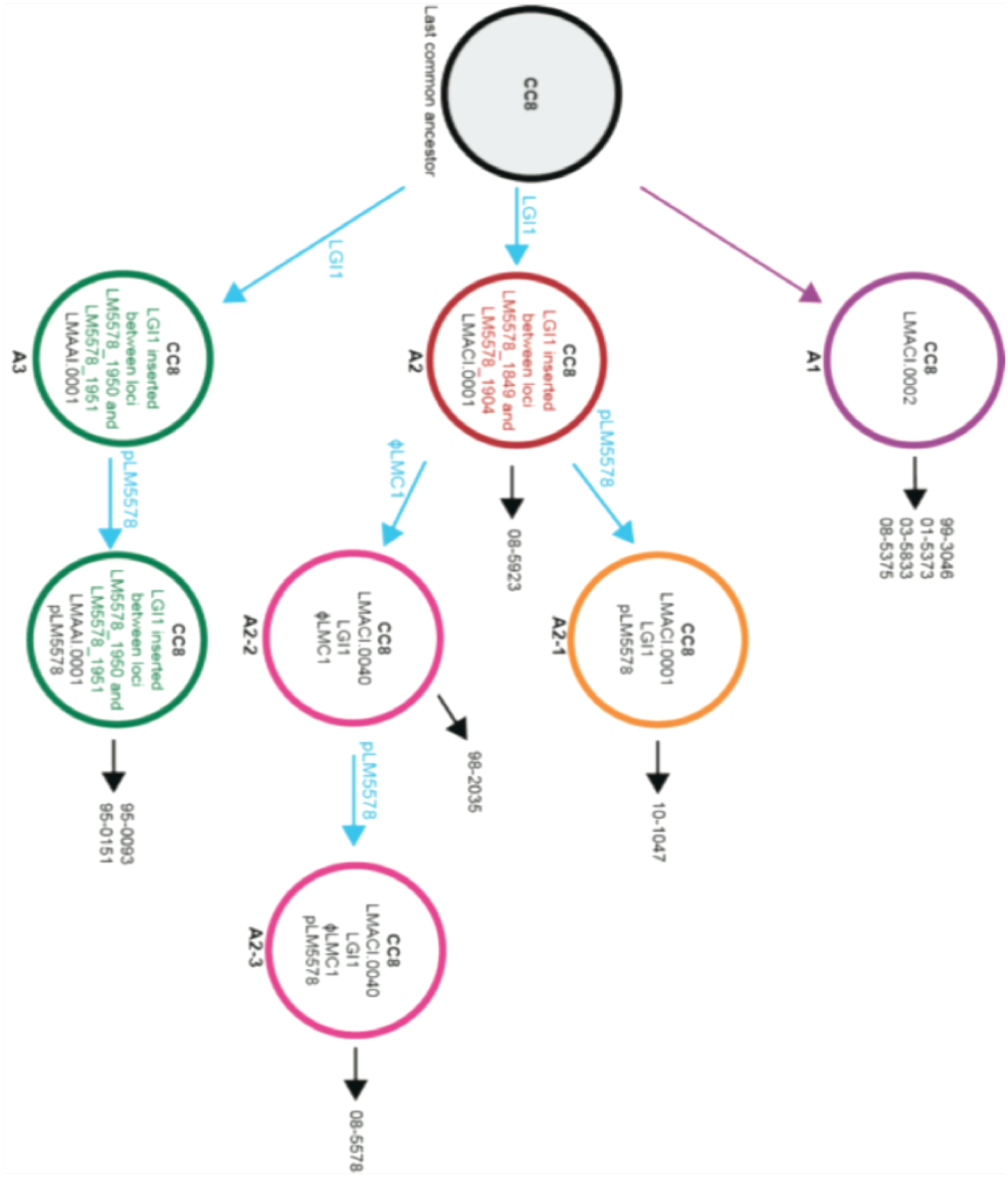
In this study, LGI1 was only identified within the genomes of serotype 1/2a and 3a *L. monocytogenes* isolates belonging to the MLST CC8 and with PFGE pattern combinations highly related to the Canadian designations LMACI.0001 and LMAAI.0001. The single serotype 3a isolate, although phenotypically serotype 3a, shares other molecular characteristics of CC8 isolates, including sequence types of virulence genes (Knabel et al., unpublished). All 67 isolates included in this study that tested positive for LGI1 also encoded each of the 16 LGI1 screening targets. These results suggest that LGI1 is present as a conserved, non-mosaic continuous genetic element. Furthermore, LGI1 was inserted at the same location within the genome in 65 out of 67 isolates that were included in this study, between the genes LM5578_1849 and LM5578_1904. These results suggest that LGI1 may have been acquired by a progenitor *L. monocytogenes* at this site and it has remained genetically stable since.

Two isolates (95-0093 and 95-0151) included in this study had LGI1 inserted approximately 100 genes downstream of the principal insertion site within the other isolates (between the loci LM5578_1950 and LM5578_1951). This LGI1 sequence was also genetically identical to that of the other isolates that had LGI1 inserted at the principal location. Furthermore, these LGI1 sequences also encode the imperfect inverted repeats at the ends of LGI1 and the site-specific serine recombinase proteins. Therefore, these 2 isolates may represent an evolutionary relative of the other 65 *L. monocytogenes* isolates that acquired LGI1 at a separate time period through horizontal gene transfer or transposition. The acquisition of identical versions of LGI1 at separate chromosomal locations (and likely at separate times) further indicates that the currently observed strains that harbor LGI1 received this element from progenitor bacterium. A larger sample size is required to definitively determine the frequency of this chromosomal mutation within Canadian *L. monocytogenes* isolates.

The 15 available whole LGI1 DNA sequences exhibited limited genetic diversity, and in the remainder of the chromosome of these strains, macro diversity was limited to phage insertions despite that these isolates were from different clusters, sources and geographical locations. Likewise, there was only one synonymous SNP detected amongst the LGI1 sequences of these isolates. This suggests that these isolates are closely evolutionarily related and likely descended from a single progenitor *L. monocytogenes* isolate.

We propose a model to describe the chromosomal evolution of strains harboring LGI1 using the distribution and segregation of genetic traits including prophages and plasmids (Figure 20). This model assumes that the last common ancestor (LCA) of all strains was a progenitor *L. monocytogenes* isolate belonging to the CC8 and it encoded

Figure 20: The evolutionary model for the acquisition of LGI1. Predicted mutational events are indicated on the horizontal arrows, genotypes of the resulting lineages are denoted within circles, and isolates representative of those lineages are indicated with black arrows from the ancestors. Mutation events are indicated with light blue arrows.


Last common ancestor
CC8
LGI1
LGI1
CC8
LMACI.0002
A1
99-3046
01-5373
03-5833
08-5375
CC8
LGI1 inserted
between loci
LM5578_1849 and
LM5578_1904
LMACI.0001
A2
pLM5578
ΦLMC1
08-5923
CC8
LMACI.0001
LGI1
pLM5578
A2-1
10-1047
CC8
LMACI.0040
LGI1
ΦLMC1
A2-2
98-2035
pLM5578
CC8
LMACI.0040
LGI1
ΦLMC1
pLM5578
A2-3
08-5578
CC8
LGI1 inserted
between loci
LM5578_1950 and
LM5578_1951
LMAAI.0001
A3
pLM5578
CC8
LGI1 inserted
between loci
LM5578_1950 and
LM5578_1951
LMAAI.0001
pLM5578
95-0093
95-0151

a similar genetic backbone as the isolate 08-5578, however the phage ϕLMC1, LGI1 and the plasmid pLM5578 were absent.

The CC8 isolates that do not encode LGI1 would have descended from the first ancestor (A1) of the LCA and these isolates would have the PFGE pattern combinations LMACI.0002 (Figure 20; A1). Descendants of the progenitor A1 include the isolates 99-3046, 01-5373, 03-5833 and 08-5375 which belong to CC8 but lack LGI1. These isolates appear to be descendants of the LCA that did not harbor LGI, but the bridging PCR results were negative for all 3 reactions with these isolates. These results could represent a different genetic mutation at that site within these CC8 LGI1 negative isolates, which could explain why LGI1 is absent in these isolates. Additionally, these isolates did all have the *AscI* PFGE pattern LMACI.0002, but the lack of LGI1 could not be attributed to the difference in PFGE pattern because LGI1 is present in the 1000kbp band and a deletion of LGI1 would not result in a significant band shift that could be detected during PFGE analysis. Whole genome sequencing of these isolates that belong to the CC8 but do not harbor LGI1 is necessary to fully elucidate the evolutionary patterns of these isolates and the reasons for the bridging PCR results that suggest that the LGI1 insertion site is absent. Similarly, 11 isolates not belonging to CC8 and not encoding LGI1 had similar inconsistencies with the expected bridging PCR results. These 11 isolates were not serotype 1/2a isolates, therefore wider variations within the genomic content are expected. The genes LM5578_1849 and LM5578_1904 may be absent, or they may be present in different location within the genomes of these isolates. Indels and SNPs at these loci could also account for the unexpected PCR results that were obtained for these isolates.

From the LCA, a second ancestor (A2) would have evolved and acquired LGI1 from an unknown source at the principle insertion site (between the loci LM5578_1849 and

LM5578_1904) (Figure 20; A2). This ancestor would have the PFGE pattern LMACI.0001. Direct descendants of this ancestor retain the PFGE pattern LMACI.0001 and include the isolate 08-5923. An ancestor A2-1 that evolved from A1 would have retained the PFGE pattern LMACI.0001 but would have acquired the plasmid pLM5578. Descendants of this ancestor A2-1 include the isolate 10-1047. The ancestor A2-2 would have evolved from the ancestor A2 by acquiring the phage ϕLMC1 and the new PFGE pattern LMACI.0040. Descendants of this ancestor A2-2 include the isolate 98-2035. Ancestor A2-2 isolates that acquired the plasmid pLM5578 would have resulted in the ancestor A2-3, and descendants include the isolate 08-5578. Additional LGI1 positive isolates differed from the LCA and the proposed ancestors by the acquisition or loss of SNPs, pLM5578, ϕLMC1 or other phages and plasmids. These diversifications would contribute to the variations in the *ApaI* PFGE pattern profiles and MLST sequence types of the CC8 isolates that encode LGI1. These genomic variations may have occurred in response to adverse environmental conditions prior to infection or host-specific conditions during infection, and may have also arisen by acquisition from other natural populations in the environment.

Lastly, a third ancestor (A3) that descended from the LCA would have also have acquired LGI1 from an unknown source, but LGI1 was inserted at the less common insertion site (between the loci LM5578_1950 and LM5578_1951) (Figure 20; A3). Descendants of this ancestor A3 that would have acquired the plasmid pLM5578 include the isolates 95-0093 and 95-0151. Similarly, the acquisition or loss of SNPs, pLM5578, ϕLMC1 or other phages and plasmids would contribute to the variations in the *ApaI* PFGE pattern profiles of these isolates.

4.2 LGI1 may be involved in an increased tolerance to sanitizers

Bioinformatic analysis of the LGI1 genes suggests that LGI1 may have a role in secretion or efflux of cationic lipophilic compounds that are targets of the SMP efflux protein encoded by LGI1. LGI1 also encodes several putative regulatory proteins, therefore the expression profile of LGI1 was analyzed to determine how LGI1 is regulated and which conditions allow for the expression of the LGI1. LGI1 was tightly regulated with expression of the gene targets only being induced by the presence of the QAC sanitizer BCl. Since the mRNA levels were detected, this implies that BCl either induces LGI1 gene expression, or BCl stabilizes the mRNA within the cell, allowing it be detected. These results suggest that LGI1 could have a role in BCl efflux and/or tolerance. Additionally, expression of the gene LM5578_1861, a putative marR repressor, was constitutive. There was also an inverted repeat found between the promoter elements of the gene LM5578_1862, a putative SMR efflux protein. These results suggest that LM5578_1861 may be a repressor the SMR efflux protein, however further studies must be undertaken to confirm this hypothesis.

The function of LGI1 was further assessed by determining the tolerance of the subset panel of isolates, which included CC8 isolates encoding LGI1, CC8 isolates not encoding LGI1 and unrelated isolates not encoding LGI1, to antibiotics as well as known target of SMR efflux proteins. The genetically unrelated isolates that do not encode LGI1 serve as a comparison for the MIC and tolerance values observed. The CC8 isolates encoding LGI1 are highly genetically related to those that do not encode LGI1, and isolates belonging to the MLST CC8 have also been proposed to descend from a single epidemic clone (Knabel et al., unpublished). Therefore, the CC8 isolates that lack LGI1 should be comparable to the LCA that lacked LGI1. Differences in the MICs and

tolerances observed between these isolates that lack LGI1 and that encode LGI1 could hence represent putative functions of LGI1.

There were few differences observed between the MICs of the isolates to the panel of antimicrobials commonly used to treat listeriosis. The isolates 08-5923, 03-5833 and EGDe had a greater resistance toward daptomycin (>2.0 µg/mL compared to 2.0 µg/mL). Daptomycin is a lipopeptide antibiotic that is used to treat skin infections, endocarditis and bacteremia caused by enterococci and staphylococci (Cantón, Ruiz-Garbajosa, Chaves, & Johnson, 2010). Daptomycin causes rapid depolarization of the bacterial cell membrane, resulting in cell death (Silverman, Perlmutter, & Shapiro, 2003). Daptomycin resistance is classified by an MIC of >4 µg/mL and it results from chromosomal polymorphisms (Palmer, Daniel, Hardy, Silverman, & Gilmore, 2011). Therefore, the *L. monocytogenes* isolates with an increased resistance to daptomycin could have a chromosomal polymorphism that increases their resistance to daptomycin, however it is difficult to elucidate without further analysis. The food isolate 09-0290 also had higher MIC values for azithromycin and erythromycin, but lower MIC values for chloramphenicol compared to the other 6 isolates within the panel. This isolate did not belong to the MLST CC8 and it did not encode LGI1, therefore genetic differences are expected, which could contribute to the different MIC values.

The CC8 isolates from the subset panel of isolates encoding LGI1 had a four times greater increase in tolerance to BCl and BeCl as compared to isolates belonging to CC8 that did not encode LGI1. The food isolate 09-0290 also had a three times greater tolerance to BCl and BeCl compared to the CC8 isolates that did not encode LGI1. In contrast, the isolate EGDe that does not belong to CC8 and does not encode LGI1 had the same tolerance to the sanitizers as the CC8 LGI1 negative isolates. Together with the expression analysis data, these results suggest a role for LGI1 in BCl and BeCl

tolerance. When the extended panel of CC8 isolates was tested for tolerance to BCl, the same tolerance to the sanitizers was observed for isolates encoding or lacking LGI1, further implying a role for LGI1 in QAC sanitizer tolerance. One isolate that lacked LGI1 and that did not belong to CC8, 08-5375, however did have a three times greater tolerance to BCl compared to the other isolates that lacked LGI1. This isolate could have additional genetic features such as a plasmid that accounts for this increased tolerance to QAC sanitizers, but it is difficult to elucidate without knowing the genetic content of this isolate.

The tolerance to sanitizers was determined at room temperature, 37°C and 4°C. All the isolates tested showed the same ability to grow at these temperatures. Therefore, the CC8 isolates are not likely to have an enhanced ability to grow at refrigeration temperatures. Temperature also did not have an effect on the tolerance to the QAC sanitizers.

4.3 LGI1 may not be horizontally mobile by conjugation

Conjugation studies were performed to determine if LGI1 could be transferred to a recipient strain via conjugation since LGI1 encodes putative mobilization genes. However, LGI1 was not transferred via conjugation under the experimental conditions used in this study, suggesting that if it is a mobile element, additional inducing agents or other environmental conditions must occur for mobility. These results could also suggest that LGI1 is not horizontally mobile, or it has lost its ability to be transferred.

4.4 The plasmid pLM5578 and the gene LM5578_1864 may enhance biofilm formation

The two isolates from the 2008 listeriosis outbreak whose genome sequences are available had differences in their abilities to form biofilms, despite being highly genetically related. The *L. monocytogenes* isolate 08-5923 was a weaker biofilm former than the isolate 08-5578, and the genomic difference between these 2 isolate are 28 SNPs, a prophage insertion within the genome of 08-5578, and the presence of the plasmid pLM5578 within 08-5578. The presence of the prophage and the SNPs are unlikely to alter biofilm formation because the prophage does not carry genes associated with biofilm formation and because the SNPs are not within genes associated with biofilm formation. In contrast, the plasmid pLM5578 encodes genes related to known T2SS and T4SS and heavy metal resistance genes, therefore the presence of this plasmid is the most likely genomic difference that could contribute to biofilm formation. Within biofilms, cells are in close proximity and the environment is ideal for the exchange of genetic material to occur. It has been shown that natural bacterial conjugation occurs readily within biofilms, and that natural conjugative plasmids can induce biofilm formation because the environment favours the transfer of the plasmid (Ghigo, 2001). Thus, plasmid-bearing strains are more likely to form a biofilm than non-plasmid bearing strains. The presence of T4SS homologs on the plasmid pLM5578 could suggest that it is involved in conjugation, therefore this could explain the differences in biofilm forming ability between these 2 closely related isolates. Further research must be conducted to prove this hypothesis, however, since the other 2 CC8 isolates that did not encode LGI1 or pLM5578 were also strong biofilm formers.

The *L. monocytogenes* 08-5578 LM5578_1864 gene deletion mutant was also a weaker biofilm former than the wild type 08-5578. The gene deletion did still encode the

plasmid pLM5578 after being subjected to the mutation treatments, therefore the mutation with the T4SS T4CP may have contributed to the difference in biofilm forming ability that was observed. LGI1 was not found to be horizontally mobile by conjugation experimentally, however conjugation may still occur in naturally, therefore as with conjugative plasmids, a conjugative genomic island could favour biofilm formation. Alternatively, the T4CP could be involved in the secretion of an effector protein or even extrapolysaccharide matrix that enhances biofilm formation. It is also plausible that another genomic mutation occurred during the creation of the gene deletion mutant that altered genes involved in biofilm formation. Further experiments must be undertaken to confirm the role of the T4SS T4CP LM5578_1864 in the formation of biofilms.

4.5 The LGI1 screening assay can be used for the rapid identification of high risk *L. monocytogenes* isolates

In Canada over the past 20 years, the CC8 subtype of *L. monocytogenes* has caused 40% of the outbreaks and 20% of clinical cases. The majority of these CC8 isolates also encode the LGI1, which may contribute to bacterial persistence by increased tolerance to sanitizers and biofilm formation. Therefore, given the problematic nature of this subtype in Canada, the LGI1 RT-PCR screening assay can be used for the rapid identification of high risk *L. monocytogenes* in both the clinical and food processing environments. In comparison to the commonly used subtyping methods, this screening assay is very rapid and targets LGI1 specifically. If an isolate encodes LGI1, additional subtyping information, such as serotype, MLST sequence type and PFGE pattern, can be predicted while these subtyping tests are being performed because LGI1 seems to be limited to serotype 1/2a isolates belonging to the MLST CC8 group with PFGE patterns highly related to the Canadian designation LMAAI.0001 and LMACI.0001.

5. Conclusions

LGI1 is a 50 kb genomic island encoding genes with predicted secretion, mobilization and efflux functions. LGI1 was only detected in Canadian isolates belonging to the CC8 with PFGE patterns highly similar to the Canadian designations LMACI.0001 and LMAAI.0001, and it was inserted in the same location within the genome of 65 of 67 isolates, and in a nearby location in the other 2 isolates. Therefore, LGI1 was acquired twice by progenitor *L. monocytogenes* ancestors and it has remained genetically stable since. Only a single synonymous SNP was observed across the LGI1 DNA sequences of 15 isolates. Expression analyses showed that LGI1 expression was induced by the presence of the sanitizer BCI, and CC8 isolates encoding LGI1 also had an increased tolerance to QAC sanitizers compared to CC8 isolates lacking LGI1. Therefore, LGI1 may have a role in tolerance to sanitizers, which could contribute to bacterial persistence within the food processing industry. Furthermore, the LGI1 screening assay can be used for the rapid identification of high-risk *L. monocytogenes* isolates due to the public health significance of this subtype in Canada and the presumptive contribution of LGI1 to sanitizer tolerance and persistence.

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Appendix

Supplementary Table 1: The complete LGI1 screening results.

Isolate	Serotype	Source	Province	AscI	ApaI	rRNA1_HKG1	1859_reg2	1865_dna1	1866_str1	1883_str2	1901_reg1	1882_pil1	1885_pil2	1889_pil3	1891_pil4	1876_virB4	1873_virB1	1864_virD4	1863_rep	1862_emrE	1861_marR	1857_recomb	Bridging PCR 1849-1904	Bridging PCR - 1849-1859	Bridging PCR - 1903-1904
01-2129	1/2a	n/a	QC	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-2417	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
07-3417	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-5080	1/2a	Human joint fluid	MB	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
03-5360	1/2a	n/a	NF	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-5379	1/2a	Human uterus swab	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
04-5457	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
07-5657	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-6771	1/2a	Human eye	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
06-6837	1/2a	Human blood	QC	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
07-7193	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-7209	1/2a	Liverwurst sausage	CFIA (BC)	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-7362	1/2a	Environmental	CFIA	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-7376	1/2a	Food processing enviro	CFIA-ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-7381	1/2a	Food processing enviro	CFIA-ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-7382	1/2a	Food processing enviro	CFIA-ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-7554	1/2a	Corsh Smoked Meat	CFIA-ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
00-0338	1/2a	Human knee fluid	MB	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
03-0402	1/2a	Human blood	AB	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-5923*	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-7374*	1/2a	Food processing enviro	CFIA-ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-7669*	1/2a	Human blood	SK	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
10-0814*	1/2a	Food processing enviro	BMH-ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
10-1046*	1/2a	Human	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
10-1047*	1/2a	Human	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
10-1321*	1/2a	Human	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
88-0478	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
88-1059	3a	Human blood	NFLD	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
95-0093	1/2a	Human blood	AB	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
96-0218	1/2a	n/a	n/a	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
96-0247	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
97-0624	1/2a	Human blood	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
97-1602	1/2a	Human urine	AB	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
98-0290	1/2a	CSF	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
98-1143	1/2a	Human blood	BC	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
98-1191	1/2a	Blood	BC	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
98-2299	1/2a	n/a	SK	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
99-6666	1/2a	CSF	ON	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
99-6871	1/2a	Human clinical	NFLD	LMACI.0001	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-3506	1/2a	Human CSF	ON	LMACI.0001	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
95-0012	1/2a	Human blood	ON	LMACI.0001	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
95-0151	1/2a	Human blood	ON	LMACI.0001	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
97-1636	1/2a	Human blood	SK	LMACI.0001	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-5373	1/2a	Human blood	ON	LMACI.0002	LMAAI.0001	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
99-3046	1/2a	Human blood	ON	LMACI.0002	LMAAI.0001	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08-5375	1/2a	Human	ON	LMACI.0002	LMAAI.0214	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
03-5833	1/2a	Human blood	AB	LMACI.0002	LMAAI.0214	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
06-4721	1/2a	Blood	ON	LMACI.0003	LMAAI.0294	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
07-5577	1/2a	Human blood	BC	LMACI.0004	LMAAI.0013	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

07-5999	1/2a	Human blood	BC	LMACI.0004	LMAAI.0013	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
09-0290	1/2a	Food	CFIA-CAL	LMACI.0004	LMAAI.0013	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
07-3998	1/2a	Human CSF	ON	LMACI.0005	LMAAI.0013	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
06-6833	1/2a	Human blood	QC	LMACI.0007	LMAAI.0068	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
10-1870	4b	Human	NS	LMACI.0009	LMAAI.0234	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08-8680	4b	Human blood	NB	LMACI.0009	LMAAI.0234	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
09-0498	1/2a	RTE Leeks	CFIA-QC	LMACI.0011	LMAAI.0015	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
06-6880	1/2a	Human blood	QC	LMACI.0014	LMAAI.0183	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
09-2089	1/2a	Human	BC	LMACI.0015	LMAAI.0024	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
09-0748	1/2a	Fish	CFIA-QC	LMACI.0027	LMAAI.0677	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
06-6846	1/2a	Human blood	QC	LMACI.0031		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08-5587	1/2a	Human	ON	LMACI.0035	LMAAI.0414	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
08-5596	1/2a	Smoked Salmon	CFIA-ON	LMACI.0036	LMAAI.0433	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
07-1873	1/2c	Food	CFIABC	LMACI.0036	LMAAI.0433	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08-8749	1/2c	Pork Loin Chops	CFIA-ON	LMACI.0036	LMAAI.0658	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08-4803	4a	Human	ON	LMACI.0038	LMAAI.0540	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
08-2593	1/2a	Human	NF	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-5243	1/2a	Human	BC	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-5319	1/2a	Human	EDM	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-5828	1/2a	Human blood	ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6011	1/2a	Corned beef AU25	CFIA-ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6040	1/2a	RTE meat	CFIA-ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6055	1/2a	RTE meat	CFIA-ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6061	1/2a	RTE meat	CFIA-ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
07-6082	1/2a	Human blood	NS	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6135	1/2a	Human CSF	ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6421	1/2a	Human blood	ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6567	1/2a	Food processing enviro	CFIA	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-7210	1/2a	Liverwurst sausage	CFIA-BC	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-5578*	1/2a	Human blood	ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6569*	1/2a	Food processing enviro	CFIA-ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
10-0815*	1/2a	Food processing enviro	BMH-ON	LMACI.0040	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-5871	1/2a	Human CSF	BC	LMACI.0040	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6056*	1/2a	Turkey Meat	CFIA-ON	LMACI.0040	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
08-6997*	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
98-2035*	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
99-6370*	1/2a	Human blood	ON	LMACI.0040	LMAAI.0003	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
06-7231	1/2a	Blood	BC	LMACI.0041	LMAAI.0033	+	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
06-6891	1/2a	Human blood	QC	LMACI.0044	LMAAI.0074	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
08-5757	1/2a	Human	ON	LMACI.0044	LMAAI.0193	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
08-8809	1/2b	Fromage Stilton Blue	CFIA-QC	LMACI.0045	LMAAI.0317	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
06-3023	4b	Human blood	MB	LMACI.0060	LMAAI.0204	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
09-5042	1/2a	n/a	CDC	LMACI.0083	LMAAI.0185	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
06-7047	1/2a	Human CSF	ON	LMACI.0084	LMAAI.0175	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
08-6880	1/2a	Human	MB	LMACI.0088	LMAAI.0448	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
01-1465	1/2a	Human blood	ON	LMACI.0096	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
01-1468	1/2a	Human brain tissue	NFLD	LMACI.0098	LMAAI.0001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
09-6935	4b	Human	BC	LMACI.0107	LMAAI.0104	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
06-6902	1/2a	Human blood	QC	LMACI.0114		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
06-4636	1/2a	Blood	BC	LMACI.0116	LMAAI.0118	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
07-0129	1/2a	Human ascitic fluid	ON	LMACI.0118	LMAAI.0005	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
02-2448	1/2a	Human blood	ON	LMACI.0122	LMAAI.0003	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
08-7538	1/2c	n/a	CFIA-SHY	LMACI.0148	LMAAI.0442	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
08-6576	1/2a	Human CSF	ON	LMACI.0149	LMAAI.0265	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

06-6878	1/2a	Human CSF	QC	LMACI.0157	LMAAI.0126	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
10-1871	1/2a	Human	NS	LMACI.0173	LMAAI.0834	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
05-7243	4b	Human blood	ON	LMACI.0207	LMAAI.0134	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08-0291	1/2a	Human	ON	LMACI.0222	LMAAI.0005	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
06-6865	3a	n/a	QC	LMACI.0226		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
07-6342	4a	n/a	NY-Cornell	LMACI.0306	LMAAI.0319	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
09-3419	4b	Human	MB	LMACI.0308	LMAAI.0555	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08-8753	1/2c	Human CSF	BC	LMACI.0316	LMAAI.0559	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
07-6350	1/2a	n/a	NY-Cornell	LMACI.0334	LMAAI.0320	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
08-6350	1/2a	Human blood	PEI	LMACI.0442	LMAAI.0551	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
09-3891	1/2a	Food	ON	LMACI.0590	LMAAI.0510	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
87-0192	1/2a	Human blood	ON			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
87-0426	1/2a	Human CSF	NS			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
88-0286	1/2a	Cooked pork	ON			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
88-0702	1/2a	Human CSF	ON			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
90-0558	1/2a	Human CSF	AB			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
91-0145	1/2a	Human blood	ON			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
92-0366	1/2a	Human blood	?			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
93-0024	1/2a	Human CSF	QC			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
93-0407	1/2a	Human blood	ON			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
94-0096	1/2a	Human blood	BC			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
94-0447	1/2a	Human blood	ON			+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
EGDe*	1/2a	Rabbit tissue				+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-