

## ABSTRACT

DUTTA, VIKRANT. Characterization of Disinfectant and Phage Resistance Determinants of *Listeria monocytogenes*. (Under the direction of Dr. Sophia Kathariou).

Environmental contamination of food plays a key role in transmission of *Listeria monocytogenes*. Adaptations such as ability to grow under refrigeration, biofilm formation, disinfectant resistance, and resistance to phage are crucial to the survival of *L. monocytogenes* in the environment. Here we have investigated some of the adaptations that may contribute towards environmental persistence of this pathogen. These include disinfectant (quaternary ammonium compounds, QAC) resistance, temperature-dependent resistance to phage among epidemic clone II strains, triphenylmethane dye reduction and resistance to heavy metals such as cadmium. Using *in silico* analysis, we have analyzed the prevalence of *cadAC* efflux systems associated with cadmium resistance among different *Listeria* genomes. Of the three distinct *cadAC* types known to date in *L. monocytogenes*, two (*cadA1C1* and *cadA2C2*) were harbored by plasmids and were encountered in diverse strains of *L. monocytogenes* and (in the case of *cadA2C2*) non-pathogenic *Listeria* spp. Large plasmids from two different epidemic-associated strains of *L. monocytogenes* harbored *cadA2C2* together with a drug efflux system (*bcrABC*), and both determinants were associated with a putative *IS1216* transposon. Plasmid-cured derivatives of these strains were susceptible both to cadmium and to BC. Genetic analyses revealed that *cadA2C2* was sufficient for restoring resistance to cadmium to a plasmid-cured derivative of *L. monocytogenes* H7550. Furthermore, *bcrABC* was sufficient for restoring resistance to high levels of QAC to the plasmid-cured strain. The transcription of *bcrABC* was temperature-dependent, with higher transcript levels at 37°C than at 25°C or below. The *bcrABC* system was encountered among *L. monocytogenes*

strains of different serotypes, origin (environmental or clinical) and genotypes and was detected more frequently in strains harboring the cadmium resistance cassette *cadA2C2* (alone or together with *cadA1C1*) than in those harboring *cadA1C1* alone. *bcrABC* was found in several different genetic arrangements, mostly on plasmids, but we also identified *L. monocytogenes* strains in which *bcrABC* appeared to have translocated into the chromosome. Transcriptional analysis revealed that transcription of *cadA2C2* and *bcrABC* was independent, and induced upon exposure to the respective compounds (cadmium and BC, respectively). On the other hand, *bcrABC* was co-transcribed with the downstream gene *tmr*, encoding a putative triphenylmethane reductase. We showed that *tmr* mediated decolorization (reduction) of toxic dyes such as crystal violet (CV) and that CV could also induce *bcrABC*. Analysis of a panel of strains of diverse serotypes revealed that those harboring *tmr* tended to also harbor *bcrABC* and *cadA2C2* (alone or together with *cadA1C1*), suggesting the presence of a co-selected multi-resistance unit that includes *cadA2C2*, *bcrABC*, and *tmr*. In previous studies, a gene (ORF 2753) was found to be responsible for the ability of *L. monocytogenes* epidemic clone II (ECII) to be resistant to phage when propagated at temperatures  $<25^{\circ}\text{C}$ . Here we have shown that ORF2753 corresponded to a putative restriction endonuclease that was a component of a type II restriction-modification (RM) system (LmoH1) that is specific for GTATCC (N6/5). This RM system is present on a chromosomal genomic region unique to ECII strains. Transcriptional analysis revealed that the putative restriction endonuclease was expressed significantly more at  $\leq 25^{\circ}\text{C}$  than at  $37^{\circ}\text{C}$ , potentially accounting for the temperature-dependent phage resistance of ECII strains, while ORF2754 (putative methyltransferase) was expressed at all tested temperatures; this was in agreement with the resistance of the DNA from cells grown at either  $25^{\circ}\text{C}$  or  $37^{\circ}\text{C}$  to BfuI, specific for GTATCC

(N6/5). Given that genetic basis for distinct adaptive attributes were elucidated in an outbreak strain (H7550), it would be of further interest to investigate the contribution of these determinants towards virulence of this pathogen. Such details would provide new avenues to control the incidence of listeriosis.

Characterization of Disinfectant and Phage Resistance Determinants of  
*Listeria monocytogenes*

by  
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A dissertation submitted to the Graduate Faculty of  
North Carolina State University  
in partial fulfillment of the  
requirements for the Degree of  
Doctor of Philosophy

Microbiology

Raleigh, North Carolina

2011

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## **DEDICATION**

I would like to dedicate this dissertation to my grandmother Tara Devi Dutta whose unconditional love, values, and vision have been and will be a sole source of inspiration throughout my life.

## BIOGRAPHY

Vikrant Dutta was born in the Western state of Rajasthan, India. He completed his high school in the Himalayan city Shimla in the state Himachal Pradesh, India. After completing high school, he joined Nagpur Veterinary College, Nagpur in the central Indian state of Maharashtra. After receiving degree in Veterinary Medicine, Vikrant worked as a veterinarian for a short duration in a small and large animal clinic at Nagpur before joining University of Arkansas, Fayetteville for his M.S. in Poultry Science. During M.S., his research was focused on analyzing the effects of stress and colibacillosis on the isolation of *Listeria monocytogenes* from synovial tissue of turkey poults.

After receiving his degree from University of Arkansas in 2008, Vikrant joined the laboratory of Dr. Sophia Kathariou at North Carolina State University, Raleigh for PhD. His doctorate work at NC State has been focused on identifying and characterizing the conditions and molecular determinants responsible for environmental persistence in *Listeria monocytogenes*.

## ACKNOWLEDGMENTS

I am forever grateful to Dr. Sophia Kathariou for believing in me and providing me with the opportunity to pursue a Ph.D. Her vision to see her students succeed and to learn from every opportunity is the driving force behind the success of our research group. For me, the tenacity of the challenges that were put forth by her during my Ph.D. has created exceptional confidence in me as a researcher. I believe attributes such as objectivity and articulate thinking that I have developed during my stay in Dr. Kathariou's lab, will help me overcome future challenges in life. Her distinctive enthusiasm for science and never ending appetite for knowledge is phenomenal and I can only imagine living by these values. I am appreciative of all the help and valuable advice from all my committee members. I am also thankful to Robin Siletzky for her phenomenal support. Her ability to find solutions even when the odds are stacked high has been a great inspiration. I would also like to thank Dr. Driss Elhanafi whose extraordinary support was essential to my research work. His willingness to help at all times was phenomenal. The support and encouragement from all my lab mates has been great. I would also like to extend my appreciation to the great Ghazal singers especially Jagjit Singh (died Oct'2011) who have often helped me get by long and tiring days. Last but not the least; I would like to thank my family in India and my forever supportive fiancée (Ashley Stephens) for their love, encouragement and faith in my abilities. My family and especially Ashley have been a great source of strength that has helped me to persevere through the challenges. I consider myself blessed to be surrounded by a loving and caring family.

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## **Chapter I: Literature Review.**

### **Introduction**

*Listeria monocytogenes* was first described by Murray, Web, and Swan in 1926 and was isolated upon necropsy from six young rabbits that died in the animal breeding establishment of the Department of Pathology at Cambridge. All the affected rabbits demonstrated abundance of mononuclear leucocytes and hence the rod-shaped organism isolated was named *Bacterium monocytogenes* (Murray et al., 1926). In 1927, the genus name was changed to *Listerella* by Pirie in honor of a British surgeon Lord Joseph Lister. Upon receiving description of the same bacterium from Pirie and Murray, the National Type Collection at the Lister Institute, London named the bacterium *Listerella monocytogenes*. It was not until 1939, the genus name *Listerella* was rejected by the International Committee on Systemic Bacteriology in order to avoid confusion with two other genus names, one for a mycetozoan named after Arthur Lister (younger brother of Lord Joseph Lister) and the other a foraminifer species named after Joseph Jackson Lister (Father of Lord Joseph Lister). Pirie in 1940 proposed the name *Listeria* (Harvey, 1940). The first human case of listeriosis was reported in a WWI soldier suffering from meningitis, even though the bacterium was later (in 1940) identified to be *L. monocytogenes*. Clinical cases of listeriosis both for humans and animals were reported in 1920s, but it was not until 1952 that *L. monocytogenes* was recognized as a major cause of neonatal sepsis and meningitis in East Germany (Potel, 1952).

### **Taxonomical, biochemical, and ecological characteristics of *Listeria monocytogenes***

There are eight environmentally prevalent species of genus *Listeria*: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L. marthii* and *L. rocourtia*

(Leclercq et al., 2010). Based on the somatic (O; I to XV) and flagellar (H; A to E) antigens, *Listeria* has been divided into number of serotypes which are ascertained on the basis of unique combinations of O and H antigens. There are at least 13 known serotypes for *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7). Serotypes for other *Listeria* sp. have also been described (Rocourt and Buchrieser, 2007). *L. monocytogenes* is the only species that is pathogenic to both humans and animals; however *L. ivanovii* has also been implicated with animal listeriosis.

*L. monocytogenes* is a facultatively anaerobic, motile, non-sporulating bacterium; cells are short gram-positive rods (0.5  $\mu\text{m}$  wide and 1-1.5  $\mu\text{m}$  long). *L. monocytogenes* is  $\beta$ -hemolytic, catalase-positive, oxidase-negative and possesses glucose oxidase and NADH oxidase activities (Patchett et al., 1991, Farber and Peterkin, 1991). *Listeria* is highly motile at low temperatures (up to 28°C) and less motile or non-motile at 37°C (Peel et al., 1988; Kamp and Higgins, 2009). Environmental distribution of *Listeria* spp. is probably due to its high adaptability and ability to grow over a wide range of temperatures (from 1° C through 45° C), pH (from 4.7 through 9.2), osmotic pressure, and high salt concentrations (Petran and Zottola, 1989; Walker et al., 1990; De Martinis et al., 1997; Stack et al., 2008). The resistant nature of *L. monocytogenes* is associated with genetic determinants that encode various components of potential stress response systems (Gandhi and Chikindas, 2007; Stack et al., 2008). *Listeria* spp. has been isolated from numerous environmental sources such as soil, sewage effluents, and a large variety of foods (Sauders and Weidmann, 2007). *L. monocytogenes* thrives as a saprophyte in decomposed organic material and as an intracellular pathogen in macrophages (Liu, 2008). The transmission route for *L. monocytogenes* in humans is primarily via foods,

however other routes of transmission have also been identified, including nosocomial and occupational (animal handlers can become infected primarily by open skin wounds) (Bell and Kyriakides, 2005). It was not until 1980s that *L. monocytogenes* was recognized as a major food-borne pathogen. Since then numerous outbreaks due to *L. monocytogenes* have been reported. Outbreak investigative efforts have led to increased understanding of this pathogen. Nonetheless more than three decades since the first major outbreak, *L. monocytogenes* remains a key food-safety threat due to the high case fatality rate associated with listeriosis (Scallan et al., 2011).

### **Listeriosis**

**General description and sources of infection.** Listeriosis is a relatively uncommon but severe clinical manifestation caused by infection with *L. monocytogenes*. Susceptible populations include pregnant women, the elderly, neonates and immunocompromised individuals (Kathariou, 2002; Kornacki and Gurtler, 2007; Painter and Slutsker, 2007; Scallan et al., 2011). Non-invasive illness is characterized by febrile gastroenteritis symptoms whereas invasive kind is characterized by the symptoms such as meningitis, abortion, and septicemia. The infectious dose for *L. monocytogenes* has not been established as it varies with strain and the immune status of the host; however, consumption of large numbers of *L. monocytogenes* has been shown to induce febrile gastroenteritis in otherwise healthy individuals (Frye et al., 2002).

**Incidence of *L. monocytogenes* as a pathogen.** The first case of listeriosis in humans was reported by Nyfeldt (1929) and later in the 1980s it was established that almost all reported human cases of listeriosis were foodborne in humans (FDA, 1992). Since then, listeriosis has

been linked to both sporadic cases and large outbreaks of human illness in various parts of the world. Even though the incidence for listeriosis has declined in the U.S., listeriosis remains a leading cause of death (16-19%) and the most common cause of hospitalizations (94%) associated with foodborne pathogens in the U.S. (Scallan et al., 2011). Contrary to the trends in the U.S., the incidence for listeriosis is on the rise in other parts of the world i.e. Canada and Europe, where increasing numbers of sporadic cases and epidemics have been reported in recent times (Pagato et al., 2006; Denny and McLaughlin, 2008; Gilmour et al., 2010; Allerberger and Wagner, 2010).

More than 95% of the human listeriosis cases are caused by strains of serotype 1/2a, 1/2b, and 4b, and strains of serotype 4b are most frequently implicated in outbreaks (Swaminathan and Gerner-Schmidt, 2007). Sources of listeriosis have been linked to widely distributed foods, demonstrating the importance of linking geographically dispersed illness. Products such as raw milk, soft cheese, and ready-to-eat meat products are usually implicated in the literature as a source of transmission (Sauders and Weidmann, 2007; Liu, 2008). Cross contamination which can occur within the food processing environment is considered to be a primary cause of *Listeria* contamination of processed food (Lin et al., 2006). However, in sporadic cases many factors such as type of food, conditions of storage, handling of food, and modes of food preparation can influence the frequency and level of contamination (Goulet, 2007), especially given the ability of *L. monocytogenes* to grow in refrigeration temperatures. Some of the major outbreaks of listeriosis are listed in Table 1.

**Intracellular life cycle of *L. monocytogenes*.** *L. monocytogenes* has the ability to invade and replicate in epithelial cells and macrophages. Virulence studies using mice and guinea pigs

have suggested that *L. monocytogenes* is taken up by enterocytes or M cells near the Peyer's Patches in the small intestine and then replicate in phagocytic cells. After entering the cell *L. monocytogenes* escapes from the phagolysosome into the cytoplasm. Presence of *L. monocytogenes* in the cytoplasm protects it from being recognized by the cell surveillance system (T cells) thereby evading the cellular immune response (Hamon et al., 2006). Once in the cytosol *L. monocytogenes* replicates and moves in the cytoplasm using actin-based motility. *L. monocytogenes* also uses this motility to spread into the neighboring cells by forming double membrane pseudopods into the adjacent cell, with the inner membrane originating from the donor cell. *L. monocytogenes* rapidly escapes (within 5 min) from the newly formed vacuole by dissolving its double membrane reaching the cytoplasm and initiates a new round of intracellular proliferation and direct intercellular spread (Hamon et al., 2006). All aspects of *L. monocytogenes*' intracellular life cycle are supported by virulence genes clustered and physically linked in a 9-kb chromosomal island formerly called the PrfA-dependent virulence gene cluster and now referred to as LIPI-1 for *Listeria* Pathogenicity Island 1 (Vázquez-Boland et al., 2001; Cossart and Arana, 2008). This cluster comprises six well-characterized genes namely *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB* that are critical for the virulence of *L. monocytogenes*.

**Cellular adherence and entry into epithelial cells.** Currently, it is known that InlA and InlB act as ligands to the cell surface receptors E-cadherin and hepatocyte growth factor, respectively. InlA-mediated internalization of *Listeria* is supported by a calcium-dependent intercellular adhesion glycoprotein host cell receptor, E-cadherin (Gieger and Ayalon, 1992; Cossart and Arana, 2008). Apart from the surfaces of intestinal epithelium, which is the major

route for *Listeria* invasion, E-cadherin is also present on hepatocytes, dendritic cells, brain microvascular endothelial cells, and the epithelial cells lining the choroid plexus and placental chorionic villi (Lecuit et al., 1999). InlB has shown to be involved with invasion in a broad range of cell lines such as hepatocytes and non-epithelial cells.

The P60 protein is encoded by the *iap* (invasion-associated protein) gene, and is considered crucial for cell viability because of its murein hydrolase activity required for normal septum formation (Wuenschel et al., 1993; Pilgrim et al., 2003). It is also required for intestinal invasion (Hess et al., 1996; Faith et al., 2007).

**Evading the phagolysosome and motility in the cytoplasm.** Listeriolysin (LLO) is a major virulence determinant as it helps *L. monocytogenes* along with two enzymes with phospholipase-C activity (PLCs) to evade the phagolysosome. LLO belongs to the family of thiol-activated, cholesterol-dependent, pore-forming toxins (Palmer, 2001). The hemolytic activity exhibited by all pathogenic *L. monocytogenes* strains is due to the expression of LLO (Geoffroy et al., 1987; Cossart et al., 1988). LLO along with the two listerial PLCs, phosphatidylinositol (PI-PLC) and phosphatidylcholine (PC-PLC) acts synergistically to destroy the primary and secondary vacuoles thereby leading to the entry of *L. monocytogenes* into the host cell cytosol (Camilli et al., 1993; Gedde et al., 2000). LLO is one of the most antigenic proteins released during intracellular growth of *Listeria*. In order to avoid the recognition of intracellular *L. monocytogenes* by the cell surveillance system, upon entry of *L. monocytogenes* into the cell cytosol the LLO production is down-regulated and LLO is rapidly degraded by the host proteasomes (Villanueva et al., 1995). ActA is an envelope protein that has a transmembrane motif in its carboxyl-terminal domain that helps in

attachment to the bacterial surface (Kocks et al., 1992). After attaching to the bacterial surface ActA polymerization causes a propulsive effect which provides motility to the bacteria and contributes to its escape (Portnoy et al., 2002).

Transcriptome analysis has suggested a number of other genes that are induced during intracellular growth of *L. monocytogenes*; however, the detailed understanding of such genes and the related regulatory pathways is limited (Chatterjee et al., 2006; Toledo-Arana et al., 2009; Camejo et al., 2009). Some of these genes are also involved in general housekeeping functions necessary for a saprophytic life such as metal ion uptake, multidrug resistance, oxidative stress responses, and quorum sensing. (Chatterjee et al., 2006; Toledo-Arana et al., 2009; Camejo et al., 2009).

**Regulation of virulence determinants of *L. monocytogenes*.** Positive regulatory factor A (PrfA) is a key regulator of *L. monocytogenes* pathogenesis and induces the expression of multiple virulence factors within the infected host. PrfA is post-translationally regulated such that the protein becomes activated upon bacterial entry into the cell cytosol. The environmental signal responsible for PrfA activation is not known yet. Nonetheless upon activation, PrfA regulates a number of genes (PrfA regulons) which directly or indirectly contribute towards the virulence and fitness of *L. monocytogenes* in the intracellular environment (Freitag et al., 2009). These genes are usually identified by a 14 bp palindrome sequence upstream of their start codon. This sequence acts as a binding site for PrfA and thereby regulates virulence genes expression. Expression of PrfA is optimal at 37°C and becomes impaired at temperatures below 30°C. The mechanism underlying this phenomenon was described by Johansson et al in 2002. Briefly, the secondary structure formed by the

*prfA* 5' untranslated mRNA region was found to change with temperature such that it transiently exposes the ribosome binding site only at 37°C.

**Genetics of *L. monocytogenes*.** Genome sequences for a number of *Listeria* strains are now available (<http://www.broadinstitute.org>, <http://www.ncbi.nlm.nih.gov/>, Francis and Thomas, 1997; Glaser et al., 2001; Nelson et al., 2004; den Bakker et al., 2010; Canchaya et al., 2010; Gilmour et al., 2010). *Listeria* spp. genomes have relatively low GC content (average, 38%), where the GC content for *L. monocytogenes* strains on average is around 37-38%. Comparative genomic analysis of *Listeria* genomes suggests a strong conservation of gene organization among members of the genus, which is suggestive of a close phylogenetic relationship (Buchrieser, 2007; Glaser et al., 2001; Nelson et al., 2004; Ragon et al., 2008; Gilmour et al., 2010). Non-pathogenic species of *Listeria* show a clear loss of core virulence genes compared to *L. monocytogenes* (Glaser et al., 2001; Buchrieser et al., 2003; Hain et al., 2006; Buchrieser, 2007). A core genome is present in all *Listeria* strains and can be speculated to play a key role in the normal physiology of the organism (Lomanco et al., 2008; Piffareti et al., 1989; Cheng et al., 2008; Ragon et al., 2008; den Bakker et al., 2010). Nonetheless, *Listeria* genomes do show genomic differences due to phage insertions, transposable elements, other unique genes and gene cassettes, and single nucleotide polymorphisms (Glaser et al., 2001, Nelson et al., 2004, Hain et al., 2006, Gilmour et al., 2010; Cheng et al., 2010).

Advancement in genome typing methods along with the availability of complete genome sequences has led to increased understanding of the ecology and evolution of *L. monocytogenes*. Typing methods have led to the identification of determinants that were

unique to a specific strain or serotype of *L. monocytogenes*. It has been hypothesized that such unique determinants will provide clues about persistence, survival, virulence, and even niche tropism for *L. monocytogenes* strains. Based on typing data, *L. monocytogenes* has been divided into three lineages. Lineage I includes serotypes 1/2b, 3b, 4b, 4d, and 4e; Lineage II includes serotypes 1/2a, 3a, 1/2c, and 3c; and Lineage III includes serotypes 4a, 4c and some 4b strains (Rasmussen et al., 1995; Call et al., 2003; Deng et al., 2010). Most human outbreaks of listeriosis have involved a small group of genetically similar strains mostly from serotype 4b referred to as “Epidemic Clones”. Even though these strains are clonal, typing analysis have suggested distinct genetic characteristics that categorize these strains into several clonal groups, including epidemic clone (EC)-I, II, and ECIa (or IV) (Cheng et al., 2008).

**Epidemic Clone I (ECI).** Strains from this clonal group have been implicated in a number of major foodborne listeriosis outbreaks both in North America and Europe including those in Nova Scotia, Canada (cole slaw, 1981), California (Mexican Style cheese, 1985), France (pork tongue, 1992) and others (Cheng et al., 2008). ECI strains were found to be widespread in the environment which may have resulted in the repeated entry of these strains into the food processing environment. It has been speculated that their better fitness compared to other serotype 4b isolates might have been responsible for their persistence in the environment (Yildirim et al., 2004, 2010).

Typing methods, sequencing of the representative strain F2365 and genetic characterization studies have highlighted some of the crucial genetic elements that were unique to this clonal group and might have contributed to their increased prevalence in the environment (Zheng

and Kathariou, 1997, Tran and Kathariou, 2002; Herds and Kocks 2001, Yildirim et al., 2004, Chen and Knabel, 2007). Using macroarray analysis Herd and Kocks (2001) identified a number of gene clusters that were unique to ECI strains. Later Nelson et al (2004) added to the list by using strain F2365 genome sequence thereby reporting the comprehensive list of genetic fragments that were unique to ECI strains: Lmof2365\_2798 to Lmof2365\_2800, Lmof2365\_2701 to Lmof2365\_2707, Lmof2365\_2347 to Lmof2365\_2748 and Lmof2365\_0687. Some of these genetic fragments were later confirmed for PCR specificity (Chen and Knabel, 2007).

Zheng and Kathariou in 1997 reported that the genomic DNA of ECI strain was resistant to Sau3AI digestion. The putative R/M system conferring this resistance to Sau3AI was found to be located in one of the gene clusters (region 85) unique to ECI strains (Yildirim et al., 2004, 2010). It wasn't until later that the same R/M system conferring Sau3AI resistance was also found in 1/2a strains as well (Yildirim et al., 2010). Detailed analysis of region 85 in a larger panel of strains has identified other previously unknown R/M systems, sometimes more than one, which have been hypothesized to act as an immigration control region (SL thesis). It can be speculated that presence of these R/M systems will provide a fitness advantage against phage infection. To what extent these systems contribute towards the persistence of the strains that harbor them remains to be seen. The functions of other ECI-unique regions are currently unknown and more work will be required to understand their contribution on the overall ecology of *L. monocytogenes*.

**Epidemic Clone II (ECII).** This clonal group was relatively rare among clinical and other isolates until it was first implicated in a major food borne listeriosis outbreak in 1998 and later

in 2002 (CDC, 1999, 2002; Evans et al., 2004; Kathariou, 2006; Cheng et al., 2010). Subsequently ECII strains have also been implicated in sporadic human listeriosis and have been isolated from environmental samples (Ducey et al., 2007; Kabuki et al., 2004, Ragon et al., 2008; Eifert et al., 2006; Sauders et al., 2006). The strains from this group were unique in their PFGE and ribotype patterns and were designated as epidemic clone II (ECII) (Evans et al., 2004; Cheng et al., 2010). Comparative genomic analysis of the ECII strains using typing techniques and the sequencing of representative strain H7858 have led to the identification of genomic regions that were unique to this clonal group (Evans et al., 2004; Nelson et al., 2004; Kathariou et al., 2006; Cheng et al., 2010). It was previously reported that a serotype 4b specific region (Region 18) was found to be diversified in ECII strains (Evans et al., 2004; Cheng et al., 2008, 2010; Chen and Knabel, 2007). This turned out to be the first genetic marker specific to ECII strains. Recently, apart from the diversified region 18 more ECII specific regions have been reported by Cheng et al, these included region 1168, region 0088, and region 2753 (Nelson et al., 2004; Cheng et al., 2010). As with the ECI strains, it can be speculated that these regions provide fitness advantage to ECII strains. Our analysis has suggested that region 2753 harbors a type II R/M system which is responsible for imparting low temperature-dependent phage resistance to ECII strains (Kim and Kathariou, 2008; JW, VD, SK unpublished). The contribution of other ECII-specific regions to the ecology of *L. monocytogenes* is still unknown.

Survey of two turkey processing plants has suggested that ECI and ECII were overrepresented among the isolates and were repeatedly isolated from the plant suggesting repeated introduction or persistence of these strains in such environments (Eifert et al., 2005). It has

also been reported that both ECI and ECII strains along with ECIIa has an increased cytopathogenicity compared to other ribotypes (Gray et al., 2004). These findings are indicative of a comparatively higher risk posed by these strains.

**Epidemic clone III (ECIII).** In 2000, a multistate outbreak of listeriosis was reported. The strain implicated belonged to serotype 1/2a which upon detailed analysis was found to be similar to another 1/2a strain implicated in case of listeriosis outbreak due to consumption of contaminated turkey deli franks in 1988. Later it was confirmed that both strains originated from the same processing plant suggesting that the strain was persistent over >12 years. The whole genome sequence of strain F6854 along with three other isolates from 2000 and 1988 has become available (<http://www.broadinstitute.org>, Nelson et al., 2004). These data will prove crucial in identifying the genetic regions unique to the ECIII strains. Comparative analysis of the genome sequence of F6854 with the previously sequenced serotype 1/2a strain EGD-e, suggested 97 genes specific for F6854 (Nelson et al., 2004)

**Epidemic clone Ia or IV (ECIIa or ECIV).** Serotype 4b isolates implicated in three outbreaks were found to share a common ribotype and these isolates were distinct enough to be designated as a separate clonal group (Piffarreti et al., 1989; Chen et al., 2007; Gray et al., 2004). These outbreaks were 1979, Boston outbreak due to vegetables; 1983 Boston outbreak due to milk and 1989 UK outbreak related to contaminated paté. This clonal group has been poorly characterized; the genetic fragments and phenotypic features unique to it have not been identified yet. Nonetheless recent availability of genome sequences for two ECIV *L. monocytogenes* strains HPB2262 and J1-220 will provide data for comparative analysis (den Bakker et al., 2010; Chen et al., 2011).

**Epidemic clone V (ECV).** The isolates from this clonal group were implicated in an outbreak due to consumption of a contaminated Mexican-style cheese in North Carolina in the year 2000 (MacDonald et al., 2005). The isolates harbored peculiar genetic features where on one hand, these isolates harbored ECII-like region 18 (a genomic region that is serotype 4b-specific but varies between ECII and other strains), and on the other hand they lacked other ECII-specific markers. These isolates also harbor at least one ECI specific genetic marker (not found in other serotype 4b strains). Subtyping analysis has suggested that ECV strains are present among the clinical and turkey processing plant environments (RS, SK unpublished). Further genome sequencing and strain typing will prove useful in understanding the prevalence of ECV strains.

#### **Bacterial transporters.**

*In silico* analysis has led to the discovery of numerous putative transporters in bacterial genomes. Based on the number of components, energy source, types of substrates, number of transmembrane-spanning regions the efflux pumps in Gram-positive bacteria has been categorized into five families namely, the resistance nodulation division (RND) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR), ATP binding cassettes (ABC) and the multidrug and toxic compound extrusion (MATE) family (Piddock, 2006a, b). Among these, the secondary transporters (MFS, SMR, RND, and MATE) drive substrate translocation by using the free energy stored in the ion/solute gradients generated by primary ABC-type transporters (Law et al., 2008). Current findings suggest that these proteins play a wide array of roles and are indispensable to the ecological and physiological adaptations of the organism.

Being a Gram-positive bacterium, *L. monocytogenes* has an overrepresentation of transporters of the ABC and MFS families, but transporters from other families such as SMR and MATE are also found. The transporter database (<http://www.membranetransport.org/>) suggests the presence of at least 226 transporters in the *L. monocytogenes* EGD-e genome with 76.87 transporters per Mb genome. Relative to other Gram-positive bacteria, the role of transporters in the physiology, virulence, and persistence of *Listeria* has been poorly studied.

### **Role of transporters in drug resistance.**

As with other bacterial pathogens, exposure to sublethal levels of antimicrobial agents is considered to be responsible for increase in *L. monocytogenes* drug resistance. Number of reports has highlighted the isolation of antibiotic resistant *L. monocytogenes* (Poyart-Salmeron et al., 1990, 1992; Hadorn et al., 1993; Charpentier et al., 1995, 1999; Walsh et al., 2001; Davis and Jackson, 2009; Morvan et al., 2010). Drug resistance for *L. monocytogenes* is not a major clinical threat as *L. monocytogenes* remains susceptible to  $\beta$ -lactams (e.g. ampicillin) and aminoglycosides (e.g. gentamicin), which are drugs of choice for listeriosis (Hof et al., 1997; Morvan et al., 2010). Given the recent trend of the rise in antibiotic-resistant bacteria, under the selective pressure of the current use of antibiotics in food production and clinics, isolation of *L. monocytogenes* with reduced antibiotic susceptibility is expected to grow.

Antibiotics resistance due to transferrable antibiotics resistance determinants on self-transferable plasmids and transposon have been described for *L. monocytogenes* (Poyart-Salmeron et al., 1990, 1992; Hadorn et al., 1993; Charpentier et al., 1995, 1999). Drug efflux as a mechanism is a crucial determinant to the intrinsic and/or acquired form of antimicrobial

resistance which often times involves flexible drug specificities. Relative to other Gram positive bacteria, the association of efflux mechanisms/transporter proteins and transferable genetic elements for *L. monocytogenes* strains is relatively less well studied. Nonetheless, efflux mechanism has been implicated for tetracycline resistance in *Listeria* sp. (Facinelli et al., 1993; Charpentier et al., 1993). Prevalence and transferability of genes encoding for other mechanisms of antibiotic resistance such as enzyme degradation or enzyme modification such as chloramphenicol acetyltransferase (encoded by *cat221*) for chloramphenicol, rRNA methylase (*ermAM*) for erythromycin and 6-*N*-aminoglycoside nucleotidyltransferase (encoded by *aad6*) for streptomycin, have been well studied instead (Charpentier et al., 1994, 1999; Charpentier and Courvalin, 1999).

Other than the acquired antibiotic resistance via plasmids and transposon, the adaptation of the chromosomal transporters that have also been linked to acquired resistance of *L. monocytogenes*. Interestingly, even though there are 26 annotated MFS transporters proteins in the genome of *L. monocytogenes* EGD-e (<http://www.membranetransport.org/>), only few have been characterized in detail. Ciprofloxacin-resistant clinical isolates of *L. monocytogenes* were shown to have enhanced transcription of a drug efflux transporter of the major facilitator superfamily named *lde* (Godreuil et al., 2003). Upon insertional inactivation of *lde*, the mutant strain was 3-4 fold more sensitive to fluoroquinolones (ciprofloxacin and norfloxacin) and 2 fold more sensitive to dyes (acridine orange and ethidium bromide) than the parental strain (Godreuil et al., 2003). Increased transcription of *mdrL* in benzalkonium chloride adapted mutants was hypothesized to be responsible for reduction in susceptibility to gentamicin, kanamycin, disinfectant BC, and the dye ethidium bromide (Romanova et al.,

2006). For strains LO28 and 10403S increased transcription level of *mdrL* was reported upon exposure to sublethal levels of rhodamine 6G (R6G) (Huillet et al., 2006; Crimmins et al., 2008). Deletion of *mdrL* in *L. monocytogenes* LO28 resulted in increased susceptibility to macrolides, cefotaxime and certain metals (zinc, cobalt and chromium) (Mata et al., 2000). Crimmins et al reported increased transcription of *mdrL* along with another MFS transporter (*mdrM*) upon exposure to tetraphenylphosphonium (TPP). R6G exposure also increased transcription of *mdrM* and *mdrT* (also a MFS transporter) along with that of *mdrL* (Crimmins et al., 2008).

Apart from the antibiotics, the affect of disinfectants especially from the family of quaternary ammonium compounds (QACs) has been extensively studied for *L. monocytogenes*. A number of studies have investigated the incidence of resistance to the QAC benzalkonium chloride (BC) in *L. monocytogenes* isolates from foods and food processing plants (Mullapudi et al., 2008; Aase et al., 2000; Soumet et al., 2005). The role of transporter systems in acquired and innate resistance to QACs including BC has been investigated for *L. monocytogenes* (To et al., 2002; Romanova et al., 2006; Elhanafi et al., 2010). The BC resistance determinants have been shown to be present on the same transposon as determinants for other toxic compounds such as cadmium and triphenylmethane dyes (Elhanafi et al., 2010; Kuenne et al., 2010). More details related to the role of the efflux systems in BC resistance and evidence for co- and cross- resistance will be discussed later in this section.

### **Role of transporters in *L. monocytogenes* virulence.**

There is increasing recognition of the role of efflux systems in the normal physiology of both Gram-positive and Gram-negative bacteria, including biofilm formation, quorum sensing and virulence. Such a role has been postulated to be beneficial to the fitness and prevalence of the organism in its environment. It has been hypothesized that it is by chance that multidrug resistance (MDR) transporters are effluxing antimicrobial compounds, as the latter are not their “natural substrates”, thereby suggesting a role of these transporters in the normal physiology. Recent studies have shed some light on some of these “natural substrates”, such as host-encoded antimicrobial compounds and c-diAMP (Woodward et al., 2010); however, our understanding of the nature of these biomolecules and role of the related transporters in bacterial physiology remains limited. One study reported the role of efflux systems (MFS family) in activation of the host cytosolic surveillance pathway. Infection of macrophages with *L. monocytogenes* 10403S mutants with inactivated repressors for three transporters (*mdrL*, *mdrM* and *mdrT*) led to increases in the amount of IFN- $\beta$  (Crimmins et al., 2008). These transporters were later found to efflux c-diAMP into the cytoplasm which in turn led to IFN- $\beta$  production (Woodward et al., 2010). The exact purpose of this phenomenon is currently unknown; however it is believed that increased knowledge of this pathway could provide links to connect innate and adaptive immunity which might prove useful for designing prophylactic and therapeutic strategies.

*In vivo* transcriptome analysis of *L. monocytogenes* has suggested differential regulation of numerous previously unidentified genes (Camejo et al., 2009; Toledo-Arana et al., 2009). *In silico* analysis of *L. monocytogenes* genomes for three strains (EGD-e, F2365 and H7858)

have suggested that homologs for most of the chromosomal transporters exist within each genome, and often these transporters and their homologs have conserved domains for one of the known transporters families. However our analysis have found three transporter proteins for which no homologs were found within the *L. monocytogenes* and a single copy of each of these transporter proteins was found in all three analyzed genomes (VD, SK unpublished). In EGD-e these transporters were annotated as: Orf lmo1300 (Arsenical pump membrane protein), Orf lmo1424 (Natural resistance-associated macrophage protein), and Orf lmo1226 (putative RND family like transporter). Presence of a unique copy of such genes might indicate their essential nature. Furthermore, *in vivo* and *in vitro* transcriptome analysis has suggested that all three genes were differentially regulated in *in vivo* systems (Chatterjee et al., 2004; Camejo et al., 2009; Toledo-Arana et al., 2009). Although unique, these transporters represent many other transporter proteins which have shown to be differentially regulated in *in vivo* environments. More work is needed to understand the details under which these transporter protein function and what contribution do they have in the intracellular life style of *L. monocytogenes*.

## **Quaternary ammonium compound resistance in *Listeria monocytogenes*: A review**

### **Introduction**

Disinfectant resistance forms a crucial part of the environmental adaptation for *L. monocytogenes* (Lm) which along with other attributes such as ability to grow in cold temperatures, biofilm formation, and phage resistance has been hypothesized to contribute to the persistence of Lm in food processing environments (Kathariou, 2002; Gandhi and Chikindas, 2007; Kornaki and Gurtler, 2007; Kim and Kathariou, 2009; Elhanafi et al., 2010).

Even though disinfectant resistance has been well recognized for *Lm*, our overall understanding of the underlying mechanisms and the environmental conditions that impact disinfectant resistance in *Lm* is currently limited.

Quaternary ammonium compounds (QACs) are among a large group of biocides that are widely used as disinfectants in multiple settings including food processing plants (Mc Donnell and Russell, 1999; Langsrud et al., 2003; Merianos et al., 1991). QACs are sometimes also referred to as cationic detergents. They have been proposed to cause the bactericidal effect by binding to the microbial cell membrane causing membrane disorganization which in turn leads to leakage of the cellular contents and eventual autolysis of the cells (Salton, 1968).

The selective pressure associated with the use of disinfectants including QACs has the potential to cause the emergence of disinfectant-resistant microorganisms in food environments (Langsrud et al., 2003, Gandhi and Chikindas, 2007; Mc Donnell and Russell, 1999). The concentrations of QACs commonly used in industrial or healthcare settings are many times higher (recommended 200-400 $\mu$ g/ml in food related environments) than the minimum inhibitory concentrations for most of the bacterial pathogens. Nonetheless, under certain conditions, mostly speculated to be improper disinfectant use, bacteria may become exposed to sublethal QAC-concentrations which in turn would lead to emergence of QACs resistance. Resistance mechanisms include physiological adaptations, genetic alteration and acquisition of new genes (Langsrud et al., 2003, Sidhu et al., 2002; Mc Donnell and Russell, 1999, Romanova et al., 2006, To et al., 2002; Aase et al., 2000; Lunden et al., 2003).

Survival of QAC-resistant foodborne pathogens present a threat to the food industry since it can lead to the unintended enrichment and long term persistence of pathogens. Although less

well known, QACs have shown to increase the gene expression of known virulence determinants in *Lm* i.e. *prfA* and *inlA* (Kastbjerg et al., 2010). Furthermore, number of reports has indicated a link between QACs and antibiotics resistance (To et al., 2000; Langsrud et al., 2003; Sidhu et al., 2002; Romanova et al., 2006). Cross-resistance between disinfectants (including QACs) and antibiotics could lead to serious public health consequences and therefore needs further investigation.

Disinfectant resistance in *Lm* has been frequently studied in association with the biofilms (Pan et al., 2006; Saá Ibusquiza et al., 2011; Purkrtova et al., 2010); however recent studies have also investigated disinfectant resistance mechanisms in planktonic cells (Aase et al., 2000; To et al., 2002; Romanova et al., 2006; Elhanafi et al., 2010). Most of these studies investigated mechanism of resistance to widely used disinfectants such as QACs, peroxide and chlorine. These studies have focussed on specific genetic determinants or metabolic pathways, and limited knowledge is available regarding the effect of disinfectant exposure on the overall physiology and ecology of this pathogen. Better understanding of the effect of disinfectant on microbial physiology at the global level can lead to novel pathogen control systems.

### **Mechanisms of Disinfectant Resistance**

In principle, all the disinfectant resistance mechanisms characterized thus far lead to reduction in intracellular disinfectant levels. The mechanisms and the underlying genetic basis for disinfectant resistance, such as cell membrane alterations, ability to efflux or degrade the biocide and induction of the cellular stress response, can be inherent or acquired. These mechanisms can make variable contributions to the overall biocide resistance of bacteria, and

even though currently unknown, may also work synergistically.

**Cell envelope, change in permeability.** Upon exposure to sub-lethal concentrations of disinfectant, changes in the membrane permeability of the adapted *Listeria* strain has been reported (Aase et al., 2000; Mereghetti et al., 2000, To et al., 2002; Lunden et al., 2003; McDonnell and Russell, 1999). Adapted Lm cells had higher BC MIC than the pre-exposure levels, and this increase in BC MIC was attributed to morphological and physicochemical changes in the cell surface of the adapted Lm strains (Mereghetti et al., 2000; To et al., 2002). Upon adapting to sublethal BC, originally BC-resistant strain of Lm (BC MIC 4 µg/ml) exhibited a 2-fold increase in BC MIC. The 2-fold change in the MIC of the originally BC-resistant strain was attributed to the alterations in surface teichoic acid and fatty acid composition (To et al., 2002). In case of originally BC-sensitive strains (in three out of four strains) upon adaptation, whereas in case of originally BC-sensitive strains, 5-fold increase in BC MIC was suggested due to efflux mechanisms.

Mechanisms responsible for intrinsic, low-level resistance (<10 µg/ml) of Lm are currently unknown. It is possible that efflux systems (chromosomal/ plasmid based) alone or in conjunction with cell membrane-associated factors are responsible for the observed overall intrinsic resistance. Interestingly, the levels of such intrinsic resistance vary among different Lm strains and it is possible that the inherent surface composition might contribute towards the differential susceptibility to QACs. Mereghetti et al (2000) reported that among 97 Lm strains tested for QACs (BC and cetrimide) susceptibility, 7 were found to be QAC-resistant, furthermore all of them belonged to serovars 1/2a and 1/2c and out of 37 serogroup 4 Lm strains none were found to be QAC-resistant. Mullapudi et al (2008) confirmed these findings

where, BC-resistant strains were overrepresented among isolates of serotype 1/2a (42-60%) and 1/2b (35-51%) and under-represented among serotype 4b isolates (7-12%). Mereghetti et al (2000) also reported that out of the 7 QAC-resistant strains, 5 were non-typeable by phage typing assay, this correlation between phage non-typeability and QAC resistance was found to be significant. The precise nature of changes in cell surface were not elucidated but cell surface structural changes such as alterations in cross-linking of peptidoglycan in the cell wall and changes in the composition of teichoic acid were rendered some of the possibilities responsible for this correlation.

**Efflux.** Drug efflux as a mechanism is a determinant crucial to the intrinsic and/or acquired form of antimicrobial resistance that may involve flexible drug specificities (Li and Nikaido, 2009). There is substantial evidence for the role of efflux pumps towards disinfectant resistance both in Gram-positive and Gram-negative bacteria (Piddock, 2006A, B; Poole, 2005). Due to the membrane-associated nature of the transport proteins, the variable nature of the substrates, and the complex regulation involved, the mechanisms by which biocide resistance transporters confer drug resistance remain poorly understood. In case of most Gram-positive bacteria, drug efflux pumps belong to either MFS or SMR family, nonetheless transporters from other families have also shown to be involved (Mc Donnell and Russell, 1999; Langsrud et al., 2003; Grkovic et al., 2002).

*In silico* analysis of the sequenced Lm strains indicate a number of efflux systems that could act as potential candidates for disinfectant efflux (<http://www.membranetransport.org/>). Even though prevalence of disinfectant resistance in Lm has been well studied, only few studies have addressed the role of efflux systems on disinfectant resistance.

Aase et al (2000) have shown that efflux of ethidium bromide (EB) was completely abolished upon addition of an oxidative phosphorylation uncoupler, carbonyl cyanide *m*-chlorophenolhydrazone-CCCP, (acts by making membrane permeable to protons) to the culture of *M. tuberculosis* (Lm) strains resistant to EB as well as BC, thereby suggesting the role of a proton motive force-dependent efflux systems (secondary transporters). However, no effect was noticed upon addition of dicyclohexyl-carbodiimide (DCCD) which is an inhibitor of F<sub>0</sub>F<sub>1</sub> ATPase (Aase et al., 2000). To et al (2002) also used EB to monitor the efflux patterns of originally BC-sensitive strains. Upon adaptation to sublethal levels of BC MIC, in three out of four strains BC MIC was increased by 5-fold. This increase in MIC was attributed to efficient efflux of EB by the BC adapted strains (To et al., 2002). Romanova et al (2006) suggested that use of reserpine, also an inhibitor of energy-dependant efflux systems, reduced the BC MIC for both parent and BC-adapted strains of *M. tuberculosis*. Increase in the transcript levels of chromosomal MFS transporters (*MdrL*, *Lde*) was attributed for the increase in BC MIC of BC adapted *M. tuberculosis* strains (Romanova et al., 2006). BC adapted strains (originally BC-sensitive) indicated significant increase in their *mdrL* transcript levels upon 30 min exposure to BC. This was in line with the previous findings where increased efflux activity was noticed upon adaptation in originally BC-sensitive strain (To et al., 2002). However findings from Romanova et al (2006) also indicated that the transcript levels for *lde* were not affected in these strains. For the originally BC resistant strains, low expression for *lde* and *mdrL* was recorded upon exposure to BC for 30 min. BC-resistant strains became significantly sensitive to BC after plasmid curing, thereby indicating the role of a putative plasmid based determinant in BC resistance. Upon exposing the plasmid cured derivatives (of originally BC-

resistant strains) to sublethal dose of BC for 30 min, significant increase (20- to 30- fold) in the transcript levels of both *mdrL* and *lde* was observed (Romanova et al., 2006).

Other than the chromosomal transporters, earlier studies have also indicated a role of plasmid based BC resistance mechanisms (Lemaître et al., 1998; Romanova et al., 2002 and 2006). Recently we have described the essential role of a plasmid-based *bcrABC* system in imparting high-level BC resistance (MIC 40 µg/ml) to the outbreak-associated Lm strain H7550 (Elhanafi et al., 2010). We have shown that the presence of *bcrABC* system renders H7550 resistant to high concentrations of BC (40 µg/ml). The transcription of *bcrABC* system was found to be increased not only in the presence of BC but also in the presence of sub-lethal dose of other QACs such as benzethonium chloride and cetyl-trimethyl ammonium bromide (VD SK unpublished). Interestingly transcription of *bcrABC* was found to be more pronounced at lower temperature, including refrigeration (Elhanafi et al., 2010). *In silico* analysis of the *bcrABC* region suggested the presence of a canonical promoter upstream of *bcrA*; an inverted repeat was identified between the -10 and -35 sequences, partially overlapping with the -10 sequence (Elhanafi et al., 2010). The *bcrA* belong to the TetR family of transcriptional regulators which regulate transcription by binding inverted repeats (Ramos et al., 2005; Grkovic et al., 1996). The molecular mechanism underlying control of expression of *bcrABC* has not been elucidated; however, based on the constituents of this system it can be speculated that *bcrA* controls the transcription of *bcrABC* by binding to the palindrome between the -10 and -35 sequences (ACCGTCCGGACGGT), as has been shown for control of QacAR system in *Staphylococcus aureus* (Grkovic et al., 1996).

**Mechanisms of acquired resistance.** Upon exposure to sublethal QACs, Lm strains have shown to adapt such that in comparison to the original strains their post-adaptation QAC MICs have shown to increase from 2 to 15 fold, where the level of fold increase varies across different studies (To et al., 2002, Aase et al., 2000, Lunden et al., 2003). Aase et al. reported that upon exposure to progressively increasing sublethal doses of BC (exposure started at 0.5 µg/ml and increased with the increment of 0.5 µg/ml until no growth was seen within a week), the BC MIC for originally BC-sensitive strains (n=6) increased by ca. 6 fold thereby equating the BC MIC to that of originally BC-resistant strains (6-7 µg/ml). The increase in BC MIC of BC-sensitive strains was maintained upon subculturing in no-BC media for up to 50 generations. No significant increase in the BC MIC of the originally BC-resistant strains (n=2) was observed (Aase et al., 2000). To et al. reported that MICs increased by 2-fold in originally BC-resistant strains versus 5-fold in strains that were originally BC-sensitive; in both cases, the increase in MICs appeared to be stable upon subsequent subculturing in BC free media for 10 months (To et al., 2002). Like Aase et al, the findings from To et al (2002) also suggested that in comparison to the originally BC-resistant strains, the adaptive increase in BC MIC upon sublethal BC exposure was significantly higher among BC-sensitive strains. Lunden et al (2003) adapted Lm by exposing the different strains of Lm to sublethal doses of two different QACs for 2h (QAC1: alkyl-benzyl-dimethyl ammonium chloride; Qac2: *n*-alkyldimethyl ethylbenzyl ammonium chloride). Post-adaptation to QAC1 and QAC2, 2-3 fold increase in the MIC for the respective disinfectants was observed. They also reported that the most increase (2-3 fold) in the disinfectant MIC was observed for the strains with low pre-adaptation MICs (0.6-1.2 µg/ml) in comparison to strains with high pre-adaptation MICs (5

µg/ml) where no post-adaptation increase in QACs MIC was reported. These were consistent with findings from previous studies (Aase et al., 2000; To et al., 2002). In the same study two Lm strains (one persistent and one non-persistent) were exposed to the progressively increasing concentrations of sublethal QAC1 and QAC2 at 10 and 37°C. Both strains were reported to be adapted post exposure to QAC1 and QAC2 where highest post-adaptive increase in MIC was reported for QAC2 (ca. 15 fold). No effect due to temperature was observed. Increased MICs were tested for stability every week for 28 days. Post-adaptive MICs levels for both QAC1 and QAC2 persisted for whole 28 days (Lunden et al., 2003).

Recently we have also performed disinfectant adaptation studies where BC-sensitive and plasmid cured derivatives of Lm strains H7550 and J0161 (BC MIC 10µg/ml) were exposed to sublethal dose of BC for 48h at 37°C. Post-adaptation 4 fold increase in BC MIC was observed for both strains (MRM et al., in review).

The mechanism responsible for increase in MIC of the adapted strains has not yet been deciphered. It has been speculated that improper use of the biocides can expose the target bacterial population to sublethal doses and that the stress caused by such exposure causes extensive DNA damage and repair leading to random mutations. Our findings have confirmed that in comparison to BC-sensitive strain (H7550-Cd<sup>s</sup>; BC MIC-10µg/ml) the transcript levels of certain chromosomal transporters (*sugE*, and *lde*) were increased in the BC adapted mutants (MIC-40 µg/ml) (MRM et al., in review). It can be speculated that these transporters have become more active in pumping out disinfectant and hence are responsible for the increased resistance to BC. Previously, Romanova et al (2006) have also reported increase in the transcription levels of *lde* along with *mdrL* in plasmid cured BC adapted mutants. Both of

these proteins belong to the MFS superfamily. Among the three (*lde*, *mdrL* and *sugE*) transporter proteins that have been implicated with increased transcriptional activity post-BC adaptation, two (*mdrL* and *sugE*) are preceded by a transcriptional repressor. For *mdrL* it has been shown that inactivation of *ladR* (*mdrL* transcriptional repressor) leads to increased transcript levels for *mdrL* (Crimmins et al., 2008). It is possible that similar regulatory mutations either in the transcriptional regulator or the transporter protein itself will cause increased transcription of the transporter protein and hence the increased efflux activity. This form of natural adaptation has been considered to be essential for the acquisition of the increased resistance to biocides by number of bacterial pathogens, in various environments (McDonnell and Russell, 1999).

Another way of acquiring resistance to the biocides is via gene transfer (McDonnell and Russell, 1999; Sidhu et al., 2002). Mechanisms describing gene transfer have been well documented for antibiotic resistance but have been poorly documented for disinfectant resistance determinants. *In silico* analysis and experimental evidence have suggested the presence of cadmium, QAC, and crystal violet detoxification gene on a putative *IS1216* transposon on the plasmid (pLM80) of Lm strain H7550 (Elhanafi et al., 2010; Kuenne et al., 2010). For Lm this was the first report suggestive of composite resistance for a disinfectant resistance determinant. Here composite resistance refers to genetic elements (such as integrons, transposons and plasmids) harboring multiple, unrelated resistance determinants that are transferred as a single unit into recipient bacterial hosts. *In silico* analysis suggested that the *IS1216* composite transposon harbored three transposase genes harboring identical sets of inverted repeats (5' GGTTCTGTTGCAAAGTTT 3'). It was hypothesized that this

element can have three transposable units (*IS1216*<sub>-left</sub> to *IS1216*<sub>-center</sub>, *IS1216*<sub>-center</sub> to *IS1216*<sub>-right</sub>, and *IS1216*<sub>-left</sub> to *IS1216*<sub>-right</sub>) which can lead to transfer of the *bcrABC* genes along with cadmium resistance efflux system, and a dye detoxification enzyme (triphenylmethane reductase) across *Lm* genomes, (Fig. 1). Even though *in silico* analysis provides a strong indication of the potential for gene transfer events, experimental evidence will be needed in support of this hypothesis.

Genomic analysis of a panel of BC-resistant *Lm* strains (MIC-40 µg/ml) suggested that the presence of *bcrABC* was correlated to the BC resistance phenotype. None of the tested BC-sensitive (MIC-10 µg/ml) strains harbored *bcrABC*, whereas the cassette was detected in majority of tested BC-resistant strains. In most of these BC-resistant strains the *bcrABC* locus resembled to that in pLM80 while in another major group *bcrABC* and the downstream transposase was flanked by novel sequences that appear to be on the chromosome (Fig. 1). It is possible that in these latter strains the transposase assists in *bcrABC* transfer. Plasmid sequences analysis for the BC-resistant strains J0161 (serotype 1/2a) and H7858 (serotype 4b) suggested that both plasmids harbor *bcrABC* (Nelson et al., 2004; Elhanafi et al., 2010; Kuenne et al., 2010). These findings suggest the ability of *bcrABC* to transfer across *Lm* genomes via plasmid mobilization or by transposition.

**Disinfectant resistance in *Listeria monocytogenes* biofilms.** It has been established that *Lm* biofilms exhibit higher resistance to QACs than their planktonic counterparts (Gandhi and Chikindas, 2007; Pan et al., 2006; Saá Ibusquiza et al., 2011; van der Veen and Abee, 2010A, B; Purkrtova et al., 2010; van der Veen and Abee., 2011). The extra polymeric substances (EPS) produced by the *Lm* biofilms has been hypothesized to act as a permeability barrier and

to be a major player in disinfectant resistance. Lm of different genomic divisions has been shown to have a different predisposition towards biofilm formation, which in turn has been attributed to their surface components (Djordjevic et al., 2002; Borucki et al., 2003). Based on molecular analyses, *L. monocytogenes* have been classified into two major genomic divisions, I (consisting of serotypes 4b and 1/2b) and II (consisting of serotypes 1/2a and 1/2c). Djordjevic et al. has reported better proclivity of Division I strains to form biofilms than Division II strains, whereas findings from Borucki et al have reported the opposite (Djordjevic et al., 2002; Borucki et al., 2003). The protocol used by both studies (microtiter plate assay) was not significantly different, furthermore the possible effect of methodology was ruled out by Borucki et al (2003). Division I serotypes were more closely associated with food-borne outbreaks whereas Division II serotypes were mostly associated with environment. In both studies no correlation between the strain serotypes and biofilm their formation ability could be established. The findings from Borucki et al where strains from Division II (serotype 1/2a, 1/2c) formed denser biofilms were later confirmed by Pan et al (2009) who showed that serotype 1/2a strains were more efficient in forming biofilms compared to serotype 4b strains (Borucki et al., 2003). It is important to mention that the protocol used by Pan et al was different from that of microtitre plate assay (used by Djordjevic et al., 2002; Borucki et al., 2003) and where biofilms were formed on stainless steel coupons instead of (Pan et al., 2009). Propensity of certain Lm strains towards efficient biofilm formation can provide superior “permeability barrier” which could in turn provide competitive advantage to related strains due to protective attributes such as enhanced disinfectants resistance.

Flagella production has also been reported to be crucial for biofilm formation of Lm (Lemon et al., 2007). This was an interesting finding because in Lm flagella are produced primarily at low temperatures (<25°C) (Peel et al., 1988; Kamp and Higgins, 2009). Given that low temperatures often prevail in the environment and in food processing plants biofilm production would be expected to be more pronounced in such environments. Lourenço et al in 2011 reported that Lm biofilms at 12°C were more resistant to treatment by non-QACs commercial dairy sanitizers than biofilms formed at 37°C (Lourenço et al., 2011). This would add to the risk of disinfectant resistance, especially when the Lm strains forming the biofilm harbor additional disinfectant resistance mechanisms some of which may be temperature dependent such as *bcrABC* (Elhanafi et al., 2010).

In nature biofilms usually exist as a multispecies entity, where complex regulatory determinants control the existence of biofilms (Wintermute and Silver, 2010). It is unknown as to how the individual bacterial species co-ordinate in this environment. It can be argued that understanding the adaptive attributes of this ecosystem such as antibiotic and disinfectant resistance will be more relevant than for the single species biofilms. For the reasons that are unknown mixed species biofilms between Lm and *Lactobacillus plantarum* were recently found to be more resistant to BC compared to the single species Lm biofilms (van der Veen and Abee, 2011).

Proteomic analysis of Lm biofilms has suggested that stress response mechanisms are intricately involved in biofilm formation (Hefford et al., 2005; Trémoulet et al., 2002). Stress response mediators including *sigB*, *hrcA*, and *dnaK* have also been associated with disinfectant resistance of Lm biofilms (van der Veen and Abee, 2010 A, B). Mechanisms

underlying correlation between stress determinants and disinfectant resistance of Lm in biofilms remain poorly understood. Mechanisms such as enzymatic inactivation of biocides, induction of efflux systems, and reduced metabolism have also been associated with increased disinfectant tolerance by bacteria in biofilm (Giwerzman et al., 1991; Huang et al., 1995; Maira-Litran et al., 2000). However, such a correlation has not been demonstrated for Lm.

It is clear that increased disinfectant resistance in biofilm-associated bacteria in comparison to planktonic cells is dependent on a complex network of cellular mechanisms. Our understanding of the genetic and environmental factors that contribute to biofilm formation and disinfectant resistance remains limited. Increased knowledge in this area will help in designing novel methods to curb the prevalence and persistence of Lm.

#### **Cross resistance to other biocides/compounds**

The possible correlation between disinfectant and antibiotic resistance and the underlying mechanisms has been poorly studied for Lm. Nonetheless, cross resistance between antibiotics such as fluoroquinolones, dyes such as EB, and disinfectants such as QACs has been reported for Lm (Romanova et al., 2006; Aase et al., 2000; Godreuil et al., 2003; Lunden et al., 2003; MRM et al., in review ).

Lunden et al. reported that upon adapting to QACs, Lm strains developed resistance to unrelated disinfectants such as tertiary alkylamine, potassium persulfate, and sodium hypochlorite (Lunden et al., 2003). Godreuil et al (2003) showed that the MFS transporter Lde was associated with fluoroquinolone resistance along with efflux of ethidium bromide and acridine orange in Lm. Aase et al (2000) reported that 50% of the BC resistant strains also exhibited EB efflux. Lde along with another transporter from the same family (MdrL) were

later implicated with the BC resistance and EB efflux by Romanova et al (2006). They found that post-BC adaptation the transcript levels of only *mdrL* were raised in adapted strains (originally BC sensitive), whereas transcript levels of both *lde* and *mdrL* were raised in plasmid cured BC adapted strains (originally BC resistant due to plasmid based determinants). They investigated the role of only two chromosomal transporters (MFS family) when there are number of other transporters, including at least 23 others MFS family transporters in *Listeria* genome that could be contributing to the increased BC MIC (<http://www.membranetransport.org/>). It is also interesting to mention that the plasmid based determinants were never investigated especially when there was a clear correlation between BC resistance and presence of plasmid (Romanova et al., 2002, 2006). BC and ciprofloxacin-adapted LM mutants show higher MIC to both BC (3-fold increase) and ciprofloxacin (4-fold increase) (MRM et al., in review). It is interesting to note that the drug action of these two compounds (BC and ciprofloxacin) is different.

The detailed mechanism for cross resistance among different compounds in Lm has not been elucidated. Genome wide transcriptome analysis of the adapted strains might provide some clues about the overall mechanisms contributing to such phenotypes. Mutations acquired by the BC-adapted mutants might lead to increased transcription of chromosomal transporters (e.g. *sugE* and *lde*), and it is possible that these transporters in adapted strains have acquired enhanced ability to efflux out compounds that may or may not be structurally related. The substrate specificity of the efflux pumps has been shown to be flexible (Piddock et al., 2006 A, B; Poole, 2003; Law et al., 2008). The underlying cause for this phenomenon has been linked to efflux regulatory proteins (usually a transcriptional repressor). Such repressors have

the ability to bind structurally similar and dissimilar compounds leading to derepression and hence increased transcription of the efflux transporter and thereby enhanced cross resistance (Law et al., 2008).

### **Prevalence of disinfectant resistance in Lm.**

The prevalence of QAC-resistant Lm isolates from food processing plants seems to vary among different studies. Aase et al (2000) reported an incidence of 10% BC resistant Lm strains (MIC 4-7 µg/ml) from fish processing plant and poultry slaughter house. Soumet et al (2005) showed that BC resistance was encountered in 42.5% of Lm isolates from fish and fish-processing environment. Strains with BC MIC of >3.75 µg/ml were considered as BC resistant in that study (Soumet et al., 2005). Mullapudi et al (2008) also reported high prevalence (46%) of BC- resistant isolates (MIC of 10 µg/ml) from turkey processing plants. Interestingly, BC-resistant strains were overrepresented among isolates of serotype 1/2a (42-60%) and 1/2b (35-51%) and under-represented among serotype 4b isolates (7-12%) (Soumet et al., 2005; Mullapudi et al., 2008). In several studies isolates of serotype 1/2a and 1/2b have been found to be overrepresented among food and environmental isolates of Lm whereas isolates of serotype 4b is overrepresented among clinical isolates (Lawrence and Gilmour, 1995; Ojeinyi et al., 1996; Soumet et al., 2005; Thevenot et al., 2005).

Among the environmental isolates of *L. monocytogenes*, cadmium resistance was also correlated to a disinfectant benzalkonium chloride (BC) resistance. Mullapudi et al (2008) reported that strains having both cadmium and BC resistance were more frequent than those with just cadmium resistance and no BC resistance (ca. 23%). All tested BC resistant strains were also cadmium resistant however reverse was not true. Incidence of joint cadmium and

BC resistance was seen more among the environmental isolates [serotype 1/2a (or 3a) (60%) and 1/2b (or 3b) (51%)] than serotype 4b complex (<10%). It was also suggested the presence of *cadA2* alone or together with *cadA1* [*cadA1* and *cadA2* were identified as two different cadmium resistance transporters in Lm (Mullapudi et al., 2010)] was significantly correlated to the BC resistant isolates (Mullapudi et al., 2010). Our analysis has confirmed these findings as a major correlation do exist between *bcrABC* (BC resistance determinant; Elhanafi et al., 2010) and *cadA2*, or *cadA1-cadA2* positive strains (VD, SK unpublished).

### **Concluding remarks**

The prevalence, mechanism, risk, and public health impact associated with resistance of Lm to QACs remain understudied. We still do not understand the mechanisms that underlie adaptation of Lm or other Gram-positive bacteria to sublethal levels QACs. Future studies involving the global effect of exposure to sublethal levels of QACs and other disinfectants on the overall physiology of Lm at the transcriptomic and proteomic level may elucidate previously unknown regulatory pathways. Further elucidation of this area will assist in understanding the ecology of Lm and will help in designing more informed disinfection protocols which can be used for the disinfection and control of Lm along with other Gram-positive pathogens.

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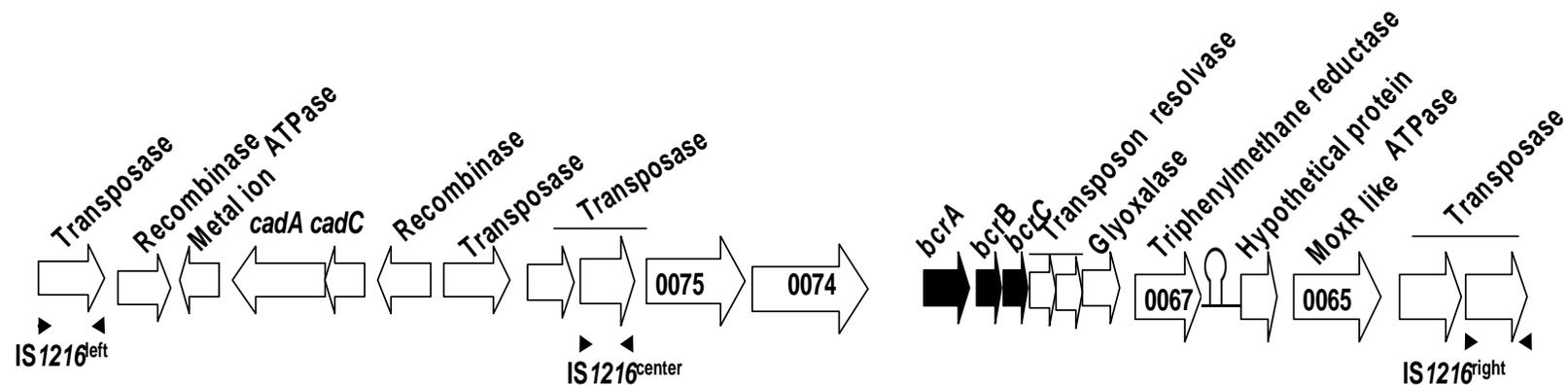
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**Table 1.** Major Epidemic Clones implicated in foodborne outbreaks of listeriosis. (Cheng et al., 2008)

<b>Epidemic Clone</b>	<b>Location, Implicated Food, Year</b>	<b>Serotype</b>	<b>Ribotype</b>	<b>Sequence Type (MVLST)</b>
Epidemic Clone I (ECI)	Nova Scotia, coleslaw, 1981 California, Mexican-style cheese, 1985 Switzerland, soft cheese, 1983-1987 Denmark, cheese, 1985-1987 France, jellied pork tongue, 1992	4b	DUP-1038B	20
Epidemic Clone II (ECII)	U.S. multistate, hot dogs, 1998-1999 U.S. multistate, turkey deli meats, 2002	4b	DUP-1044A	19
Epidemic Clone III (ECIII)	U.S. multistate, turkey deli meats, 2000	1/2a	DUP-DUP-1053A	1
Epidemic Clone IV (ECIV) (formerly known as EC1a)	Boston, vegetables, 1979 Boston, milk, 1983 U.K., pate, 1989	4b	DUP-1042B	21
Epidemic Clone V (ECV)	North Carolina, Mexican-style cheese, 2000-2001	4b	DUP-1042B	30
Not designated	Illinois, chocolate milk, 1994	1/2b	DUP-1051B	32



**Figure 1.** Genetic organization of *bcrABC* cassette on putative *IS1216* transposon on pLM80 in *L. monocytogenes* H7858. Three copies of *IS1216* are indicated as *IS1216*<sup>left</sup>, *IS1216*<sup>center</sup>, and *IS1216*<sup>right</sup> with solid arrows indicating the orientation of *IS1216* inverted repeats (modified from Elhanafi et al., 2010).

**Chapter II. Genetic Characterization of Plasmid Associated Benzalkonium Chloride Resistance Determinants in a Strain of *Listeria monocytogenes* from the 1998-1999 Outbreak.**

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**Author's contribution.**

**DE:** recombinant plasmids constructs with *bcrABC*, and manuscript writing ; **VD:** all other recombinant cloning, RT-PCR work, remaining experiments, data analysis and manuscript writing; **SK:** mentor, data analysis, manuscript writing

**Publication status.**

Published in Applied and Environmental Microbiology, 2010 Dec; 76(24):8231-8.

## ABSTRACT

Quaternary ammonium compounds such as benzalkonium chloride (BC) are widely used as disinfectants in both food processing and medical environments. BC-resistant strains of *Listeria monocytogenes* have been implicated in multistate outbreaks of listeriosis and have been frequently isolated from food processing plants. However, the genetic basis for BC resistance in *L. monocytogenes* remains poorly understood. In this study, we have characterized a plasmid (pLM80)-associated BC resistance cassette in *L. monocytogenes* H7550, a strain implicated in the 1998-1999 multistate outbreak involving contaminated hot dogs. The BC resistance cassette (*bcrABC*) restored resistance to BC (MIC, 40 µg/ml) in a plasmid-cured derivative of H7550. All three genes of the cassette were essential for imparting BC resistance. The transcription of H7550 BC resistance genes was increased under sublethal (10 µg/ml) BC stress and was higher at reduced temperatures (4, 8, or 25 °C) than at 37 °C. Level of transcription was higher at 10 µg/ml than at 20 or 40 µg/ml. *In silico* analysis suggested that the BC resistance cassette was harbored by an *IS1216* composite transposon along with other genes whose function is yet to be determined. The findings from this study will further our understanding of the adaptations of this organism to disinfectants such as BC and may contribute to elucidating possible dissemination of BC resistance in *L. monocytogenes*.

## INTRODUCTION

*Listeria monocytogenes* is a foodborne pathogen associated with severe illness (listeriosis) in at-risk individuals, including those in extremes of age, pregnant women and their fetuses, and those with compromised immunity. Environmental contamination with this pathogen plays a

key role to eventual contamination of ready-to-eat foods and subsequent foodborne illness (16, 19, 30). Biofilm formation and persistence, resistance to disinfectants, resistance to *Listeria*-specific viruses and ability to replicate at low temperatures are among the attributes contributing to the organism's prevalence and persistence in food processing environments (7, 18, 19).

Resistance to quaternary ammonium disinfectants such as benzalkonium chloride (BC) is especially relevant to *Listeria*'s adaptations in food-related environments, as these compounds are extensively used in food processing, at retail, and for household or personal use (24, 26). BC resistance of *L. monocytogenes* from foods and from the processing plant environment has been found to range from 10% (1) to as much as 42-46% (25, 27, 37). A study of strains from turkey processing plants revealed that resistance to BC was especially high among those of serotype 1/2a (or 3a) and 1/2b (or 3b) (60 and 51%, respectively) and that all BC-resistant strains were also resistant to the heavy metal cadmium (27).

Mechanisms underlying BC resistance in *L. monocytogenes* remain poorly understood. Several studies have provided evidence for chromosomal determinants (6, 34, 35, 37, 38), and evidence for plasmid-mediated resistance to BC also exists (21, 34, 35), even though plasmid-associated genes mediating BC resistance were not identified. Genome sequencing of several *L. monocytogenes* strains has identified numerous efflux systems on the chromosome as well as on plasmids (8, 9, 28, [http://www.broadinstitute.org/annotation/genome/listeria\\_group/MultiHome.html](http://www.broadinstitute.org/annotation/genome/listeria_group/MultiHome.html)), and some of these are potential candidates for BC resistance.

In this study we have identified a plasmid-based BC resistance system on an *IS1216* composite transposon in *L. monocytogenes* H7550, implicated in the 1998-1999 multistate outbreak of listeriosis.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table 1. *L. monocytogenes* strain H7550 was isolated from the 1998-1999 hot dog outbreak (4). Its pulsed-field gel electrophoresis profile with *AscI* and *ApaI* was indistinguishable from that of the sequenced strain H7858 (28; R.M. Siletzky and S. Kathariou, unpublished). Both H7550 and H7858 are resistant to BC and to cadmium (27, data not shown). H7550-Cd<sup>s</sup> was a cadmium-susceptible, plasmid-free derivative of *L. monocytogenes* H7550. The plasmid-cured derivative was obtained following repeated passages of the bacteria at 42°C. H7550-Cd<sup>s</sup>S was a spontaneous mutant of H7550-Cd<sup>s</sup> with resistance to streptomycin and was isolated on plates with streptomycin (150 µg/ml). *L. monocytogenes* was grown either in trypticase soy broth with 0.6% yeast extract (TSBYE) (Becton, Dickinson & Co., Sparks, MD) or in brain heart infusion (BHI) broth (Becton, Dickinson & Co.). Agar media were BHI agar (1.2% Bacto-agar, Becton, Dickinson & Co.) or TSBYE with 1.2% agar (1.2% Bacto-agar). *Escherichia coli* strains were grown at 30°C in Luria Bertani (LB) broth (Becton, Dickinson & Co.) or on LB broth supplemented with 1.2% Bacto-agar.

**BC susceptibility and determinations of MIC.** BC susceptibility of *L. monocytogenes* was assessed as described (27). Strains H7550 and H7550-Cd<sup>s</sup> were used as resistant and susceptible controls, respectively. For MIC determinations, a single colony from a blood agar plate (Remel, Lenexa, KS) was suspended in 100 µl of Mueller Hinton broth (Becton,

Dickinson & Co.) and 5  $\mu$ l of the suspension was spotted in duplicate on Mueller Hinton agar (1.2% agar) plates with variable concentrations of BC (0, 5, 10, 15, 20, 25, 30, 35, and 40  $\mu$ g/ml) and the plates were incubated at 30°C for 48 h. MIC was defined as the lowest assessed concentration of BC that prevented growth. All MICs were determined in at least two independent trials. For cadmium susceptibility a single colony from a blood agar plate (Remel, Lenexa, KS) was suspended in 100  $\mu$ l of Mueller Hinton broth (Becton, Dickinson & Co.) and 5  $\mu$ l of the suspension was spotted in duplicate on Iso-sensitest agar (ISA; Oxoid Ltd., Hampshire, England) plates supplemented with cadmium chloride 70  $\mu$ g/ml. For cadmium MIC determinations variable concentrations of cadmium chloride (0, 35, 70, 100, 140, 150, and 160  $\mu$ g/ml) were added to ISA. After spotting all plates were incubated at 37°C for 48 h.

**Recombinant plasmid constructs.** Primers used to construct the recombinant plasmids are listed in Table 2, and their locations are indicated in Fig. 1. PCR employed the Takara Ex Taq kit (Takara, Madison, WI) and a T1 thermal cycler (Biometra, Goettingen, Germany). Primers BcF and BcR were used to produce a PCR fragment containing the entire *bcrABC* cassette along with the ca. 800 nt upstream intergenic region. Primers BcF2 and BcR were employed to amplify the *bcrABC* cassette with 105 nts upstream of the putative start codon of *bcrA*, including the putative promoter region. The *bcrABC* cassette without the putative promoter was amplified with primers BcF5 and BcR. A PCR fragment containing the ca. 800 nt upstream intergenic region, *bcrA* and *bcrB*, but lacking *bcrC* was amplified with BcF and BcR2 (Table 2 and Fig. 1). The PCR fragments obtained with BcF and BcR, BcF2 and BcR, BcF5 and BcR, BcF and BcR2 were digested with BamHI and EcoRI (New England Biolabs,

Beverly, MA), and ligated into the temperature-sensitive shuttle vector pCON-1 (3) similarly digested with BamHI and EcoRI, resulting in pDS195, pDS202, pBEC59, and pDS201, respectively. The recombinant plasmids were electroporated into *E. coli* SM10 (36) and transformants were selected on LB agar supplemented with ampicillin (100 µg/ml). For the plasmid construct pBEC57 harboring an in-frame deletion of *bcrB*, pDS202 was used as template for inverse PCR with primers BcF7 and BcR3 (both harboring a KpnI restriction site) (Fig. 1). The PCR amplicon was digested with KpnI, self-ligated with T4 DNA ligase (Promega, Madison, WI) and electroporated into *E. coli* SM10.

Recombinant plasmids were mobilized into H7550-Cd<sup>S</sup> via conjugation with *E. coli* SM10 harboring the recombinant plasmids, as described (20). Transconjugants were selected on BHI agar plates supplemented with chloramphenicol (6 µg/ml) and streptomycin (1,200 µg/ml) at 30° C for 2-3 days, and confirmed using PCR. BC susceptibility of the transconjugants was assessed as described above.

**Inactivation of accessory gene regulator (*agr*) system.** *agr*-system inactivation construct (H7550A) was created as described before with modifications (33a). Briefly, using primers AgrD-A F and AgrD-B R sequence upstream to *agrD* and primers AgrD-C F and AgrD-D R were used to amplify sequence downstream to *agrD* from the genomic DNA of wild type strain H7550 (Table 2; 33a). Two amplicons thus generated were then joined by SOE PCR (33a). The resulting fragment was then digested with HindIII and XbaI and was ligated into a similarly digested temperature sensitive shuttle vector plasmid pCON1. The ligation mixture was electroporated into DH5α and the recombinant clones were selected on LB agar supplemented with ampicillin (100 µg/ml). The recombinant plasmid (pAgrD) was confirmed

using PCR and restriction analysis. This plasmid was then electroporated into competent *L. monocytogenes* strain H7550. The *L. monocytogenes* competent cell preparation and electroporation conditions were same as described before (26a). The recombinant *L. monocytogenes* strain H7550 harboring pAgrD was selected on BHI agar supplemented with Cm (6 µg/ml) at 30°C. Two putative transformant colonies were then inoculated into BHI supplemented with Cm (6 µg/ml) and the cultures were incubated overnight at 42°C. Dilutions ( $10^{-2}$  and  $10^{-3}$ ) of the overnight cultures were then plated on BHI agar supplemented with Cm (6 µg/ml) following which the plates were incubated overnight at 42°C. The recombinant colonies with insertional inactivation of *agr* system (H7550A) were confirmed using primers flanking the site of insertion, agrB\_F and agrC\_R (Table 2). The efforts to perform a clean in-frame deletion were not successful. The stability of the insert was tested at 37°C. Overnight culture of H7550A was grown at 37°C and the dilutions ( $10^{-5}$  and  $10^{-6}$ ) were plated on BHI agar and BHI agar supplemented with Cm (6 µg/ml) following which the plates were incubated at 37°C.

**Complementation of pLM80 *cadA2C2* in H7550Cd<sup>s</sup>.** Primers compCadA3\_F and compCadA3\_R were used to produce ca. 3.2 kbp PCR fragment containing the entire *cadA2C2* cassette along with the entire upstream and downstream intergenic region (Table 2). The PCR amplicon and the shuttle vector plasmid pPL2 were digested with XmaI and KpnI (New England Biolabs). The plasmid and the insert were ligated as described above. The recombinant plasmid pPLcad was first electroporated into DH5α and then into *E. coli* SM10 (36), and for both *E. coli* strains, transformants were selected on LB agar supplemented with chloramphenicol (25 µg/ml). Recombinant plasmids were mobilized into H7550-Cd<sup>s</sup> via

conjugation with *E. coli* SM10 harboring the recombinant plasmids, as described (20). Transconjugants were selected on BHI agar plates supplemented with chloramphenicol (6 µg/ml) and Nalidixic acid (20 µg/ml) at 30° C for 2-3 days, and confirmed using PCR. Cadmium susceptibility and cadmium MIC of the transconjugants were assessed as described above.

**BC dose and cell number effect on the survival of BC resistant strain H7550 in the liquid**

**medium.** Overnight cultures of H7550 cultivated in BHI at 37°C (ca. 10<sup>8</sup>cfu/ml) were diluted 1:10 or 1:100 fold in fresh BHI. These culture (2 mls each) were inoculated with BC (0, 2.5 and 5 µg/ml) in duplicates and then incubated at 25°C for ca. 12 hrs and the OD<sub>600</sub> for these cultures was recorded using a spectrophotometer (SmartSpec 3000; Bio-Rad, Hercules, CA) every 2.5 hrs. The findings were confirmed independently at least twice.

**Assessing the effect of H7550Cd<sup>s</sup> spent culture media on survival of H7550 in sublethal**

**BC.** Overnight culture of *L. monocytogenes* H7550Cd<sup>s</sup> cultivated in BHI at 37°C was centrifuged at 14000g for 2 min. The supernatant was aspirated using a sterile 10 ml syringe (Beckton and Dickinson & Co.). The medium was then filtered through 0.2 µm nylon filter (Fisherbrand, UK) and the filtrate was immediately used as a spent medium. Overnight cultures of H7550 cultivated in BHI at 37°C (ca. 10<sup>8</sup>cfu/ml) were washed twice (14000g for 2 min) and after washing the bacterial pellet was resuspended in 1 ml of fresh BHI. Dilution (1:40) of the washed bacterial culture was inoculated to fresh BHI, BHI+ 10% spent medium and BHI+20% spent medium in duplicates, each media were then supplemented with BC 2.5 µg/ml. The inoculations were incubated at 25°C and the OD<sub>600</sub> was recorded using a spectrophotometer (SmartSpec 3000; Bio-Rad) at the intervals of 5 hrs up to 55 hrs. The

effect of H7550Cd<sup>s</sup> spent medium on the survival of H7550 was tested twice in independent trials.

**RNA isolation and reverse transcription PCR (RT-PCR).** *L. monocytogenes* H7550 was grown in TSBYE at 4, 8, 25, and 37°C until mid to late logarithmic phase (OD<sub>600</sub> value~0.7-0.9), with growth phase being monitored with a spectrophotometer (SmartSpec 3000; Bio-Rad). Cultures were then divided into two portions, one of which was treated at the indicated temperature for 30 min with sublethal concentrations (10 µg/ml) of BC or other concentrations, as indicated. The other portion (control) remained untreated at the indicated temperature for 30 min. Total RNA was isolated using the SV Total RNA Isolation System (Promega). RNA was then subjected to DNase treatment using Turbo DNA-free<sup>TM</sup> (Ambion, Austin, TX). The concentration and quality of the RNA was determined by measuring the absorbance at 260nm (Nanodrop, Wilmington, DE). RNA was stored at -80°C when necessary. RT-PCR for *cadA* involved a similar approach. H7550 cultures grown at 25°C were divided into two portions, one of which was treated for 30 min with sublethal concentrations (10 µg/ml) of cadmium chloride. The other portion (control) remained untreated at the indicated temperature for 30 min. Reverse transcription used primer c2 and PCR was done with primers c1, c2 (Table 2).

RT-PCR experiments included *spoVG* as housekeeping gene control as previous studies indicated that expression of this gene was constitutive in *L. monocytogenes*, including at low temperature (23). Total RNA was reverse-transcribed to produce complementary DNA (cDNA) using 200ng of RNA and the ImProm-II<sup>TM</sup> Reverse Transcription System (Promega) according to the manufacturer's protocol using extension temperature of 46 °C and 43 °C for

*bcrABC* and *spoVG* transcripts, respectively. Self priming controls (reverse transcription without gene-specific primer) and negative controls (reverse transcription with no RNA) were included with all RT-PCR reactions. Sense and antisense primers for *bcrABC* RT-PCR were p1 and p2 respectively (Table 2), whereas for *spoVG* RT-PCR they were s1 and s2, respectively (Table 2). Each RT-PCR was done in duplicate and in at least two independent experiments. For band quantifications we used the image processing software ImageJ (<http://rsbweb.nih.gov/ij/>). In order to calculate the fold increase in the transcript levels of the genes in response to presence of BC or cadmium the gel density values obtained from the RT-PCR gel images using ImageJ were normalized to those of the control (culture without BC or cadmium). The transcript levels of the reference gene *spoVG* in the presence and absence of BC or cadmium were similarly normalized. The ratio of the normalized *bcrABC* levels to that of the normalized *spoVG* levels corresponded to fold increase. The same approach was employed to assess increase in gene transcript level of *bcrABC* following exposure to different concentrations of BC. To determine impact of temperature, *bcrABC* levels were first normalized to those of *spoVG* from the same culture and the fold increase upon addition of BC was determined as the ratio of the normalized levels in the absence or presence of BC.

## RESULTS

**Identification of BC resistance determinants on *L. monocytogenes* plasmid pLM80.** *L. monocytogenes* strain H7550 from the 1998-1999 hot dog outbreak was resistant to both cadmium and BC, and harbored a large, ca. 80 kb plasmid, pLM80 (28). Plasmid curing of H7550 rendered the bacteria susceptible to the heavy metal cadmium. This was expected

since pLM80 harbored genes for cadmium resistance (28). Testing of H7550-Cd<sup>s</sup> for BC resistance revealed that the derivative was also susceptible to BC (Table 1). These findings suggested that, in addition to cadmium resistance determinants, pLM80 also harbored determinants for resistance to BC. However, unlike BC-resistant derivatives of originally BC-susceptible strains obtained following prolonged exposure to BC, which were resistant both to BC and to ethidium bromide (34, 35), the ethidium bromide MICs for H7550 and H7550-Cd<sup>s</sup> were the same (both strains grew at 32 but not at 64 µg/ml).

Analysis of the annotation of pLM80 (Refseq No.NZ\_AADR000000000), identified three open reading frames (orfs) as possible determinants for BC resistance (Fig 1). These included a putative transcriptional regulator of the TetR family harboring a helix-turn-helix DNA binding motif (*bcrA*; Pfam: PF00440). This orf was followed by two putative small multidrug resistance (SMR) genes, *bcrB* and *bcrC* (Pfam: PF00893). Sequence analysis of the region suggested a long (850 bp; GC-36%) intergenic region between *bcrA* and its upstream orf (LtrC-like protein). Upstream (31 nts) of the putative start codon of *bcrA* we identified a canonical promoter (-10: TATAAT and -35: TTGACA) (Fig1). A perfect palindrome (ACCGTCCGGACGGT) was identified between the -10 and -35 promoter sequences (Fig. 1), suggesting a possible binding site for a transcriptional regulator. Highly similar (99-100% identity) *bcrABC* sequences (including the upstream region harboring the putative promoter) were detected in two *L. monocytogenes* strains among those with sequenced genomes: J0161, of serotype 1/2a (NZ\_AARW02000017.1) and FSL F2-515, also of serotype 1/2a (NZ\_AARI02001718). The genes were located on a large plasmid in J0161 (19a); even

though strain FSL F2-515 harbored plasmid, the genes were absent from that plasmid (19a), suggesting chromosomal location in that strain.

With the exception of J0161 and FSL F2-515, no other sequences with significant homology to *bcrABC* were detected in nucleotide database. The deduced BcrB and BcrC polypeptides, however, exhibited similarity (44-73%) to multiple proteins of the SMR family, in diverse Gram-positive and Gram-negative bacteria. Furthermore, a chromosomal cassette (SugE1-E2) with 33-34% identity at the amino acid sequence level was identified in the genome of all screened *L. monocytogenes* strains including H7858 (data not shown).

Sequence analysis of the *bcrABC* region provided evidence for a composite transposon flanked by two *IS1216* elements (*IS1216* center and *IS1216* right, each containing a transposase (75.3% nt identity; 83.2% amino acid identity to each other) flanked by *IS1216* inverted repeats (5' GGTTCTGTTGCAAAGTTT 3') (12) (Fig 1). The putative composite transposon was ca. 12.4 kb long. In addition to the two *IS1216* elements and the *bcrABC* cassette, the transposon harbored genes with putative conjugative functions: plm80\_0074 and plm80\_0075 were annotated as LtrC-like protein and a member of the P-loop NTPase domain superfamily, respectively. Downstream of the *bcrABC* cassette we identified one orf with 87-99% identity to a putative resolvase gene in plasmids of *Bacillus cereus* and in Tn1546 of *Enterococcus faecium*. The putative resolvase gene was followed by four orfs encoding a putative glyoxalase superfamily protein, a putative triphenylmethane reductase, a hypothetical protein, and a MoxR-like protein, respectively. A stem-loop structure ( $\Delta G = -18.7$  kcal/mol)

was identified 110 nt downstream of plm80\_0067 stop codon, possibly corresponding to a Rho-independent transcriptional terminator (Fig. 1).

The overall GC content of the ca. 12.4 kb region is 40%; however, pronounced diversity in GC content was observed within this region: the GC content of *bcrABC* cassette was 34%, significantly lower than the average for the genome of *L. monocytogenes* (38%); other orfs had widely variable GC contents, ranging from 34 to 58% (Fig. 1). It was of interest that plm80\_0067, downstream of the putative glyoxalase and encoding a putative triphenylmethane reductase (TMR), had unusually high GC content (45%) and 99-100% identity to putative TMR genes from *Pseudomonas* sp., *Aeromonas* sp., and *Citrobacter* sp.

The *IS1216* composite transposon harboring *bcrABC* was immediately downstream of another putative transposon harboring the cadmium resistance cassette *cadAC* and flanked by *IS1216* left and *IS1216* center (Fig. 1A). The genomic region in pLM80 suggests the presence of three possible transposable units, with one carrying both cadmium and BC resistance genes (Fig 1C-I) and others carrying either cadmium (Fig 1C-II) or BC resistance genes (Fig1C-III).

**The *bcrABC* cassette confers BC resistance to a plasmid-cured derivative of *L. monocytogenes* H7550.** Subcloning of the entire *bcrABC* cassette along with 900 nts upstream of the *bcrA* start codon in pCON-1 (including the 850 bp intergenic region between *bcrA* and LtrC-like protein) resulted in pDS195, whereas pDS202 harbored *bcrABC* and 105 nts upstream of *bcrA*, including the canonical promoter (Fig. 1B). Transfer of pDS195 and pDS202 into H7550-Cd<sup>S</sup> rendered the bacteria able to grow at 35 µg/ml, whereas MICs of BC were markedly lower for H7550-Cd<sup>S</sup> harboring the empty vector (Table 3). Constructs

pDS201 (lacking *bcrC*), pBEC57 (lacking *bcrB*) and the pBEC59 (*bcrABC* with only 11 nts upstream of *bcrA* and thus lacking the canonical promoter) (Fig. 1B) were not able to complement BC resistance (Table 3). In spite of repeated efforts, a construct harboring *bcrB* and *bcrC*, but lacking *bcrA*, could not be obtained. These data suggested that BC resistance could be conferred by the *bcrABC* cassette, and that the 105 nt upstream region that included the canonical -10 and -35 promoter sequences was both required and sufficient for expression.

**Co-transcription of *bcrABC* with downstream genes, and increase in *bcrABC* transcript levels in response to BC.** RT-PCR data suggested that *bcrA*, *bcrB* and *bcrC* were co-transcribed, and were also transcribed together with the downstream orfs encoding a putative transposon resolvase, putative glyoxalase and putative triphenylmethane reductase (Fig. 2). The orf upstream of *bcrA*, plm80\_0074, was not in this transcriptional unit, neither was plm80\_0066, downstream of the putative triphenylmethane reductase (Fig. 2). These results were confirmed in five independent trials and are in congruence to the GC rich stem loop structure found downstream of plm80\_0067 (Fig 1). Thus, *bcrABC* was part of a polycistronic message of ca. 3.8 kb, with the putative triphenylmethane reductase gene being the last gene in the operon.

The RT-PCR data also suggested that levels of the transcript that included *bcrABC* were higher in the presence of BC (10 µg/ml) than without the disinfectant (Fig. 3). To further assess the impact of BC on expression of *bcrABC*, transcript levels were assessed via RT-PCR at different concentrations of BC (0, 10, 20, 40 µg/ml) using the housekeeping gene *spoVG* as

reference. The RT-PCR data suggested that transcript levels of *bcrABC* were increased in the presence of 10 µg/ml BC (ca. 1.4-2 fold), but were similar to baseline levels at higher BC concentrations (20 and 40 µg/ml) (Fig. 3). RT-PCR with *spoVG* suggested that there were no significant changes in levels of *spoVG* transcripts when cells were grown in the presence of different concentrations of BC (0, 10, 20, 40 µg/ml) (Fig. 3).

#### **Transcription of *bcrABC* is higher at lower temperatures (4, 8, 25°C) than at 37°C.**

Temperature was found to impact *bcrABC* transcript levels, with levels at 4-25°C being greater than those at 37°C. Following normalization (ratio of *bcrABC* band density to the *spoVG* band from the same sample) levels in the absence of BC (baseline levels) at 37°C were ca. 57% of those at 4°C (Fig. 4). The impact of temperature was even more noticeable in the presence of BC, normalized levels at 8, 25 and 37°C being ca. 83, 92 and 42%, respectively, of those at 4°C (Fig. 4). Comparison of normalized values in absence or presence of BC revealed that the presence of the disinfectant resulted in higher transcript levels at all tested temperatures. However, the impact of BC was greater at 4 and 25°C (ca. 1.8 and 2.0 fold increase, respectively), than at 8 and 37°C (ca. 1.2 fold increase each) (Fig. 4). The observed impact of temperature on increased and baseline levels of *bcrABC* transcription was consistently observed in independent experiments (data not shown).

#### **Lack of cross-induction of pLM80 genes mediating resistance to BC and to cadmium.**

As described above, pLM80 harbors genes mediating resistance to BC (*bcrABC*) as well as to cadmium (*cadAC*), and the *cadAC* cassette (orfs plm80\_0082 and plm80\_0083) is in the vicinity of *bcrABC* (Fig 1A). To determine whether *bcrABC* may be also enhanced by

cadmium, and reversely whether BC may increase expression of *cadAC*, RT-PCR was employed again using *spoVG* as reference. The data clearly indicated that *bcrABC* expression was increased (ca. 2 fold) by BC but not by sublethal exposure to cadmium (10 µg/ml CdCl<sub>2</sub>), and similarly *cadA* expression was enhanced by cadmium (ca. 4 fold) but not by BC (Fig. 5). RT-PCR data indicated that *spoVG* expression was stable under the conditions tested (Fig. 5).

**Cadmium resistance phenotype was partially complemented in H7550Cd<sup>s</sup>.** Subcloning of the entire *cadA2C2* cassette along with its entire upstream and downstream intergenic region resulted in pPLcad. Transfer of this plasmid in H7550Cd<sup>s</sup> resulted in partial complementation of the cadmium resistance phenotype, where the parent strain was able to grow in presence of cadmium 160 µg/ml, whereas the recombinant strain (H7550pPLcad) was not able to grow in presence of cadmium 140 µg/ml.

**Effect of BC dose, and cell number on the survival of BC resistant strain H7550 in the liquid medium.** *L. monocytogenes* strain H7550 does not propagate or sustain survival in the presence of BC-10 µg/ml in the planktonic state (data not shown). In a liquid medium containing low dose of BC (2.5 and 5 µg/ml), survival of *L. monocytogenes* strain H7550 was sustained upon inoculating cells in high cell concentrations (10<sup>8</sup> cfu/ml) (Fig 6A). The survival and growth of 10<sup>7</sup> cfu/ml cells was severely effected in the medium containing BC 2.5 µg/ml whereas in the BC 5 µg/ml no growth was seen at all (Fig 6B). At lower cell concentration (10<sup>6</sup> cfu/ml), no signs of survival or growth were seen in liquid medium containing either BC 2.5 or BC 5 µg/ml (Fig 6C).

**H7550Cd<sup>s</sup> spent media had a protective effect on the survival of H7550 in presence of sublethal dose of BC.** Strain H7550 sustained sublethal BC stress better in spent media than in just BHI. In the presence of 20% spent media in BHI, H7550 growth entered log phase around 10 hrs post-inoculation. This was followed by 10% spent media in BHI and in just BHI where H7550 growth entered log phase around 25 and 40 hrs post-inoculation, respectively (Fig. 7).

**BC-MIC of H7550A was lower than the wild type strain.** BC MIC of the strain with inactivated *agr* system (H7550A) was 30 µg/ml compared to the wild type strain H7550 (40 µg/ml) (Table 3). This assay was confirmed twice in two independent trials. The growth rate and morphology of the mutant and wild type strain were the same (data not shown). The insert in *agr* locus was confirmed to be stable. The dilutions of overnight culture of H7550A propagated at 37°C were plated on BHI agar and BHI agar supplemented with Cm-6 µg/ml and number of colonies on both plates after overnight incubation at 37°C was the same (data not shown). Also the mutant strain was tested for the presence of *cadA2* and *bcrABC* using PCR using with primers c1, c2 and p1, p2 respectively with a goal to confirm no plasmid (pLM80) loss during the mutant construction (data not shown).

## DISCUSSION

In this study we have described a BC resistance mechanism of *L. monocytogenes* H7550 (1998-1999 multistate outbreak strain) associated with a gene cassette harbored on the plasmid of this strain, pLM80. This gene cassette was also detected in two other sequenced *Listeria* genomes. Evidence for chromosomal efflux pumps implicated in quaternary

ammonium compound resistance was provided for *L. monocytogenes* (6, 34, 35, 37, 38). Even though there was also evidence for plasmid-mediated resistance to BC (21, 34, 35), the plasmid-associated genes responsible for such resistance have not yet been identified.

The BC-resistance cassette described here was composed of one TetR family transcriptional regulator (*bcrA*) with a helix-turn-helix (HTH) DNA binding motif and two SMR genes (*bcrB* and *bcrC*). SMR proteins are proton-dependent multidrug efflux systems that typically require co-expression of two genes (2, 31) and have been characterized in several Gram-positive and Gram-negative bacteria, including *E. coli* EmrE and *Bacillus subtilis* EbrA and EbrB (14, 17, 29).

Members of the TetR family of regulators control transcription of multidrug efflux systems (33), suggesting a likely role for *bcrA* in transcriptional control of the *bcrABC* cassette. The palindrome between the putative -10 and -35 promoter regions of *bcrABC* may serve as recognition sequence for the repressor. This palindrome overlaps with the putative -10 promoter sequence, thus possibly interfering with the transition of the RNA polymerase-promoter complex into a transcribing state. A palindrome overlapping the -10 region (but of different length and sequence content) was also described upstream of *qacR*, implicated in transcriptional control of the multidrug efflux pump *qacA* of *Staphylococcus aureus* (10, 32). Further studies are needed to confirm that *bcrA* functions as a repressor by binding to this region of dyad symmetry. A deletion mutant of this gene could not be obtained suggesting that constitutive overexpression of *bcrBC* might be lethal to the cells.

Transcription of *bcrABC* was increased by sub-lethal levels of BC. No increase in the gene transcript level was noted in response to treatment with cadmium. Inversely, increase in gene transcript level of *cadA* was observed upon exposure to cadmium, but not BC. Thus, in spite of their genomic proximity on pLM80 the cassettes mediating resistance to BC and to cadmium are not cross-effected.

In spite of the increased *bcrABC* transcript levels upon exposure to BC, transcripts were also readily detected even in the absence of the disinfectant. This may suggest either that repression of transcription is weak or that other, currently unidentified molecules result in the observed baseline transcription levels in the absence of BC. Further studies are needed to determine how transcription is enhanced in the presence of sublethal levels of BC (e.g. by binding to BcrA and prevention of repression, as described in other efflux systems) (13, 40) and to assess whether additional molecules also increase transcript levels. Preliminary data suggest that besides BC other quaternary ammonium disinfectants (benzethonium chloride, cetyl-trimethyl ammonium bromide) also enhance the transcription of *bcrABC* in H7550 (V. Dutta and S. Kathariou, unpublished), but it is not known whether transcript levels can also be increased by other, structurally unrelated molecules that may be exported by this efflux system.

Transcript levels of *bcrABC* were increased at sublethal concentrations (10 µg/ml) of BC, but not at higher concentrations (e.g. 20 or 40 µg/ml). It is possible that BcrA conformation and ability to bind to operator sites is different at low (10 µg/ml) than at high concentrations (20 or 40 µg/ml) of BC. It is also possible that general toxicity associated with exposure to high

concentrations of BC (even with a relatively short exposure time of 30 minutes) resulted in the observed loss of transcript level increase. However, we think this is less likely, as *bcrABC* transcripts were at baseline levels, and *spoVG* levels were not impacted.

*Listeria's* resistance to BC is of special relevance to the pathogen's ecology in food processing plants, where BC and other quaternary ammonium disinfectants are used extensively. As noted before, two classes of BC-resistant strains can be recognized in *L. monocytogenes*: resistant derivatives of previously BC-susceptible strains, resulting from adaptation to sublethal BC levels, and strains that are naturally resistant to BC, such as H7550 (34, 35, 38). Resistant derivatives of previously susceptible strains appear to involve chromosomal efflux systems such as *mdrL* that also mediate efflux of ethidium bromide, and that are inhibited by the efflux inhibitor reserpine (35). The adapted derivatives may result from mutations leading to overexpression of these efflux systems, even though such mutations still need to be identified. On the other hand, in strains such as H7550 resistance to high levels of BC is mediated by *bcrABC*, a plasmid-associated system which show increased transcript levels following exposure to BC and at low temperatures (4-25°C). In naturally resistant strains resistance to BC is not accompanied by resistance to ethidium bromide, neither is it inhibited by reserpine (35). We have indeed found that in strain H7550 the BC MIC was not affected by this efflux inhibitor, whereas MICs of the plasmid-cured strains were reduced in the presence of reserpine (M. Rakik-Martinez and S. Kathariou, unpublished).

The impact of temperature on transcription of *bcrABC* may be of special relevance to *Listeria*'s environmental adaptations in food processing plants where low temperatures often prevail. Temperature has profound impact on gene expression of *L. monocytogenes*, including genes involved in virulence and in environmental adaptations (5, 11, 15, 22, 39). Further studies are needed to characterize the mechanism responsible for temperature-dependent expression of *bcrABC* in *L. monocytogenes*, and to also determine whether a similar impact of temperature is observed at the proteomic level.

Given that quaternary ammonium compounds such as BC are man-made reagents, it will be of interest to identify compounds in nature that can be exported by *bcrABC*, and conditions responsible for acquisition and retention of this gene cassette by *L. monocytogenes*. The cassette was found to be on putative composite transposable units flanked by *IS1216* inverted repeats. In addition to genes mediating resistance to BC, these putative composite transposable units harbor a cadmium resistance cassette, a putative glyoxalase, and a putative triphenylmethane reductase. The diversity of GC content within this region of pLM80 suggests acquisition of these genes through horizontal gene transfer, possibly from varied sources. The role of *IS1216* transposition in gene transfer has been well-described in another Gram-positive bacterium, *Enterococcus faecium* (12).

The presence of *bcrABC* on a putative *IS1216* composite transposon harbored by pLM80 suggests possible mechanisms for the transfer of these genes among different *Listeria* genomes via transposition or plasmid mobilization. The presence of *bcrABC* in the vicinity of, and possibly in the same transposable unit as *cadAC*, may also partially explain the finding

that BC-resistant strains of *L. monocytogenes* were also resistant to cadmium (27). Further work is needed to assess the ability of *bcrABC* to disseminate from H7550 to BC-susceptible strains of different serotypes of *L. monocytogenes*, and different *Listeria* species.

Partial complementation of the cadmium resistance phenotype in the plasmid free derivative H7550Cd<sup>s</sup> suggests that *cadA2C2* was not sufficient and other factors on the plasmid (pLM80) might be involved with the cadmium resistance. Alternatively, presence of more than one copy of *cadA2C2* systems in the wild type strain, due to multiple copies of pLM80, versus one copy of the chromosomally integrated *cadA2C2* in the H7550pPLcad, can be implicated with the lower MIC of H7550pPLcad.

The protective effect of high cell concentration on BC tolerance was intriguing. One possibility that might confer such a phenotype is quorum sensing. Role of accessory gene regulator (*agr*) system as a quorum sensing mechanism has been well explained for other Gram positive bacteria (23a). Riedel et al have suggested the role of *agrD* as a quorum sensing protein in *L. monocytogenes* strain EGDe (33a). Accumulation of AgrD protein in the extracellular medium up to certain levels results into activation of the cascade of reactions which in turn leads to differential regulation of number of metabolic reactions including the *agr* system (23a, 33a). We have shown the protective effect of the spent culture medium on the ability of H7550 to sustain BC stress. Our findings have clearly indicated that compared to the wild type strain, the mutant strain (H7550A) with inactivated *agr* system was less efficient in dealing with BC stress. In order to relate the *agr* system to the quorum sensing based BC resistance mechanism, it is imperative to perform in-frame isogenic deletion of the genes

involved with the *agr* system. Alternatively the comparative efficiency of the mutant strain (with inactivated *agr* system), relative to the wild type strain, to deal with BC stress in liquid medium will also provide clues about the possible role of this system in BC resistance.

In conclusion, we have characterized a plasmid-based BC resistance gene cassette on a putative composite transposon in a strain of *L. monocytogenes* associated with the 1998-1999 multistate outbreak. Such a resistance cassette draws attention to the possible role of disinfectants in adaptations and persistence of *L. monocytogenes* in food processing and other environments with frequent disinfectant use. Further studies are needed to elucidate the evolution and dissemination of these BC resistance genes in *L. monocytogenes*, and to assess the possible role of these genes in environmental persistence as well as in virulence of this pathogen.

#### ACKNOWLEDGEMENTS

This work was partially supported by a grant from the American Meat Institute Foundation and USDA grant 2006-35201-17377. We thank Mira Rakic-Martinez for information on MICs for ethidium bromide and on impact of reserpine. We are thankful to all other members of our laboratory for their support and encouragement.

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**Table 1.** Bacterial strains used in this study.

Strain	Serotype	BC <sup>R*</sup>	Source (Reference)
<i>L. monocytogenes</i>			
H7550	4b	+	1998-99 hot dog outbreak (4)
H7550-Cd <sup>s</sup>	4b	-	Plasmid-cured derivative of H7550
H7550-Cd <sup>s</sup> S	4b	-	Streptomycin resistant mutant of H7550-Cd <sup>s</sup>
H7550-Cd <sup>s</sup> S (pDS195)	4b	+	This study
H7550-Cd <sup>s</sup> S (pDS202)	4b	+	This study
H7550-Cd <sup>s</sup> S (pDS201)	4b	-	This study
H7550-Cd <sup>s</sup> S (pBEC59)	4b	-	This study
H7550-Cd <sup>s</sup> S (pBEC57)	4b	-	This study
H7550-Cd <sup>s</sup> S (pCON-1)	4b	-	This study
H7550pPLcad	4b	+	This study
H7550A	4b	+	This study
<i>Escherichia coli</i>			
SM10 thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km			(36)

\* Resistance to BC was determined on MHA plates with added BC concentration of 20 µg/ml as described in Materials and Methods.

**Table 2.** Primers used in this study.

Primer	Sequence (5' to 3')*
BcF	GAAT <u>GGATCCTT</u> CAATTAGATCGAGGCACG
BcR	GTAT <u>GAATTC</u> GTATAATCCGGATGCTGCCC
BcR2	GTAT <u>GAATTC</u> CATCAACCTTCTTTAATGAGG
BcF2	GACT <u>GGATCC</u> GATTCTGGAACATCCCTATC
BcF5	GAAT <u>GGATCC</u> GGAGGGTAATCATGTCAG
BcF7	GACT <u>GGTACC</u> CTCATTAAGGAAGGTTGATGG
BcR3	GACT <u>GGTACC</u> CATATGGATTCATGTG
AgrD-A F:	GTAA <u>AAGCTT</u> CTGTCATATTTATGGCTCAGACG
AgrD-B R:	TTTATTCATATTTTTTCATAGTCCGATAACC
AgrD-C F	<u>GAACTATGAAAAATATGAATAAA</u> TAAA TTTAAACTGTATAACATCAAAATTC
AgrD-D R	TATAT <u>CTAGAGTT</u> CAAGCCCGTACTAAAATAAAACC
agrB_F	GCTGATTTTCGAAAGACCGC
agrC_R	TCT ATG CAG GGA CTC TAA TG
compCadA3_F	GACT <u>CCCGGG</u> ACC TAT TCT ATA TTC GCC TAC TTC
compCadA3_R	GACT <u>GGTACC</u> GCT TTT ATA ATG GTG GTG CTC
p1	CATTAGAAGCAGTCGCAAAGCA
p2	GTTTTCGTGTCAGCAGATCTTTGA
p3	GTTATCAGGATCTACGACTGTC
p4	GTAATTCCTGCATTACGCATAACTG
p5	GTCATAGATCGCTGCCGTAATTTTCAG
p6	CAATATGGCTATTGTATTCCCATGCTG
p7	CAGAGTGAACGTGATCAGCATC
s1	ATTACAGATGTGAGATTACGACG
s2	ACGTTTACTTGGCATAGCTAC
c1	ACAAGTTAGATCAAAAGAGTCTTTTATTAAACG
c2	ATCTTCTTCATTTAGTGTTCCCTGCAAATACTTC

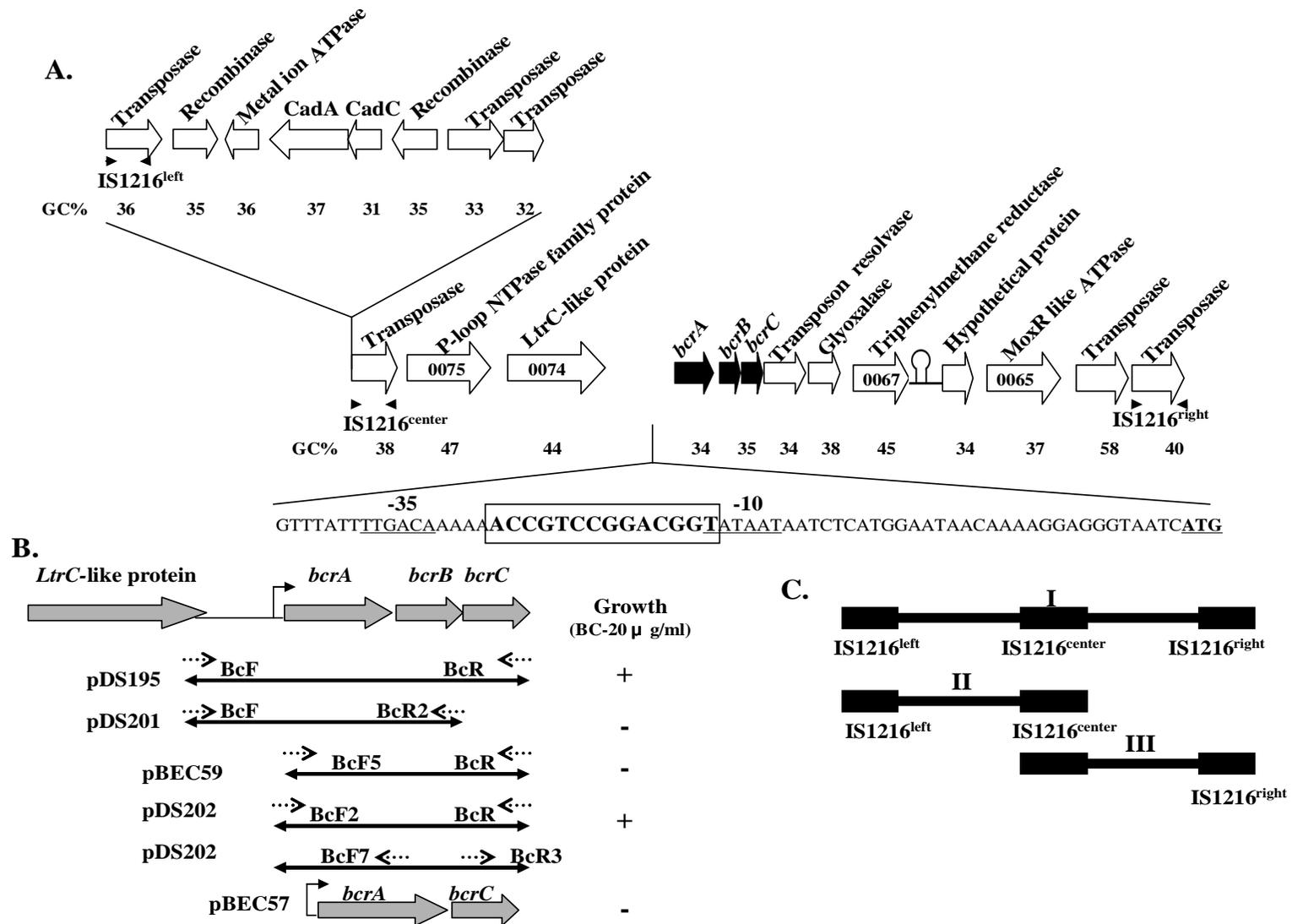
\* underlined sequences correspond to restriction enzyme sites: GGATCC, BamHI; GAATTC, EcoRI; GGTACC, KpnI; CCCGGG, XmaI

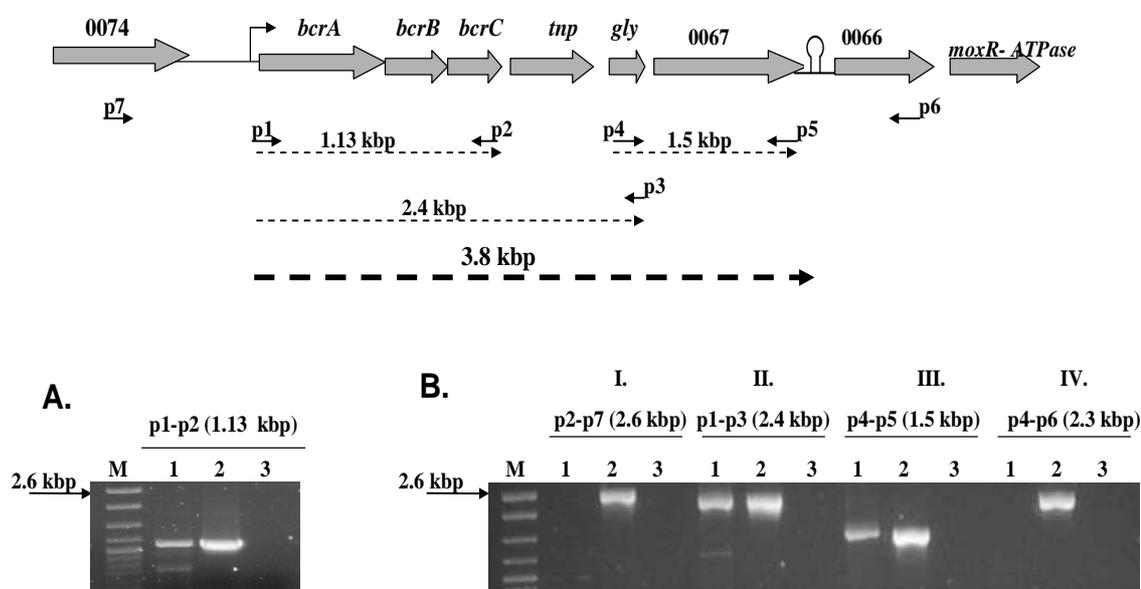
**Table 3.** Benzalkonium chloride (BC) MIC for *L. monocytogenes* strain H7550 and derivatives used in this study.

Strain	MIC* ( $\mu\text{g/ml}$ )
H7550	40
H7550-Cd <sup>s</sup>	10
H7550-Cd <sup>s</sup> S	10
H7550-Cd <sup>s</sup> S (pCON-1)	10
H7550-Cd <sup>s</sup> S (pDS195)	40
H7550-Cd <sup>s</sup> S (pDS201)	10
H7550-Cd <sup>s</sup> S (pDS202)	40
H7550-Cd <sup>s</sup> S (pBEC59)	10
H7550-Cd <sup>s</sup> S (pBEC57)	10
H7550A	30

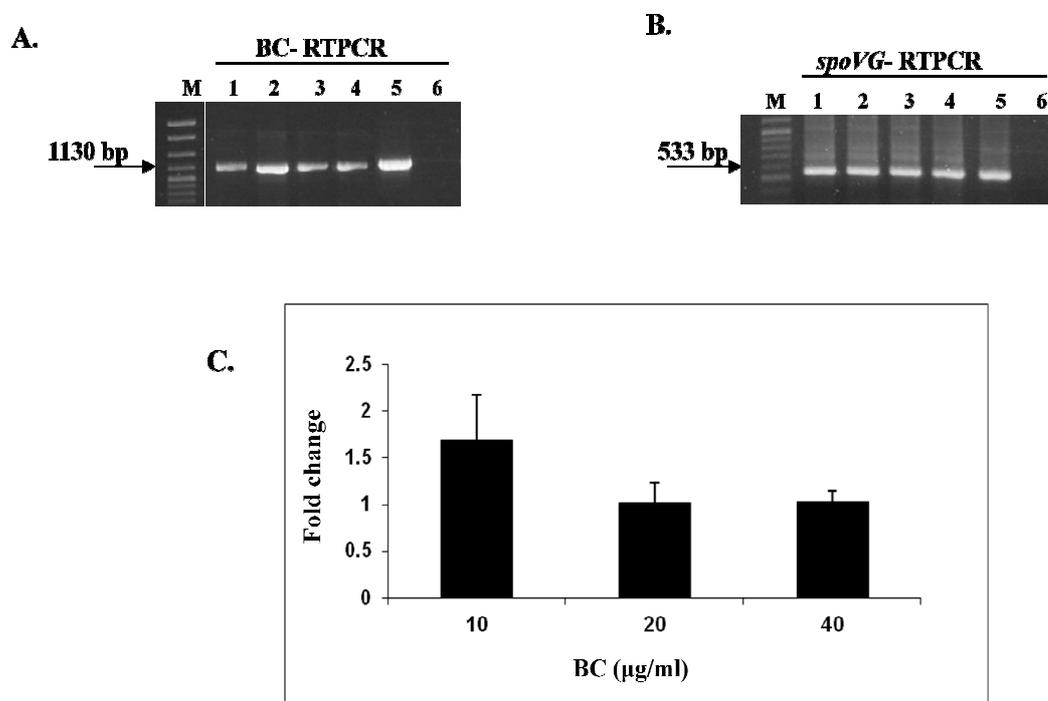
\*MIC was defined as the lowest assessed concentration of BC that prevented growth. MICs were determined as described in Materials and Methods at 30°C. Strains with MIC 10  $\mu\text{g/ml}$  had impaired growth at 2.5 and 5  $\mu\text{g/ml}$  and did not grow at all at 10  $\mu\text{g/ml}$ .

**Figure 1.** BC resistance cassette *bcrABC* in *L. monocytogenes* H7858. **A.** Genetic organization and putative annotation of the *bcrABC* resistance cassette region in plasmid pLM80. Arrows indicate direction of transcription. Genes implicated in BC resistance (*bcrABC*) are in black. The 67 nts sequence upstream of the start codon of *bcrA* gene harboring a canonical promoter is shown along with the -10 and -35 regions (underlined). A palindrome sequence between the -10 and -35 regions, and partially overlapping with the -10 region, is shown in bold and in rectangle. Three copies of *IS1216* are indicated as *IS1216*<sup>left</sup>, *IS1216*<sup>center</sup>, and *IS1216*<sup>right</sup> with solid arrows indicating the orientation of *IS1216* inverted repeats. **B.** Recombinant plasmids harboring different *bcrABC* segments. Dotted arrows indicate the position and orientation of the primers used for recombinant plasmid construction. The plasmids were introduced in the pLM80-cured derivative H7550-Cd<sup>S</sup>S and the resulting strains harboring these plasmids were tested for growth on BC (20 µg/ml), as described in Materials and Methods. Growth or lack of growth are indicated by + and -, respectively. **C.** Schematic diagram of the possible transposable units harboring both cadmium and BC resistance genes (I), cadmium resistance genes (II), and BC resistance genes (III).



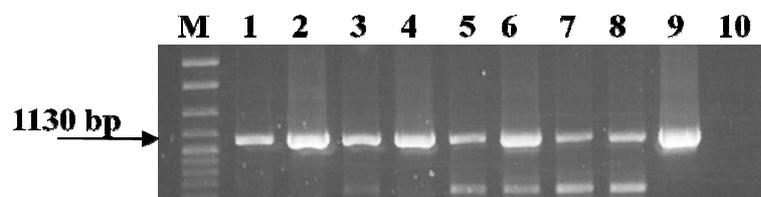
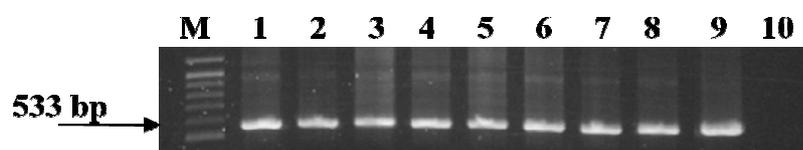
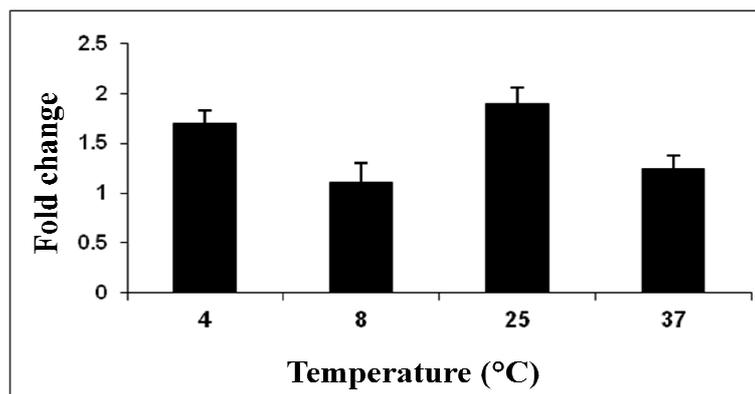


**Figure 2.** Co-transcription of BC resistance cassette and downstream genes assessed by reverse transcription PCR in *L. monocytogenes* strain H7550 grown at 25°C. **A.** Total RNA was reverse transcribed into cDNA using primer p2 and PCR was performed using cDNA template and primers p1, p2. **B.** I) Reverse transcription with primer p2 and PCR with cDNA template and primers p7, p2. II) Reverse transcription with primer p3 and PCR with cDNA template and primers p1, p3. III) Reverse transcription with primer p5 and PCR with cDNA template and primers p4, p5. IV) Reverse transcription with primer p6 and PCR with cDNA template and primers p4, p6. Lanes: 1, RT-PCR of H7550 exposed to BC (10 µg/ml); 2 and 3, H7550 genomic DNA and total RNA, respectively, used as a positive and negative control for RT-PCR. M, 100 bp to 2686 bp DNA molecular marker XIV (Roche, Indianapolis, IN).

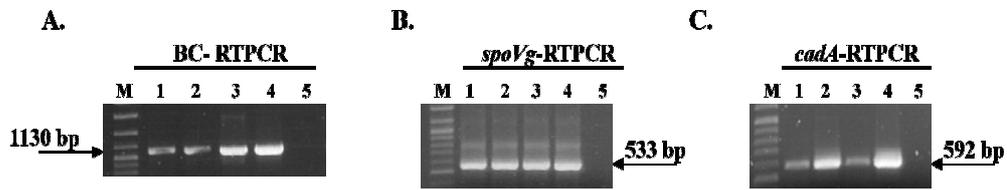


**Figure 3.** Impact of BC concentration on transcription of *bcrABC*. **A.** RT-PCR of *bcrABC* using primers p2 (for cDNA) and p1, p2 for PCR. Lanes: 1-4, H7550 exposed to 0, 10, 20, and 40 µg/ml of BC, respectively, at 25 °C for 30 min; 5 and 6, H7550 genomic DNA and total RNA, respectively, used as a positive and negative control for RT-PCR. M, is a 100 bp to 2686 bp DNA molecular marker XIV (Roche). Arrow points to the expected *bcrABC* PCR product of 1,130 bp **B.** Transcript levels of housekeeping gene *spoVG* in the same cultures as shown in A. RT-PCR using primer s2 (for cDNA) and s1, s2 for PCR. Lanes are as described for A. Arrow points to the expected *spoVG* PCR product of 533 bp. **C.** Fold change in the transcript levels of *bcrABC* after exposure to 10, 20 and 40 µg/ml of BC. Fold change was determined as described in Materials and Methods and the data are averages from two independent trials.

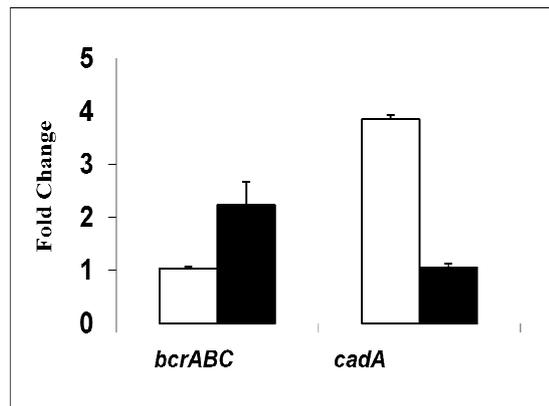
**Figure 4.** Temperature regulation of *bcrABC*. **A.** RT-PCR of *bcrABC* using primers p2 (for cDNA) and p1, p2 for PCR. Lanes: 1, 3, 5, and 7, H7550 grown at 4, 8, 25 and 37 °C, respectively. Lanes: 2, 4, 6, and 8, H7550 grown at 4, 8, 25 and 37 °C and exposed to BC (10 µg/ml) for 30 min as described in Materials and Methods; Lanes: 9 and 10, H7550 genomic DNA and total RNA, respectively, used as a positive and negative control for RT-PCR. M, is a 100 bp to 2686 bp DNA molecular marker XIV, Roche. Arrow points to the expected *bcrABC* PCR product of 1,130 bp. Lower bands in lanes 5-8 represents unspecific PCR products (confirmed with southern blot). **B.** Transcript levels of housekeeping gene *spoVG* in the same cultures as shown in A. RT-PCR using primer s2 (for cDNA) and s1, s2 for PCR. Lanes are as described for A. Arrow points to the expected *spoVG* PCR product of 533 bp. **C.** Fold change in transcript levels of *bcrABC* at 4, 8, 25 and 37°C after exposure to BC (10 µg/ml). Fold change was determined as described in Materials and Methods and the data are averages from two independent trials.

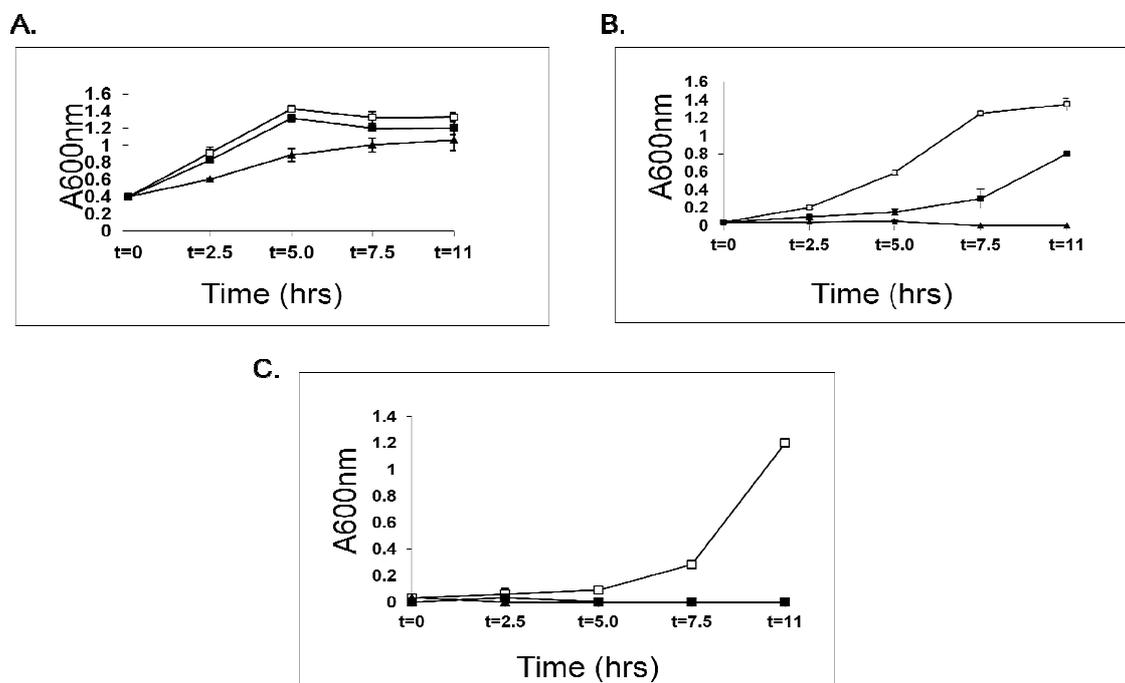
**A.****B.****C.**

**Figure 5.** Increased transcript levels of *bcrABC* and *cadA* by BC and cadmium in *L. monocytogenes* H7550. **A.** RT-PCR of *bcrABC* using primer p2 (for cDNA) and primers p1, p2 for PCR. Lanes: 1, H7550 in the absence of BC or cadmium; 2, H7550 exposed to cadmium; 3, H7550 exposed to BC; 4 and 5, H7550 genomic DNA and total RNA, respectively, used as a positive and negative control for RT-PCR. M, is a 100 bp to 2686 bp DNA molecular marker XIV (Roche). Arrow points to the expected *bcrABC* PCR product of 1,130 bp. Cadmium and BC exposures were for 30 min as described in Materials and Methods. **B.** Transcript levels of housekeeping gene *spoVG* in the same cultures as shown in A. RT-PCR using primer s2 (for cDNA) and s1, s2 for PCR. Lanes are as described for A. Arrow points to the expected *spoVG* PCR product of 533 bp. **C.** RT-PCR of *cadA* using primer c2 (for cDNA) and primers c1, c2 for PCR. Lanes are as described for A. Arrow points to the expected *cadA* PCR product of 592 bp. **D.** Fold change in the transcript levels of *bcrABC* and *cadA* after exposure to BC (black bars) and cadmium (white bars). Fold change was determined as described in Materials and Methods and the data are averages from two independent trials.

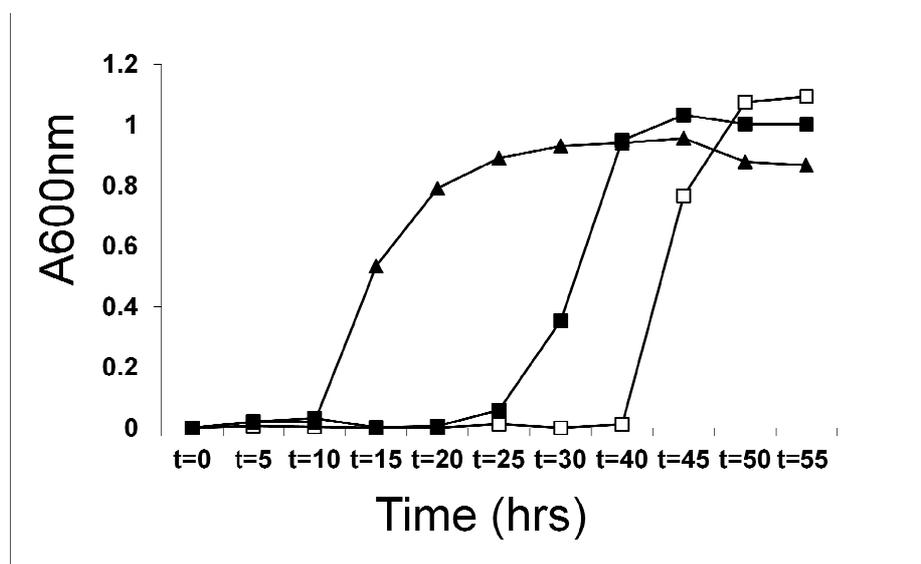


**D.**





**Figure 6.** Impact of cell density on survival and growth *L. monocytogenes* strain H7550 in liquid medium. Cultures in the presence of the indicated BC concentrations were initiated at (A),  $10^8$  cfu/ml, (B),  $10^7$  cfu/ml, and (C),  $10^6$  cfu/ml. Open ( $\square$ ) and closed squares ( $\blacksquare$ ) represent cultures in the absence of BC and exposed to BC (2.5  $\mu$ g/ml), respectively; closed triangles ( $\blacktriangle$ ) represent cultures exposed to 5  $\mu$ g/ml BC. Data are from one representative experiment. The experiments were performed in two independent trials.



**Figure 7.** Protective effect of H7550Cd<sup>s</sup> spent medium on survival and growth of *L. monocytogenes* strain H7550 in liquid cultures in the presence of BC (2.5 μg/ml). H7550 cultures were exposed to BC (2.5 μg/ml) in presence of 20% H7550-Cd<sup>s</sup> spent medium (closed triangles, ▲), 10% H7550Cd<sup>s</sup> spent medium (closed squares, ■) and no spent medium (open squares, □). Data are from one representative experiment. The experiments were performed in two independent trials.

### **Chapter III. Conservation and Distribution of the Benzalkonium Chloride Resistance Cassette *bcrABC* in *Listeria monocytogenes*.**

#### **Authors.**

Vikrant Dutta, Driss Elhanafi, Robin M. Siletzky and Sophia Kathariou

#### **Author's contribution.**

**VD:** PCR analysis of *bcrABC* loci, RT-PCR work, phenotypic characterization, data analysis and manuscript writing; **DE:** *bcrABC* sequencing, phenotypic characterization, data analysis and manuscript writing; **RM:** Strain analysis, phenotypic characterization, data analysis and manuscript writing **SK:** mentor, data analysis, and manuscript writing

#### **Publication status.**

To be submitted to Applied and Environmental Microbiology.

## ABSTRACT

Analysis of a panel of *Listeria monocytogenes* of diverse serotypes revealed that all benzalkonium chloride((BC)-resistant (BC<sup>R</sup>) isolates harbored *bcrABC*, previously identified on a large plasmid (pLM80) of the 1998-1999 hot dog outbreak strain H7858. The *bcrABC* sequences were highly conserved among strains of different serotypes, but variability was noted in sequences flanking *bcrABC*. With only one exception, in strains that also harbored a specific cadmium resistance efflux cassette (*cadA2C2*) *bcrABC* was flanked by the same genes as in pLM80. Most isolates had either this type of organization or appeared to harbor *bcrABC* on the chromosome, flanked by novel sequences. Transcription of *bcrABC* was induced by sublethal levels of BC in strains of different serotypes and flanking sequence organization.

## INTRODUCTION

*L. monocytogenes* is a food-borne pathogen associated with infections that can result in severe illness and death in susceptible individuals (pregnant women and their fetuses, the elderly, and patients in immunosuppressed states) (Painter and Slutsker, 2007; Scallan et al., 2011). An array of adaptations of *L. monocytogenes* including biofilm formation, cold tolerance, and resistance to disinfectants as well as to listeriophage can contribute to this pathogen's persistence in food processing environments, thereby increasing the potential to contaminate the ready-to-eat food products (Kathariou, 2002; Gandhi and Chikindas, 2007; Kornaki and Gurtler, 2007; Kim and Kathariou 2009; Elhanafi et al., 2010). However, our understanding of the mechanisms and factors affecting such adaptations remains limited.

Quaternary ammonium compounds such as benzalkonium chloride (BC) are extensively used in food processing and medical environments (Merianos 1991; Mc Donnell and Russell, 1999). Recently we described the role of a plasmid-based transporter system (*bcrABC*) in BC resistance of strains implicated in the 1998-1999 hot dog outbreaks (epidemic clone II, serotype 4b) (Elhanafi et al., 2010). BC resistance has been detected in *L. monocytogenes* strains of different serotypes, and from diverse sources (Aase et al., 2000; Mereghetti et al., 2000; To et al., 2002; Romanova et al., 2002, 2006; Soumet et al., 2005; Mullapudi et al., 2008). Characterization of *L. monocytogenes* from turkey processing plants revealed that resistance to BC was high among isolates of serotype 1/2a (or 3a) and 1/2b (or 3b) (60 and 51%, respectively) compared to those of the serotype 4b complex (4b, 4d and 4e) (7%). All BC-resistant isolates were found to be also resistant to the heavy metal cadmium, although the reverse was not the case (Mullapudi et al., 2008). Two distinct cadmium resistance determinants were identified among these BC-resistant isolates; one (*cadA1C1*) corresponded to a Tn5422-associated cassette (Lebrun et al., 1994) while the other (*cadA2C2*) corresponded to the cassette identified on pLM80 of *L. monocytogenes* H7858, implicated in the 1998-1999 hot dog-associated outbreak of listeriosis (Nelson et al., 2004; Mullapudi et al., 2010). Even though *bcrABC* has been characterized and shown to be associated with BC resistance in pLM80-harboring strain H7858 (Elhanafi et al., 2010), limited information is available on BC resistance determinants of other BC-resistant *L. monocytogenes* isolates of diverse serotypes and lineages. The objectives of this study were to determine whether such strains harbored *bcrABC* and, if indeed this would be the case, to characterize the genomic location

and transcriptional control of *bcrABC* among diverse BC-resistant strains of *L. monocytogenes*.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *L. monocytogenes* strains used in this study were from our laboratory's *Listeria* collection at North Carolina State University and included 75 BC-resistant (BC<sup>R</sup>) and 36 BC-susceptible (BC<sup>S</sup>) strains. Of the 75 BC<sup>R</sup> isolates, 60 were from the environment of processing plants (Mullapudi et al., 2008), 13 were of human clinical origin and two were from foods. The 36 BC<sup>S</sup> isolates included 17 from the processing plant environment (Mullapudi et al., 2008), 13 of human clinical origin and 15 from foods. Strains used for characterization of the *bcrABC* locus are listed in Table 1. Bacteria were grown either in trypticase soy broth with 0.6% yeast extract (TSBYE) (Becton, Dickinson & Co., Sparks, MD) or in brain heart infusion (BHI) broth (Becton, Dickinson & Co.). Agar media were BHI agar (1.2% Bacto-agar, Becton, Dickinson & Co.) or TSBYE with 1.2% agar (1.2% Bacto-agar). For long term storage bacterial strains were kept at -80°C in BHI supplemented with 20% glycerol (Fischer Scientific, Fair Lawn, NJ).

**BC susceptibility and determinations of MIC.** BC susceptibility of *L. monocytogenes* was assessed as described (Mullapudi et al., 2008; Elhanafi et al., 2010). Strains H7550 and H7550-Cd<sup>S</sup> were used as resistant and susceptible controls, respectively (Elhanafi et al., 2010). For MIC determinations, a single colony from a blood agar plate (Remel, Lenexa, KS) was resuspended in 100 µl of Mueller Hinton broth (Becton, Dickinson & Co.) and 5 µl of the suspension was spotted in duplicate on Mueller Hinton agar (1.2% agar) plates with variable concentrations of BC (0, 5, 10, 15, 20, 25, 30, 35, and 40 µg/ml) and the plates were

incubated at 25°C for 48 h. MIC was defined as the lowest assessed concentration of BC that prevented growth. All MICs were determined in at least two independent trials.

**PCR conditions.** Primers used in this study are listed in Table 2, and their locations are indicated in Fig. 1. PCR employed the Takara Ex Taq kit (Takara, Madison, WI) and a T1 thermal cycler (Biometra, Goettingen, Germany). Primers *cadA2\_F-0076R*, *0076F-bcrA\_R*, and *LtrC\_F-p2* were used to assess the genomic region upstream of *bcrA*, while primers *p1-0067R* and *0067F-0065R* were used to assess the region downstream of *bcrC* (Fig. 1B and Table 2). PCR protocol consisted of 1 cycle at 95°C for 3 min, 32 cycles each at 95°C for 30 sec, 54°C for 1 min, and 72°C for 1 min, and one cycle at 72°C for 10 min. For longer (>2.6 kb) amplicons, extension time was increased by 1 min per kb increase in size of the expected PCR product.

**Sequencing of *bcrABC* amplicons.** For sequencing, *bcrABC* was amplified using Bc-F2 and BC-R (Table 1) followed by double digestion with BamHI/EcoRI and then cloning in pGEM-T in *Escherichia coli* (DH5 $\alpha$ ). The cloned fragment was sequenced using SP6 and T7 primers at Davis sequencing LLC (Davis, CA).

**Reverse transcription PCR (RT-PCR) of *bcrABC* and cadmium resistance determinants.**

Transcription of *bcrABC* and cadmium resistance determinants was assessed for eight BC<sup>R</sup> isolates (Table 1). Bacteria were grown at 25°C and RNA extraction was performed as described before (Elhanafi et al., 2010). Primers and conditions used for *bcrABC*, *cadA2*, and *spoVG* RT-PCR were as described before (Elhanafi et al., 2010). For *cadA1* RT-PCR, primer *c1R* was used for reverse transcription, and *c1F* and *c1R* were used for PCR (Table 2).

**Nucleotide sequence accession numbers.** The *bcrABC* sequences from *L. monocytogenes* (Table 1) have been deposited in Genbank (accession numbers to be obtained).

## RESULTS and DISCUSSION

**Correlation between presence of *bcrABC* and BC resistance in *L. monocytogenes* of diverse serotypes** A panel of 111 *L. monocytogenes* isolates (75 BC<sup>R</sup> and 36 BC<sup>S</sup>) was chosen for screening for *bcrABC* using PCR with primers p1 and p2. PCR results suggested that *bcrABC* was harbored by all 75 BC<sup>R</sup> isolates; in contrast, none of the 36 BC<sup>S</sup> isolates yielded the expected PCR product (Fig. 2A and data not shown).

***bcrABC* sequences were highly conserved among different isolates.** Nine BC<sup>R</sup> strains of different serotypes, Pulsed-field gel electrophoresis (PFGE) types and sources (Table 1) were chosen to determine the sequence of *bcrABC* along with the 106 nt intergenic region upstream of *bcrA* and 70 nt downstream of *bcrC*. The sequences exhibited 99-100% identity (nucleotide and amino acid sequence level) to each other and to *bcrABC* from pLM80 of strain H7858 (Nelson et al., 2004). No amino acid substitutions were detected among the deduced BcrA and BcrB polypeptides, and substitutions in the deduced BcrC were rare: at residue 13 glycine was replaced by serine in three strains and in one strain valine at residue 78 was replaced by alanine. In all strains the canonical promoter and inverted repeats between the putative -10 and -35 sequences were identical to those of H7858 (Elhanafi et al., 2010).

**Diversity among sequences flanking *bcrABC* suggests at least seven categories of *bcrABC* loci.** All BC<sup>R</sup> isolates in our panel were also resistant to cadmium, as described before for isolates from turkey processing plants (Mullapudi et al., 2008). With only two exceptions (*L.*

*monocytogenes* 2877 and 3170, both of serotype 4b) all other BC<sup>R</sup> isolates harbored one or both of the previously detected cadmium resistance cassettes *cadA1C1* and *cadA2C2* (Table 1; Mullapudi et al., 2010). To determine whether the organization of the region harboring *bcrABC* differed among the isolates, we employed PCR with primers derived from sequences flanking *bcrABC* in pLM80 to screen a panel of 55 BC<sup>R</sup> isolates of different serotypes and harboring different cadmium resistance determinants (Table 1, 2). PCR results suggested the presence of seven categories of *bcrABC*-harboring regions (Fig. 1). Two categories (I and VI) accounted for the majority of the isolates. Category I (n= 29, 53% of the screened BC<sup>R</sup> isolates) consisted of isolates of diverse serotypes that harbored *bcrABC* flanked by the same IS1216-associated sequences as present on pLM80, including *cadA2C2* (Table 1; Fig 1B, 2A and 2B). Category VI (n=17, 31% of the screened BC<sup>R</sup> isolates) consisted exclusively of isolates of serotype 1/2a (or 3a). In these isolates downstream to *bcrABC*, transposon resolvase (orf 0070 in pLM80) and a truncated version of orf 0069 was present. Flanking to *bcrABC* and the truncated transposon resolvase (orf 0069), no other pLM80 orfs were found (Fig. 2G, F, Ha, Hb).

Each of the remaining five categories (II to V and category VII) consisted of a small number of isolates (1-4 each) and harbored *bcrABC* flanked by some, but not all, of the IS1216-associated orfs flanking *bcrABC* in pLM80 (Fig. 1B, 2C, 2D, 2E, 2G; Table 1). *L. monocytogenes* isolates of these categories were of various serotypes and with the exception of the sole strain of category VII (strain 258a-1) they lacked *cadA2C2*. With the exception of the previously mentioned strains 2877 and 3170 that lacked any of the known cadmium resistance determinants, these isolates harbored *cadA1C1* (Table 1). Strains 2877 and 3170

along with 181 and 884 of serotypes 1/2a (or 3a) which harbored *cadA1C1* comprised a unique category (V) that harbored three of the orfs downstream of *bcrABC* in pLM80, but not any of those upstream (Table 1; Fig. 1B, 2E).

As mentioned above, of all the isolates that harbored *cadA2C2*, all but one were in category I, with *bcrABC* flanked by the same sequences present in pLM80 (Table 1). The sole exception, the category VII strain 258a-1 (serotype 1/2b or 3b) lacked any of the known sequences upstream of *bcrABC* and appeared to harbor a shorter variant of transposon resolvase (orf 0070) (Fig. 2G).

**Category VI isolates harbor a chromosomal *bcrABC* cassette.** Genome sequence information is available for one of the category VI isolates, F2-515 (<http://www.broadinstitute.org>). Even though this strain harbors a plasmid with *cadA1C1*, *bcrABC* was not with this plasmid (Kuenne et al., 2010; Elhanafi et al., 2010). The chromosome of F2-515 harbored 279 nts upstream of *bcrA* start codon and the two downstream transposon resolvase orfs (orf 0070 and orf 0069). Orf 0069 appeared to lack 129 nts at its 3' end missing compared to its homolog in H7858 (Fig. 2G; data not shown).

In strain F2-515 the *bcrABC*-orf 0069 cluster was flanked on both sides by sequences that appeared to be unique to this strain. PCR with primers *fsl\_F* and *fsl\_R* derived from these F2-515 flanking sequences and primers internal to *bcrABC* indicated that the location of *bcrABC* was conserved among all category VI isolates, and the same as in the sequenced genome of F2-515 (Fig. (Fig. 2Ha, 2Hb; data not shown). These findings suggest that in category VI isolates *bcrABC* was in the chromosome, possibly associated with a transposable element (as evidenced by the transposon resolvase orfs adjacent to *bcrABC*). Further sequence

information on the *bcrABC* region in these strains will be needed to characterize their presence in the genome of these strains.

MIC for BC was 40 µg/ml for strains in categories I-V, as reported before for the pLM80-harboring strain H7550 (Elhanafi et al., 2010). However, lower MICs (35 µg/ml) were observed for strains in categories VI and VII (Table 3). Similar MIC (35 µg/ml) were noted with a recombinant derivative of H7550-Cd<sup>s</sup> in which *bcrABC* with its upstream intergenic region but not other orfs had been integrated into the chromosome (V. Dutta and S. Kathariou, unpublished findings). The lower MIC of category VI isolates may be associated with the chromosomal nature (and hence single copy) of *bcrABC* in isolates of this category. In addition, some of the sequences flanking *bcrABC* in other categories but absent from category VI and VII isolates may themselves contribute to the observed levels of resistance to BC. MICs for all tested BC<sup>S</sup> strains were 10 µg/ml (data not shown), similar to the previously reported MIC for H7550-Cd<sup>s</sup> (Elhanafi et al., 2010).

**Organization of *bcrABC*-harboring regions suggests putative mechanisms for *bcrABC* dissemination across *Listeria* genomes.** The strong association between *cadA2C2* and the pLM80-type flanking gene arrangement of *bcrABC* in category I isolates suggests the dissemination of a pLM80-like plasmid harboring *cadA2C2* and *bcrABC* among *L. monocytogenes* of diverse serotypes. Indeed, the category I serotype 1/2a strain J0161 harbors a large plasmid highly similar to pLM80 (Kuenne et al., 2010). To obtain evidence for similar plasmids in other category I strains we employed PCR with DNA from three strains (201A, serotype 1/2a; 171D, serotype 1/2b; and 4MI-16, serotype 1/2b) and primers derived from four different orfs of pLM80 (pLM80\_0010, a putative secreted protein; pLM80\_0040,

encoding a putative adenine methylase; pLM80\_0058, encoding a putative restriction endonuclease; and pLM80\_0093, encoding a putative plasmid replication protein) (Table 2). Strains 201A and 171D yielded the expected amplicons with all four primer pairs, while 4MI-16 failed to produce a product with the pLM80\_0010 and pLM80\_0040 primers (Fig. 3). Such data constitute tentative evidence for the presence of pLM80-like plasmids in at least some of the category I strains. Representative strains from category II (283a-3 and 516b-1, both of serotype 1/2c) and category IV strain LWA111 (serotype 1/2b) yielded a PCR product with primers 0058F-0058R (encoding a putative restriction endonuclease). Strain LWA111 also yielded a PCR product with primers 0093F-0093R (putative plasmid replication protein). PCR using DNA from selected strains from the other categories showed that primers derived from pLM80\_0010 and pLM80\_0040 only yielded a PCR product with category I strains (Fig. 3).

Since the majority of the isolates belonged to either Category I or VI, it can be hypothesized that *bcrABC* dissemination in *L. monocytogenes* has taken place either via plasmids that also harbor *cadA2C2* (category I) or via chromosomal integration events (category VI). Evidence for other possible mechanisms for *bcrABC* dissemination was provided by the *bcrABC* locus in isolates of category II-V. Category-II strains harbored a part of the pLM80-like IS1216 transposon (IS1216<sup>center</sup> to IS1216<sup>right</sup>; Fig 1A). This genetic arrangement represents a putative self-transposable unit that may be able to mobilize *bcrABC* along with its flanking genes. Category V strains harbored *bcrABC* and the downstream genes up to orf 0067, but lacked sequences upstream of *bcrA*. This genetic arrangement was identical to the *bcrABC* transcriptional unit as described before (Elhanafi et al., 2010). Relatively few strains

represented these categories; it is possible that these genetic arrangements represent relatively recent trends, or that environmental fitness of these strains may be relatively low, for various reasons.

**Transcription of *bcrABC* was induced by sublethal levels of BC in BC<sup>R</sup> strains, regardless of serotype or sequences flanking *bcrABC*.** Transcription of *bcrABC* was analyzed using RT-PCR (Table 1). With all tested strains, transcription of *bcrABC* increased upon exposure to sublethal BC (10 µg/ml) and was not effected by the type of cadmium resistance genes harbored by the strain (Fig. 4 and data not shown). Similarly, transcription of *cadA* (*cadA1*, *cadA2*, or both *cadA1* and *cadA2*) increased in the presence of cadmium (10 µg/ml CdCl<sub>2</sub>) but not following exposure to BC (Fig. 4 and data not shown). No significant changes were observed in the levels of transcripts of the reference housekeeping gene *spoVG* when cells were exposed to either cadmium or BC (data not shown). The transcriptional data were similar to those observed before for strain H7550, harboring *bcrABC* on pLM80 (Elhanafi et al., 2010).

In conclusion, these findings highlight the dissemination of *bcrABC* in *L. monocytogenes* strains of different serotypes. Our findings suggest mobility of *bcrABC* on pLM80-like plasmids that also harbor the cadmium efflux cassette *cadA2C2*, as observed in *L. monocytogenes* H7858 (1998-1999 hot dog outbreak) and J0161 (2000 turkey deli meats outbreak) (Nelson et al., 2004; Kuenne et al., 2010). Sequencing of other plasmids from category I isolates will be needed to adequately assess their similarity to pLM80 or the plasmid harbored by J0161. On the other hand, in strains harboring only the earlier described Tn5422-associated cadmium efflux cassettes *cadA1C1* (Lebrun et al., 1994), *bcrABC* was

flanked by genes other than those on pLM80 and appears to be on the chromosome. Additional sequencing of chromosomal regions will be needed to elucidate the genomic location of *bcrABC* in these strains. Regardless of their location, the pronounced conservation of the *bcrABC* cassettes suggests their relatively recent acquisition from sources that remain to be identified. Such acquisition events likely were in response to selection pressures associated with disinfectant use in processing plants and other environments.

#### ACKNOWLEDGEMENTS

This work was partially supported by a grant from the American Meat Institute Foundation and USDA grant 2006-35201-17377. We are thankful to all other members of our laboratory for their support and encouragement.

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**Table 1.** *L. monocytogenes* strains used for analyzing *bcrABC* locus.

Code	Strain <sup>1,2</sup>	Isolation date	Serotype	Cadmium determinant type	Source
<b>Category I (n=29)</b>					
83	171D	4/13/2004	1/2b, 3b	A1, A2	Processing Plant
84	176A	4/13/2004	1/2a, 3a	A1, A2	Processing Plant
93	192A	6/15/2004	1/2a, 3a	A1, A2	Processing Plant
162	<b>201A</b>	6/15/2004	1/2a, 3a	A1, A2	Processing Plant
163	201B	6/15/2004	1/2b, 3b	A1, A2	Processing Plant
378	128b-2	2/10/2004	1/2b, 3b	A1, A2	Processing Plant
677	223a-1	8/23/2004	1/2b, 3b	A1, A2	Processing Plant
720	231b-1	8/23/2004	1/2a, 3a	A1, A2	Processing Plant
1077	326b-5	12/14/2004	1/2b, 3b	A1, A2	Processing Plant
1104	34-2a	9/24/2003	1/2b, 3b	A1, A2	Processing Plant
1134	360b-1	4/5/2005	1/2b, 3b	A1, A2	Processing Plant
2405	414a-1	10/13/2005	1/2b, 3b	A1, A2	Processing Plant
1308	91a-1	12/3/2003	1/2b, 3b	A1, A2	Processing Plant
1481	<b>J2446</b>	1/1/2003	4b	A1, A2	Clinical
3041	14LA	1994	1/2a, 3a	A1, A2	Clinical
3053	26LA	1995	1/2b, 3b	A1, A2	Clinical
130	2 MI 40	7/27/2004	1/2c, 3c	A2	Processing Plant
171	210A	6/15/2004	1/2a, 3a	A2	Processing Plant
172	210B	6/15/2004	1/2b, 3b	A2	Processing Plant
197	<b>4 MI-16</b>	12/16/2004	1/2b, 3b	A2	Processing Plant
1117	<b>34-6a</b>	9/24/2003	4b	A2	Processing Plant
1354	F6825	1/1/1989	1/2a, 3a	A2	Processing Plant
1355	F6972	1/24/1989	1/2a, 3a	A2	Processing Plant
1450	H7550	Outbreak 1998-99	4b	A2	Clinical
1452	H7738	Outbreak 1998-99	4b	A2	Clinical
1453	H7762	Outbreak 1998-99	4b	A2	Clinical
1461	J0161	Outbreak 2000	1/2a	A2	Clinical
1907	L1712a	6/6/2005	1/2a, 3a	A2	Processing Plant
2503	H7858	Outbreak 1998-99	4b	A2	Clinical

Table 1 Continued.

<b>Category II (n=2)</b>					
961	<b>283a-3</b>	10/21/2004	1/2c, 3c	A1	Processing Plant
2642	<b>516b-1</b>	3/29/2006	1/2c, 3c	A1	Processing Plant
<b>Category III (n=1)</b>					
2716	LWA131	7/28/2006	1/2a, 3a	A1	FDA
<b>Category IV (n=1)</b>					
2696	LWA111	2/10/2006	1/2b, 3b	A1	FDA
<b>Category V (n=4)</b>					
181	266A	10/21/2004	1/2a, 3a	A1	Processing Plant
884	266a-1	10/21/2004	1/2a, 3a	A1	Processing Plant
2877 <sup>3</sup>	J3916	1/1/2006	4b	unknown	Clinical
3170 <sup>3</sup>	J5202	9/5/2008	4b	unknown	Clinical
<b>Category-VI (n=17)</b>					
180	265E	10/21/2004	1/2a, 3a	A1	Processing Plant
273	101b-4	12/3/2003	1/2a, 3a	A1	Processing Plant
408	139a-5	2/10/2004	1/2a, 3a	A1	Processing Plant
513	176b-1	4/13/2004	1/2a, 3a	A1	Processing Plant
600	201a-4	6/15/2004	1/2a, 3a	A1	Processing Plant
627	206a-5	6/15/2004	1/2a, 3a	A1	Processing Plant
653	<b>210b-1</b>	6/15/2004	1/2a, 3a	A1	Processing Plant
686	223b-4	8/23/2004	1/2a, 3a	A1	Processing Plant
754	239a-2	8/23/2004	1/2a, 3a	A1	Processing Plant
869	26-1a	9/24/2003	1/2a, 3a	A1	Processing Plant
933	279a-2	10/21/2004	1/2a, 3a	A1	Processing Plant
1096	339b-5	12/14/2004	1/2a, 3a	A1	Processing Plant
1171	367b-1	4/5/2005	1/2a, 3a	A1	Processing Plant
1178	369a-1	4/5/2005	1/2a, 3a	A1	Processing Plant
1845	<b>L1624a</b>	5/18/2005	1/2a, 3a	A1	Processing Plant
2622	<b>494b-1</b>	3/29/2006	1/2a, 3a	A1	Processing Plant
2813	SL F2-515	-	1/2a1	A1	Cornell

Table 1 Continued.

<b>Category VII (n=1)</b>					
854	258a-1	10/21/2004	1/2b, 3b	A2	Processing Plant

<sup>1</sup>Strains 4MI-16, 34-6a, L1624a, 494b-1, 201A, 210b-1, 283a-3, J2446, and 516b-1 listed in bold were used for sequencing *bcrABC*.

<sup>2</sup>Strains 14LA, 26LA, 201A, 171D, J0161, 4MI-16, 516b-1 and 210b-1 were used to assess *bcrABC* transcription. Bacterial culture conditions, RNA extraction, and reverse transcription method used for *bcrABC*, *cadA2*, *spoVG* and *cadAI* RT-PCR were as described before (Elhanafi et al., 2010). For *cadAI* RT-PCR primer c1R was used for reverse transcription, and c1F and c1R were used for PCR (Table 2).

<sup>3</sup>Strains 2877 and 3170 were negative for all *cadAC* determinants.

**Table 2.** Oligonucleotides used in this study.

Primer	Sequence (5'-3')
BcF2 <sup>1</sup>	GACTGGATCCGATTCTGGAACATCCCTATC
BcR <sup>1</sup>	GTATGAATTCGTATAATCCGGATGCTGCCC
cadA2_F	GCACCAAAGTTTACTGTTGC
c1F	CAGTCATATTCAGTTTCTCCTTCC
c1R	GACAATAGCGGTAATTAATCTTTCAC
fsI_F	CCAATGCTTCATGCGTTAC
fsI_R	CGCTTAACTTCTCGTAAACT
0076R	CTCTGTAAC TTTCTAAAGGCG
0076F	CGCCTTTAGAAAGTTACAGAG
bcrA_R	GATTACCCTCCTTTTGTTATTC
LtrC_F	CAGAGTGAACGTGATCAGCATC
p1	CATTAGAAGCAGTCGCAAAGCA
p2	GTTTTCGTGTCAGCAGATCTTTGA
tnpR	CTAATTGGTTAACACCAGCC
0067R	CTGAAATTACGGCAGCGATCTATGAC
0067F	GTCAAATCATTGCCATCGTGCGTA
0065R	CTTCACTCGTTCTACCGTGTTGG
0093F	GGACACCGAATACATACCG
0093R	CTACTTCGTCTATAATCGTTGC
0040R	CAGTATATGCTTCAAACCGTTG
0040F	GTA TGT GTG ATC GAT GCG AC
0010F	CTGCAAGAAACGTTCTATCG
0010R	ATGACTGTAGATCCACATGC
0058F	TCCAGCATCACTCGCAAAG
0058R	TCAACTAGATGCCGTAGAGG

Table 2 Continued.

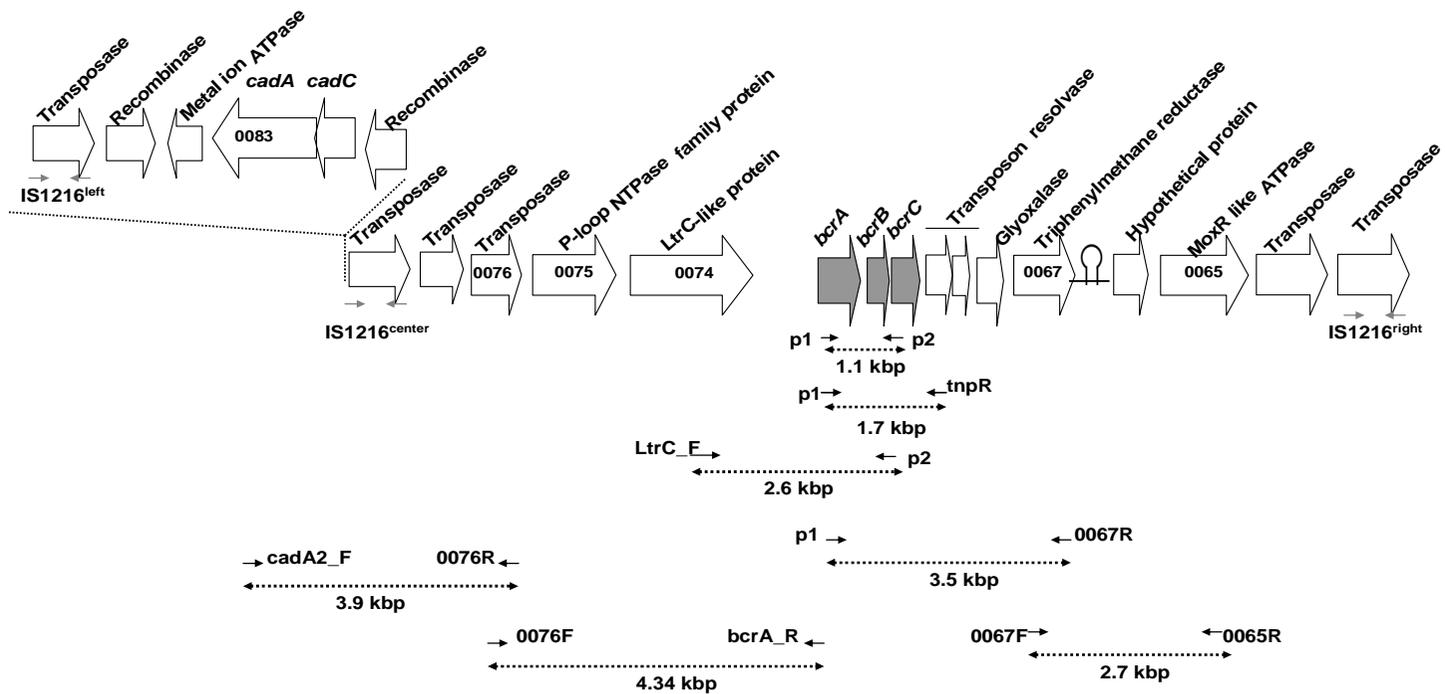
<sup>1</sup> underlined sequences correspond to restriction enzyme sites: GGATCC, BamHI; GAATTC, EcoRI. Primers p1, p2, 0067F, 0067R, BcF2, BcR have been reported before (Elhanafi et al., 2010).

**Table 3.** BC MIC for representative strains from different categories

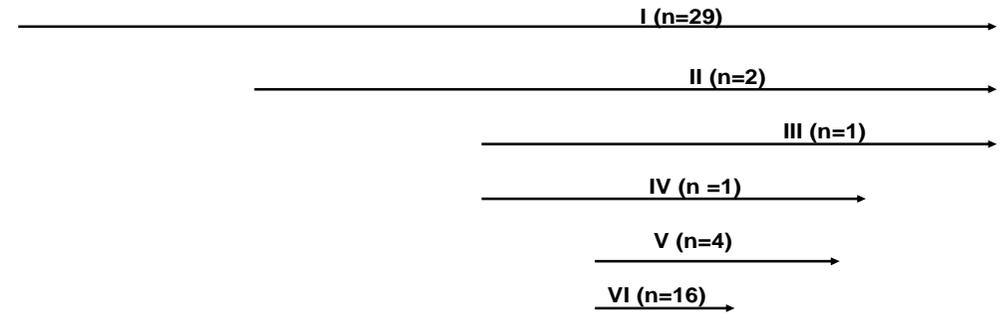
<b>Strains</b>	<b>BC MIC (<math>\mu\text{g/ml}</math>)</b>
Category I	
201A	40
4MI-16	40
210B	40
26LA	40
Category II	
516b-1	40
283a-3	40
Category III	
LWA131	40
Category IV	
LWA111	40
Category V	
J3916	40
J5202	40
Category VI	
F2-515	30
494b-1	30
210b-1	30
Category VII	
258a-1	30

**Figure 1.** Detection of *bcrABC* and neighboring orfs in BC-resistant *L. monocytogenes*. **A.** Organization of the *bcrABC* region in pLM80. *IS1216*<sub>center</sub>, *IS1216*<sub>right</sub> and *IS1216*<sub>left</sub> indicate putative *IS1216* elements, and inverted repeats associated with these elements are indicated with solid gray arrows (figure modified from Elhanafi et al., 2010). Primers are indicated by solid black arrows, and the size of expected PCR products is indicated (in kb). **B.** Diagrammatic representation of different categories of *bcrABC*-harboring regions in *L. monocytogenes*. Category designation was based on PCR data using primers listed in Table 2.

**A.**

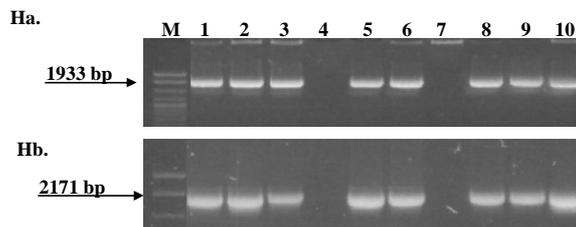
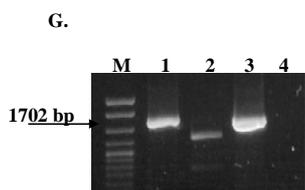
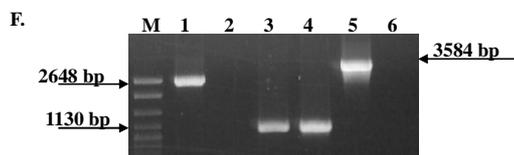
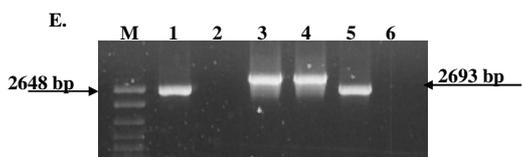
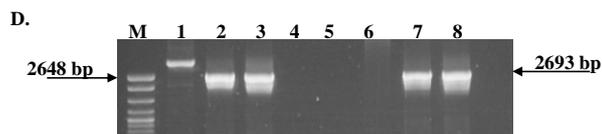
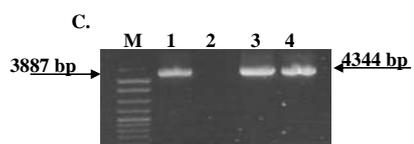
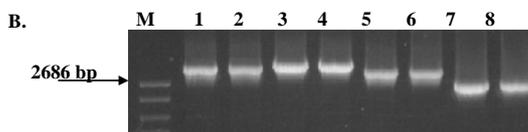
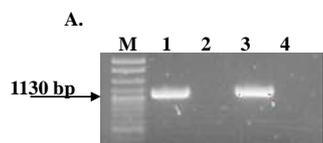


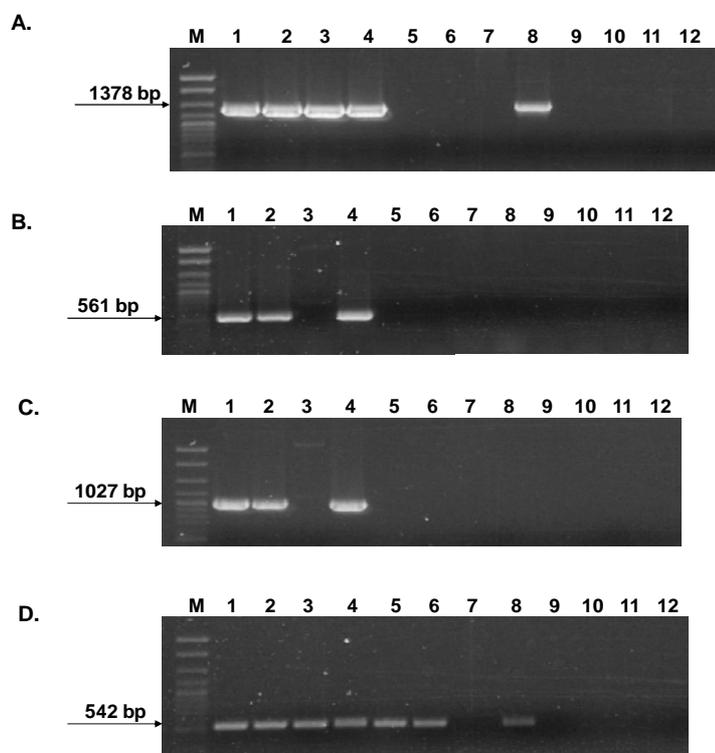
**B.**



**Figure 2.** PCR-based analysis of *L. monocytogenes* *bcrABC* regions. **A.** *bcrABC* was present in BC<sup>R</sup> but not in BC<sup>S</sup> strains. Lanes: 1 and 3, BC<sup>R</sup> strains 4MI-16 and H7550, respectively; 2 and 4, BC<sup>S</sup> strains 24-1b and H7550-Cd<sup>s</sup>, respectively. PCR was done using primers p1 and p2. **B.** Category I strains with pLM80-like *bcrABC* locus. PCR was done using primers *cadA2\_F* and 0076R (lanes 1 and 2), 0076F and *bcrA\_R* (lanes 3 and 4), p1 and 0067R (lanes 5 and 6), 0067F and 0065R (lanes 7 and 8). Lanes: 1, 3, 5, and 7, DNA from strain H7550; 2, 4, 6, and 8, DNA from strain 4MI-16. **C.** Category II strains. PCR was done using primers *cadA2\_F* and 0076R (lanes 1 and 2) and primers 0076F and *bcrA\_R* (lanes 3 and 4). Lanes: 1 and 3, strain H7550; 2 and 4, strain 516b-1. **D.** Category III and IV. PCR was done using primers *LtrC\_F* and p2 (lanes 1-4) and primers 0067F and 0065R (lanes 5-8). Lanes: 1 and 5, strain LWA111 (category II); 2 and 6, strain LWA131 (category III); 3 and 7, strain H7550; 4 and 8, H7550-Cd<sup>s</sup> (negative control). **E.** Category V. PCR was done using primers *LtrC\_F* and p2 (lanes 1 and 2), primers p1 and 0067R (lanes 3 and 4) and primers 0067F and 0065R (lanes 5 and 6). Lanes: 1, 3, and 5 strain H7550; Lanes 2, 4, and 6, strain J5202. **F.** Category VI. PCR was done using primers *LtrC\_F* and p2 (lanes 1 and 2), primers p1 and p2 (lanes 3 and 4) and primers p1 and 0067R (lanes 5 and 6). Lanes: 1, 3, and 5, strain H7550; 2, 4, and 6, strain 210b-1. **G.** Evidence for a truncated transposon resolvase immediately downstream of *bcrABC* in strain 258a-1 (category VII). Lanes 1-4 represent PCR with p1 and *tnpR* primers using genomic DNA from strains F2-515, 258a-1, H7550 and H7550-Cd<sup>s</sup> (negative control), respectively. **H.** Sequences upstream to *bcrABC* and the downstream transposon resolvase are novel and conserved in category VI isolates but not in the category VII strain 258a-1. **a.** Lanes 1-10, PCR with (a) primers *fsl\_F*, p2 and (b)

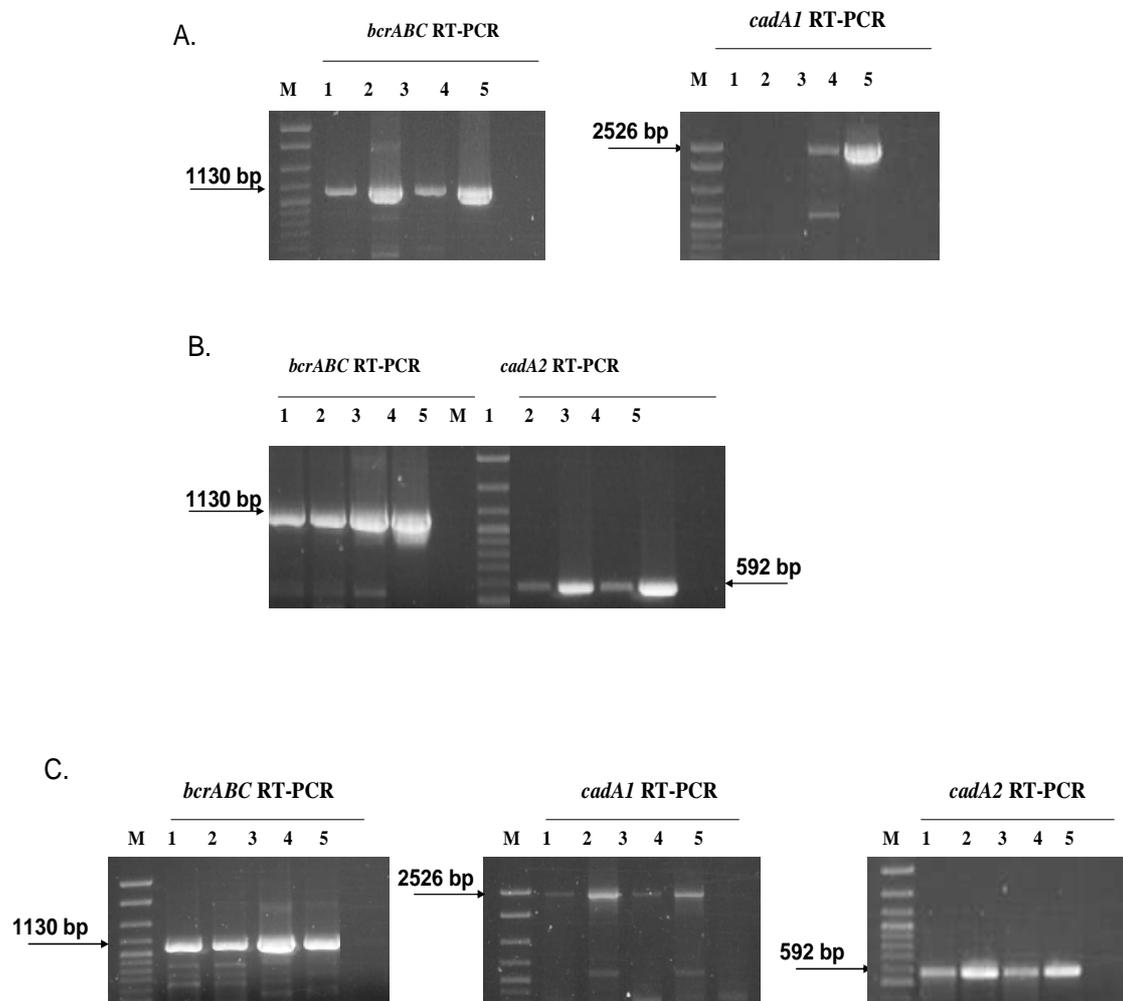
primers p1, fsl\_R . Template was genomic DNA from strains 223b-4, 279a-2, 210a-4, 258a-1, 210b-1, 239a-2, H7550 (negative control) , 265E, 339b-5, and 367b-1, respectively. With the exception of H7550 (negative control) and 258a-1 (category VII) all other isolates are category VI, M, 100 bp to 2686 bp DNA molecular marker XIV (Roche). Arrows indicate the expected band size of the PCR amplicons.





**Figure 3.** PCR specific to pLM80 markers performed with representative strains from each category. **A, B, C, D:** PCR using primers 0093F-0093R, 0010F-0010R, 0040F-0040R and 0058F-0058R, respectively (Table 2). Lanes:1-4, category I strains (201A, 171D, 4MI-16, H7550); 5 and 6, category II strains (283a-3, 516b-1); 7, category III strain LWA131; 8, category IV strain LWA111; 9 and 10, category V strains (J3916, J5202); 12, negative control (H7550-Cd<sup>s</sup>); M, 100 bp to 2686 bp DNA molecular marker XIV (Roche). Arrows indicate the expected band size of the PCR amplicon.

**Figure 4.** Increased transcript levels of *bcrABC* upon exposure to sublethal BC (10 µg/ml) and *cadA* (*cadA1*, *cadA2*, and both) upon exposure to sublethal cadmium (10 µg/ml) in *L. monocytogenes* isolates. **A.** RT-PCR of *bcrABC* and *cadA1* for category II strain 516b-1. **B** and **C**, *bcrABC* and *cadA1* RT-PCR for category I strains 4MI-16 and 201A, respectively. Lanes: 1, bacteria exposed to no BC or cadmium ; 2 and 3, bacteria exposed to cadmium and BC, respectively (30 min, 25°C). Lanes 4 (genomic DNA) and 5 (total RNA) represent positive and negative RT-PCR controls, respectively. M, 100 bp to 2686 bp DNA molecular marker XIV (Roche). Arrows point to the expected PCR product for *bcrABC* (1130 bp), *cadA1* (2526 bp) and *cadA2* (592 bp). RT-PCR was done as described in Materials and Methods.



**CHAPTER IV: A Novel Restriction- Modification System is Responsible for Temperature- Dependant Phage Resistance in *Listeria monocytogenes* ECII.**

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**Author's contribution.**

**JWK:** Transposon mutant library construction, screening of phage susceptible mutant, ORF2753 deletion, and manuscript writing; **VD:** All other recombinant cloning, RT-PCR work, remaining experiments, data analysis and manuscript writing; **DE:** Transposon mutant library construction, screening of phage susceptible mutant; **SL:** *In silico* analysis to identify the recognition site for LmoH7; **JO:** Statistical analysis; **SK:** mentor, data analysis, and manuscript writing

**Publication status.**

Under review in Applied and Environmental Microbiology. Submitted in October 2011.

## ABSTRACT

*Listeria monocytogenes* epidemic clone II (ECII) strains are unusual in being completely resistant to phage when grown at low temperatures ( $\leq 30^{\circ}\text{C}$ ). In the current study we constructed and characterized a *mariner*-based mutant (J46C) of the ECII strain H7550-Cd<sup>s</sup> that lacked temperature-dependent resistance to phage. The transposon was localized in LMOh7858\_2753 (ORF 2753), a member of a 12-ORF genomic island unique to ECII strains. ORF 2753 and ORF 2754 exhibited homologies to restriction endonucleases and methyltransferases associated with type II restriction restriction-modification (RM) systems. *In silico*-based predictions of the recognition site for this putative RM system were supported by resistance of DNA from ECII strains to digestion by BfuI, a type II restriction enzyme specific for GTATCC (N6/5). Similarly to J46C, a mutant harboring an in-frame deletion of ORF 2753 was susceptible to phage regardless of temperature of growth ( $25^{\circ}\text{C}$  or  $37^{\circ}\text{C}$ ). Genetic complementation restored phage resistance in  $25^{\circ}\text{C}$ -grown cells of ORF 2753 mutants. Reverse transcription and qRT-PCR data suggested enhanced transcription of ORF 2753 at low temperatures ( $\leq 25^{\circ}\text{C}$ ) than at  $37^{\circ}\text{C}$ . In contrast, available transcriptional data suggested that the putative methyltransferase (ORF 2754) was constitutively expressed at all tested temperatures ( $4$ - $37^{\circ}\text{C}$ ). Thus, temperature-dependent resistance of *L. monocytogenes* ECII to phage is mediated by temperature-dependent expression of the restriction endonuclease associated with a novel RM system (LmoH7) unique to this epidemic clone.

## INTRODUCTION

*Listeria monocytogenes* remains a major foodborne pathogen for at-risk populations including pregnant women and their fetuses, the elderly, and patients with compromised immunity.

Even though listeriosis is relatively infrequent, it is accompanied with high mortality and severe symptoms such as septicemia, meningitis and stillbirths (Painter and Slustker, 2007; Scallan et al., 2011).

A number of genotypic tools have revealed that strains from different outbreaks can be closely related, constituting epidemic clones (Cheng et al., 2008; Chen and Knabel 2007; denBakker et al., 2010; Kathariou, 2002; Ragon et al., 2008; Swaminathan et al., 2007). Epidemic clone I (ECI) and epidemic clone II (ECII) have been most extensively characterized. ECI strains have been responsible for numerous outbreaks of listeriosis in North America and Europe, with the first documented outbreak being the coleslaw-associated outbreak in the Maritime Provinces, Canada (Cheng et al., 2008; denBakker et al., 2010; Kathariou, 2002; Ragon et al., 2008). In contrast, ECII was not recognized until the 1998-1999 multistate outbreak of listeriosis in the United States, attributed to contaminated hot dogs. ECII was subsequently implicated in another multistate outbreak in 2002, involving contaminated turkey deli meats, as well as in an outbreak in Belgium (CDC, 1999, 2002; Kathariou et al., 2006; Yde et al., 2010; M. Yde, personal communication).

A special phenotypic characteristic of ECII strains is their temperature-dependent resistance to phage. In contrast to all other screened *Listeria* strains, for which susceptibility to phage was not significantly influenced by the temperature of growth of the bacteria, ECII strains failed to form plaques when the bacteria were grown at temperatures below 30°C but did so following growth at 37°C (Kim and Kathariou, 2009). This temperature-dependent resistance of ECII strains was observed with all tested phages, including the wide host range phages A511 (Loessner and Scherer, 1995) and 20422-1 (Kim et al., 2008), the serotype 4b specific

phage A500 (Zink and Loessner, 1992), and other serotype 4b specific phages (Kim et al., 2008). The observed temperature-dependent phage resistance of ECII strains was specific to the temperature of growth of the bacteria, and was not affected by the temperature during infection or subsequent incubation (Kim and Kathariou, 2009).

The apparent resistance of ECII strains to phage when the bacteria are grown at low temperature has important implications for the adaptations of this clonal group in food processing plants, foods, and other environments. However, the mechanisms underlying temperature-dependent phage resistance of this clonal group have remained elusive. Resistance did not simply reflect absence of a receptor, since phages adsorbed similarly onto bacteria grown at 25 and 37°C (Kim and Kathariou, 2009). In this study, we describe genetic determinants required for temperature-dependent phage resistance of the ECII strain H7550, implicated in the 1998-1999 multistate outbreak of listeriosis in the United States.

## MATERIALS AND METHODS

**Bacterial strains and growth media.** Bacterial strains used in this study are listed in Table 1. *L. monocytogenes* H7550-Cd<sup>s</sup> is a plasmid (pLM80)-cured derivative of strain H7550 from the 1998-1999 hot dog-associated outbreak (CDC, 1999; Elhanafi et al., 2010; Evans et al., 2004). It has the same pulsed-field gel electrophoreses profile as strain H7858 which was involved in the same outbreak and the genome of which has been sequenced (Nelson et al., 2004). Listeriophage 20422-1 was used for phage susceptibility tests as described (Kim et al., 2008). *L. monocytogenes* strains were routinely grown in brain heart infusion broth (BHI, Difco, Sparks, MD), BHI supplemented with 1.2% agar (Difco) or trypticase soy broth with 0.6% yeast extract (TSBYE; Becton Dickinson & Co, Sparks, MD). *Escherichia coli* DH5 $\alpha$

was grown in Luria-Bertani broth (LB) or LB supplemented with 1.2% agar (Difco). Antibiotics, when indicated, were kanamycin (Km), erythromycin (Em), chloramphenicol (Cm), ampicillin (Amp) and nalidixic acid (NA) and were purchased from Sigma-Aldrich (St. Louis, MO).

**Mariner-based transposon mutagenesis.** A transposon mutant library of H7550-Cd<sup>s</sup> was constructed with a *mariner*-based transposition system using pMC39 as described (Cao et al., 2007; Azizoglu and Kathariou, 2010). Each putative mutant was separately tested for growth on BHI agar supplemented with Em and Km; mutants that were resistant to erythromycin but susceptible to kanamycin were individually inoculated in 250  $\mu$ l of BHI in 96-well microtiter plates (Corning, Inc., Corning, NY), grown overnight at 37°C and stored at -80°C.

**Identification of phage-susceptible mutants.** To identify transposon mutants susceptible to phage following growth at 25°C, 45  $\mu$ l from each mutant was mixed with 10 mM CaCl<sub>2</sub> and 5  $\mu$ l phage 20422-1 solution (10<sup>7</sup> PFU/ml) in individual wells of 96-well plates (22). After 30 min of incubation at 37°C, 5  $\mu$ l of the phage-bacterial mixture from each well was spotted onto LB-CaCl<sub>2</sub> plates (air-dried in a laminar flow cabinet for 30 min prior to use) and the plates were incubated at 37°C overnight (spot assay). Phage-susceptible mutants (no or visibly reduced growth on the inoculated spot) were confirmed individually with the standard plaque assay as described (Kim et al., 2008).

**Molecular methods.** DNA was extracted as described (Yildirim et al., 2004) and polymerase chain reactions (PCR) were done using the Takara ExTaq kit (Takara, Madison, WI) and a T1 thermal cycler (Biometra, Goettingen, Germany). Primers (Table 2) were purchased from Eurofin MWG Operon (Huntsville, AL). DNA probes for Southern blots were prepared with

PCR and the amplicons were labeled with digoxigenin as described (Yildirim et al., 2004). A probe (*ermC*) specific to the transposon was constructed with primers Maq206 and Maq254 (Cao et al., 2007) and used to determine the number of transposon copies, as described (Cao et al., 2007). The transposon insertion site was determined by two rounds of PCR as described (Cao et al., 2007), followed by sequencing of the PCR product (Genewiz, Inc., South Plainfield, NJ).

**DNA and protein sequence analysis.** Sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology. *In silico* identification of the recognition site for the putative restriction-modification system was performed using REBASE ([rebase.neb.com/rebase/rebase.html](http://rebase.neb.com/rebase/rebase.html)) (Roberts et al., 2010).

**Determination of methylation at GTATCC (N6/5) sites.** Genomic DNA from bacteria grown at the indicated temperatures was digested with BfuI (Fermentas, Glen Burnie, MD) as per manufacturer's protocol. DNA was also digested with HindIII (New England Biolabs, Ipswich, MA) as control.

**Construction of in-frame deletion of ORF 2753.** Primers Delcon54-F (5' SacI site) and Delcon54-R (5' PstI site) (Table 2) were used to amplify a 619 nt fragment including 614 nt from the 3' end of ORF 2754 and 5 nt from the 5' end of ORF 2753. Similarly, primers Delcon52-F (5' PstI site) and Delcon52-R (5' BamHI site) (Table 2) were used to amplify a 601 nt fragment including 170 nt from the 3' end of ORF 2752 and 431 nt of the intergenic space between ORF 2752 and ORF 2753. The two amplicons were digested with SacI/PstI and PstI/BamHI (New England Biolabs), respectively, and cloned in *E. coli* strain DH5 $\alpha$  using the temperature-sensitive shuttle vector pCON1 (Behari and Youngman, 1998) digested

with SacI and BamHI. The recombinant plasmid (pCON1\_5452) was introduced into *L. monocytogenes* H7550-Cd<sup>s</sup> by electroporation (Park and Stewart, 1990). Transformants were selected on BHI agar plates supplemented with Cm (6 µg/ml) at 30° C for 2-3 days, and were confirmed using PCR. A single colony of a transformant was grown at 42°C for 24 h followed by four serial passages at 42°C (Behari and Youngman, 1998), and subsequent steps to isolate chloramphenicol-susceptible deletion mutants lacking the plasmid vector were as described (Yildirim et al., 2010). Putative mutants were tested with the spot assay to determine phage susceptibility. The in-frame deletion of ORF 2753 was confirmed with PCR using primers Delcon54-F and Delcon52-R (Table 2) and with DNA sequencing; a deletion mutant designated J25FII-7 was chosen for further study.

**Genetic complementation.** PCR with primers comp2752\_2R and comp2755\_F (Table 2) resulted in a fragment (ca. 4.0 kb) that included ORFs 2755, 2754 and 2753 as well as the intergenic regions upstream of ORF 2755 and downstream of ORF 2753. The PCR fragment was digested with XmaI and SacI (New England Biolabs), cloned into the integration vector pPL2 (Lauer et al., 2002) that was also digested with XmaI and SacI, and electroporated into *E. coli* DH5α. Transformants were isolated on LB agar with Cm (25 µg/ml) following incubation for 24-48 h at 30°C as described (Lauer et al., 2002). The presence of insert was confirmed with PCR using primers Delcon54F and Delcon54R (Table 2). The recombinant plasmid (designated pPLØ) was electroporated into *E. coli* S17-1, mobilized into J46C and J25FII-7 via conjugation, and transconjugants were selected on BHI agar plates supplemented with Cm (6 µg/ml) and NA (20 µg/ml) at 30° C for 2-3 days as described (Yildirim et al.,

2010). Plasmid integration was confirmed with PCR using primers PL95 and NC16 (Lauer et al., 2002).

#### **Cloning and expression of ORF 2754 and upstream ORF 2755 in heterologous hosts.**

PCR with primers comp2753R and comp2755F (Table 2) resulted in a fragment (ca. 2.5 kb) that included ORFs 2755, and 2754 and the intergenic region upstream of ORF 2755 as well as 102 nts downstream of ORF 2754. The PCR fragment was digested with XmaI and SacI (New England Biolabs), cloned into the integration vector pPL2 (Lauer et al., 2002) that was also digested with XmaI and SacI, and electroporated into *E. coli* DH5 $\alpha$ . Transformants were isolated on LB agar with Cm (25  $\mu$ g/ml) as described (Lauer et al., 2002). The presence of insert was confirmed with PCR using primers Delcon54F and Delcon54R (Table 2). The recombinant plasmid (designated pPLmet) was electroporated into *E. coli* S17-1, mobilized into non-pathogenic *L. innocua* CLIP11262 and *L. welshimeri* L1225 via conjugation, and transconjugants were selected on BHI agar plates supplemented with Cm (6  $\mu$ g/ml) and NA (20  $\mu$ g/ml) at 30° C for 2-3 days as described (Yildirim et al., 2010). Transconjugants were named CLIP11262pPLmet and L1225pPLmet (Table 1). Plasmid integration was confirmed with PCR using primers PL95 and NC16 (Lauer et al., 2002).

#### **RNA isolation, reverse transcription PCR (RT-PCR) and quantitative real-time PCR**

**(qRT-PCR).** *L. monocytogenes* strain H7550-Cd<sup>s</sup> was grown on TSBYE at 4, 8, 25, and 37°C (37°C for 7 h; 25°C for 14 h, 8°C for 72 h and 4°C for 168 h) until mid to late logarithmic phase in BHI (OD<sub>600</sub>, 0.7-0.9, measured using a spectrophotometer [SmartSpec 3000, Bio-Rad, Hercules, CA]). Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI) and subjected to DNase treatment using Turbo DNA free (Ambion,

Austin, TX). Concentration of RNA was determined by measuring absorbance at 260nm (Nanodrop, Wilmington, DE). Transcriptional assessments were made relatively to *spoVG*, used as reference; previous studies indicated that expression of this gene was constitutive in *L. monocytogenes*, including at low temperature (Elhanafi et al., 2010; Liu and Ream, 2008).

To produce cDNA, 200ng of total RNA and the ImProm-II<sup>TM</sup> Reverse Transcription System (Promega) were used according to the manufacturer's instructions. RNA was stored at -80°C when necessary. Self-priming controls (reverse transcription without added gene-specific primer), controls with total RNA as template (to exclude presence of contaminating DNA) and controls without RNA were included. Gene-specific cDNA was reverse transcribed using CompL2p\_2753-R for the putative endonuclease (ORF 2753), Delcon54-R for the putative methyl transferase (ORF 2754), and s2 for housekeeping gene *spoVG* (Liu and Ream, 2008) (Table 2). PCR used cDNA as template and primers CompL2p\_2753-F and CompL2p\_2753-R for ORF 2753 cDNA, Delcon54-F and Delcon54-R for ORF 2754 cDNA, and s1-s2 for *spoVG* cDNA (Table 2; Elhanafi et al., 2010). Fold increases were determined using band quantifications obtained with the image processing software ImageJ (<http://rsbweb.nih.gov/ij/>) as described (Elhanafi et al., 2010).

The mRNA expression levels of ORF 2753 were also analyzed by qRT-PCR using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) as per manufacturer's instructions. Gene-specific TaqMan probe and primer sets were designed using AlleleID 7.0 software (Premier Biosoft, Palo Alto, CA). The gene expression mix employed 18µM forward primers (q2753\_F and qspoVG\_F) and reverse primers (q2753\_R and qspoVG\_R), and 5µM Taqman probe (q2753\_P and qspoVG\_P) (Table 2).

Each reaction (20  $\mu$ L total volume) included 1  $\mu$ L of gene expression mix, 10 $\mu$ L of Taqman Fast Universal PCR Master Mix (2X), No AmpErase UNG (Applied Biosystems), and 1  $\mu$ L of cDNA. Thermocycling parameters were 95°C for 20 sec followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Fluorescence measurements were collected every cycle during the extension step (60°C). Cycle threshold (Ct) values were calculated for each sample corresponding to the cycle with maximal amplification rate. Three technical replicates were included per cDNA sample, and the experiments were done independently at least three times.

**Statistics.** The qRT-PCR experiments used a randomized complete block design, with biological replicates as complete blocks. The Ct values of ORF 2753 for each treatment were normalized to that of *spoVG* and were designated as  $\Delta$ Ct. The fold change in expression of ORF 2753 per temperature treatment was expressed relative to 37°C and was designated as  $\Delta\Delta$ Ct. Fold change was described as a log<sub>2</sub> transformation of  $\Delta\Delta$ Ct values assuming a gene amplification efficiency for ORF 2753 and *spoVG* as 2 (Pfaffl, 2001). The log fold change values were analyzed with a linear model using the GLM procedure of the SAS statistical software (SAS, Cary, NC).

## RESULTS

***Mariner-based* transposon insertion in a putative restriction endonuclease gene abolishes temperature-dependent phage resistance.** To identify genes responsible for temperature-dependent phage resistance of ECII strains, a *mariner*-based transposon library was constructed in H7550-Cd<sup>s</sup>. The frequency of integration of the transposon into the genome of this strain was 92 % (1,906 of 2,080 tested colonies).

The phage spot assay was used to screen the mutants for susceptibility to phage 20422-1 following growth of the bacteria at 25 and 37°C, and one mutant (J46C) was found to be susceptible to the phage following growth at either temperature. Phage susceptibility of J46C following growth at either 25 or 37°C was confirmed by standard plaque assays (Table 3). Sequencing of the insertion site revealed that the transposon was inserted in the chromosomal locus Lmoh7858\_2753 (ORF 2753; 1191 nts) (Fig. 1A). Southern blots using a probe derived from ORF 2753 confirmed the location of the transposon in this coding sequence. Furthermore, southern blots using the *ermC* transposon-specific probe showed that J46C harbored a single copy of the transposon (data not shown).

Nucleotide sequences with significant homology to ORF 2753 were not identified in the NCBI database. However, the deduced polypeptide was similar (40-42% identity) to putative restriction endonucleases from *Erwinia* spp. (including *E. amylovora* and *E. pylorifora*) and *Lactobacillus ruminis*. Lower similarity was also found with restriction endonucleases from numerous other bacteria. Three sets of inverted repeats were identified at the 5' end of ORF 2753, within 79 nts downstream of the start codon (Fig. 1B).

Immediately upstream of ORF 2753, and overlapping with it by 14 nts, was Lmoh7858\_2754 (ORF 2754) encoding a putative adenine-specific DNA methyltransferase with homologs in numerous bacteria, including *E. amylovora*, *E. pylorifora* and *L. ruminis* (59-62% aa identity). As with ORF 2753, nucleotide sequences with significant homology to ORF 2754 were not identified in the NCBI database. Interestingly, the putative methyltransferase ORFs in *Erwinia* spp. and *L. ruminis* were adjacent to the ORFs encoding the cognate endonucleases described above, suggesting that the genes were members of homologous cassettes both in

these organisms and in *L. monocytogenes* H7858. These findings suggested that ORF 2753 and ORF 2754 are members of a type II restriction modification (RM) system, designated LmoH7 after the designation of the strain (*L. monocytogenes* H7858) that was first found to harbor this system.

The ORF upstream of ORF 2754 (Lmoh7858\_2755; ORF 2755) belonged to the helix-turn-helix DNA-binding xenobiotic response element family (XRE) of transcriptional regulators (accession number: cl09100), and may correspond to the regulatory control (C) protein associated with several RM systems (Knowle et al., 2005; McGeehan et al., 2011; Rimseliene et al., 1995; Sorokin et al., 2009; Vijesurier et al., 2000; Yildirim et al., 2010). The GC content of ORF 2753, ORF 2754 and ORF 2755 was 31-32 %, noticeably lower than the average for the genome of *L. monocytogenes* H7858 (38 %) (Nelson et al., 2004). Size of the deduced C protein (83 amino acids) is within the range of other C proteins (Sorokin et al., 2009).

Comparative analysis of the region harboring ORF 2753-ORF 2755 revealed that these three ORFs were the proximal members of a genomic island (12 ORFs) in the chromosome of the ECII strain H7858 (Nelson et al., 2004; Fig. 1A). In a previous study this entire genomic island was shown to be ECII-specific (Cheng et al., 2010). This ECII-specific region was flanked by Lmoh7858\_2752 and Lmoh7858\_2765 that were conserved among different *L. monocytogenes* strains and encode a putative lipoprotein and ribosomal protein S9, respectively (Fig. 1A). A set of direct repeats (5'TATATCAAC/TGTTT3' and 5'TTGAACCAAC/TTTGA/CACCAAAA3') were found in the intergenic space between ORF 2753 and the putative lipoprotein gene (ORF 2752) as well as in the intergenic space

between ORF 2764 and the putative ribosomal protein S9 gene *Lmoh7858\_2765* (ORF 2765) (Fig. 1A).

The last ORF (ORF 2764) in this ECII-specific genomic island was annotated as a putative site-specific phage integrase protein (tyrosine recombinase family; pfam00589), suggesting a prophage origin for this ECII-specific gene cluster. In addition to ORF 2753- ORF 2755 discussed above, most of the other ORFs in this ECII-specific region had GC content lower than average for the chromosome (Fig. 1A).

To confirm the role of ORF 2753 in temperature-dependent phage resistance of ECII strains, an in-frame deletion in this ORF was constructed in H7550-Cd<sup>s</sup> and the deletion mutant was designated J25FII-7. The deletion was confirmed by PCR and DNA sequencing (data not shown). The deletion mutant was indistinguishable from J46C in being susceptible to phage when grown at either 25 or 37°C (Table 3).

ECII bacteria grown at 25°C were found to be susceptible to phage that had been propagated in J46C or J25FII-7, regardless of the temperature of growth for these mutant strains. The same results were obtained with phage propagated in the parental strain H7550-Cd<sup>s</sup> grown at 37°C (Table 4). These findings suggested that DNA was modified via a DNA methyltransferase (ORF 2754) expressed at both 25 and 37°C, while the cognate restriction endonuclease (ORF 2753) was expressed at 25°C but not at 37°C.

**ORF 2753 (putative restriction endonuclease) exhibits enhanced transcription at low temperatures ( $\leq 25$  °C) while ORF 2754 (putative methyltransferase) is constitutively expressed.** To characterize the impact of growth temperature on expression of ORF 2753 we determined transcript levels at 4, 8, 25 and 37°C using RT-PCR with *spoVG* as reference

gene. A temperature-associated gradient in transcript levels of ORF 2753 was observed, with the highest levels observed at 4°C while expression at 37°C could not be detected (Fig. 2). In contrast, ORF 2754 transcript levels were not significantly impacted by growth temperature (Fig. 2).

The effect of temperature on expression level of ORF 2753 was also quantified using qRT-PCR with *spoVG* as an internal control. At 4, 8 and 25°C transcript levels were approx. 16, 8 and 4 times higher than at 37°C. Significantly higher transcript levels of ORF 2753 (relatively to *spoVG*) were noted when bacteria were grown at 4°C than following growth at 25°C ( $p=0.0150$ ; S.E. 0.53;  $df=4$ ) (Fig. 3).

RT-PCR data suggested that when the bacteria were grown at low temperature ORF 2753 was co-transcribed with ORF 2754 and ORF 2755 but not with the upstream ORF 2756, which thus appeared to be a part of a transcriptional unit distinct from that of the LmoH7 system (ORF 2753 – ORF 2755) (Fig. 4). A putative Rho-independent transcriptional terminator ( $\Delta G=-12.3$  kcal/mol) was identified downstream (242 nts) of the ORF 2753 stop codon (Fig. 1A).

**The ORF 2753-ORF 2755 RM system (LmoH7) has specificity for GTATCC (N6/5).**

RM systems homologous to ORF 2753 and ORF 2754 included some with specificity for GGTGA(N)8 and CATG. However, genomic DNA of H7550 was found to be readily digestible with restriction enzymes HphI and NlaIII, specific for GGTGA(N)8 and CATG, respectively (data not shown), suggesting that these sites were not methylated and that LmoH7 had a different recognition site. On the other hand, recent information on RM systems of *Listeria* spp. in REBASE indicated GTATCC (N6/5) as the recognition site for the

putative methyltransferase encoded by ORF 2754 (<http://rebase.neb.com/rebase/rebase.html>).

Indeed, genomic DNA from H7550 grown at either 25 or 37°C was resistant to digestion by BfuI, a restriction enzyme specific for GTATCC (N6/5) (Fig. 5). Identical results were obtained with a panel of eight additional ECII strains of environmental or clinical origin (Table 1). In all cases, genomic DNA of the bacteria was resistant to BfuI (data not shown).

As mentioned earlier, ECII strains exhibit temperature-dependent resistance not only to phage 20422-1 but to all other tested phages (Kim et al., 2008). The sequenced genome of A511 (accession no. NC\_009811) and P100 (accession no. DQ004855) contained numerous BfuI recognition sites (70 and 66, respectively, corresponding to 0.51 and 0.50 sites per kb, respectively), distributed throughout the phage genome (data not shown).

In certain RM systems, methylation of sites in the promoter region of the genes has been shown to impact gene expression (Nagornykh et al., 2008). However, no BfuI recognition sites were found upstream of ORF 2755 or anywhere else in the ECII-specific cassette harboring the putative RM system.

**Heterologous expression of the putative methyltransferase.** Chromosomal integration of ORFs 2754, 2755, and the intergenic region upstream of ORF 2755 into the non-pathogenic strains *L. innocua* CLIP11262 and *L. welshimeri* L1225 resulted in CLIP11262pPLmet and L1225pPLmet, respectively. Genomic DNA of CLIP11262pPLmet and L1225pPLmet was found resistant to BfuI digestion, whereas DNA from the parental strains was readily digested by BfuI (Fig. 6). In addition to demonstrating that ORF 2754 functions as a methyltransferase for GTATCC (N6/5) sites, these findings also suggest the presence of a promoter in the intergenic region upstream of ORF 2755.

**Temperature-dependent phage resistance is restored in genetically complemented ORF 2753 mutants.** Integration of pPLØ (pPL2 with the ca. 4.0 kb PCR fragment including ORFs 2755, 2754 and 2753, and the upstream and downstream intergenic regions) in the chromosome of J46C and J25FII-7 resulted in strains J46CcompØ and J25FII-7compØ, respectively. PCR using primers NC16 and PL95 confirmed the integration (data not shown). Resistance to phage following growth at 25°C was partially restored in both complemented mutants (Table 3).

## DISCUSSION

In this study, we have shown that insertional inactivation or deletion of ORF 2753, encoding a putative restriction endonuclease specific for GTATCC (N6/5), abolished the ability of *L. monocytogenes* ECII to be resistant to phage following growth at low temperatures ( $\leq 30^{\circ}\text{C}$ ). The restriction endonuclease encoded by ORF 2753 was accompanied by a DNA methyltransferase and a putative regulatory C protein (ORF 2754 and ORF 2755, respectively). The role of ORF 2754 in methylation of GTATCC (N6/5) sites was confirmed by the fact that cloning of ORF 2754 and ORF 2755 in heterologous hosts (*L. innocua* and *L. welshimeri*) rendered the genomic DNA of these strains resistant to BfuI. The findings suggest that ORF 2753 – ORF 2755 constitute a novel type II RM system (LmoH7) harbored by *L. monocytogenes* ECII. Earlier genomic analysis of *L. monocytogenes* strains suggested that these ORFs were part of a genomic island (12 ORFs totally) unique to ECII strains (Cheng et al., 2010). This is in agreement with the fact that temperature-dependent phage resistance was a characteristic of all tested ECII strains but was not encountered among other strains of *L. monocytogenes* (Kim et al., 2008).

The mechanisms responsible for the presence of this genomic island in the chromosome of ECII strains remain unknown. RM systems are known for their ability to disseminate among bacterial genomes (Furuta et al., 2010; Jeltsch and Pingoud, 1996). It is possible that the genomic island harboring LmoH7 was acquired by an ancestral ECII lineage via horizontal gene transfer from an unidentified source and maintained in ECII strains, as phage resistance genes can confer fitness advantages to the bacteria that harbor them. Horizontal gene transfer is supported by the unusually low GC content of the LmoH7 ORFs and several other ORFs in this genomic island. *In silico* analysis revealed a putative site-specific phage integrase as the last gene of the ECII-specific genomic island. Phage integrases can mediate site-specific DNA recombination using specific DNA recognition sequences such as inverted or direct repeats (Groth and Calos, 2004). It is tempting to speculate that one or both of the two sets of direct repeats in the intergenic regions flanking the ECII-specific genomic island (Fig. 1A) may have facilitated the integration of the island at this locus in the chromosome of *L. monocytogenes* ECII.

LmoH7 fits the criteria of a typical type-II restriction system specific for GTATCC (N6/5). The relatively high number (>60) of GTATCC sites in the genome of virulent phages (e.g. A511 and P100) suggests that unmodified DNA of such phage would be highly vulnerable to digestion following injection into ECII cells. This may explain the finding that 25°C-grown cells remained resistant to phage even when infections and subsequent plaque formation were performed at 37°C. Restriction endonuclease produced during growth at 25°C was apparently sufficient to destroy the injected phage DNA before it could become methylated. It was

earlier shown that 25°C-grown cells of H7550 remained resistant to phage for up to 5.5 h following transfer to 37°C (Kim et al., 2008).

LmoH7 appears to be critical for the temperature-dependent phage resistance phenotype of ECII, since inactivation or deletion of ORF 2753 (putative restriction endonuclease) rendered the bacteria susceptible to phage infection regardless of growth temperature, and the phenotype was at least partially restored in genetically complemented mutants. Constitutive expression of the putative methyltransferase (ORF 2754) at 37°C and at  $\leq 25^\circ\text{C}$  was suggested by transcriptional data as well as by the BfuI restriction assays which showed that DNA of cells grown at either 25 or 37°C was resistant to BfuI, and thus expected to be methylated at GTATCC sites. In contrast, the putative restriction endonuclease (ORF 2753) was expressed at low temperature ( $\leq 25^\circ\text{C}$ ) but down-regulated at 37°C, in agreement with the observed resistance of  $\leq 25^\circ\text{C}$ -grown bacteria to phage.

In *Listeria*, temperature-dependent phage resistance with bacteria being resistant to phage when grown at low temperature has only been encountered in ECII strains (Kim et al., 2008). Temperature-dependent resistance to phage that is mediated by thermoregulated RM systems has been rarely reported in other bacterial systems. A plasmid (pNP40)-associated RM system (LlaJ1) was shown to mediate temperature-dependent resistance of *Lactococcus lactis* to phage, with resistance being most pronounced when the plaque assays were performed at 19°C and declining with assays done at either lower or higher temperatures (O'Driscoll et al., 2004). The genetic components, sequence and recognition site of LlaJ1 are unrelated to those of LmoH7; furthermore, phage resistance mediated by LlaJ1 depended on temperature during plaque assays. In contrast, in ECII strains resistance to phage was dependent on the

temperature of growth of the bacteria and was not impacted by the temperature during infection and subsequent plaque formation (Kim et al., 2008).

The fact that ECII grown at 25°C remained resistant to phage even when shifted to 37°C for infection and plaque forming assays can be attributed to pre-formed restriction endonuclease, as discussed earlier. On the other hand, when infection and plaque forming assays of 37°C-grown cells were done at 25°C the bacteria remained phage-susceptible, and plaques were readily formed (Kim et al., 2008). These findings suggest that phage DNA injected into the cells became methylated before sufficient restriction endonuclease could be synthesized.

The mechanisms responsible for expression of ORF 2753 at low temperature and down-regulation at 37°C remain to be elucidated. Our findings indicated that ORF 2753 was co-transcribed with ORF 2754 (the putative MT) and ORF 2755 (putative C protein) at 4, 8, and 25°C), but transcript levels of ORF 2753, the last gene in this operon, were low or undetectable at 37°C. This could be mediated by several mechanisms, including inhibition of transcription of ORF 2753 at 37°C (but not  $\leq 25^\circ\text{C}$ ) or a highly unstable message at 37°C.

One possible scenario for inhibition of transcription may involve the binding of a repressor (possibly the product of ORF 2755) at one or more of the three sets of palindromic sequences identified in the 5' of ORF 2753 (Fig. 1B). One of these palindromes (AAAATAAC-N13-GTTATTTTT) is highly AT-rich, suggesting the possibility that secondary structure may be especially susceptible to changes in temperature, allowing binding of a putative repressor at 37°C but not at  $\leq 25^\circ\text{C}$ . Binding of C-protein to the 5' end of RE would interrupt ORF 2753 transcription without affecting transcription of the putative methyltransferase. The region containing the operators for the C-protein in another RM system was recently also shown to

be rich in polyA and polyT tracts (McGeehan et al., 2011). Further studies are needed to identify the binding sites for the putative C-protein of LmoH7, and to assess the potential roles of the observed AT-rich palindromes.

In *L. monocytogenes* the extensively documented down-regulation of flagellar genes at 37°C (Grundling et al., 2004; Kamp and Higgins, 2009; Mauder et al., 2008; Peel et al., 1998; Shen and Higgins, 2006) has been shown to be mediated by the binding of a repressor (MogR) to AT-rich palindromic sequences (TTTT-N5-AAAA) in promoter regions so that transcription initiation was prevented at 37°C (Shen and Higgins, 2006). The impact of temperature on nucleic acid secondary structure has also been shown to be responsible (at the RNA level) for thermoregulated expression of the key virulence regulator *prfA*, expressed at 37°C but not below 30°C (Jphansson et al., 2002; Loh et al., 2009). Further studies are needed to characterize the mechanism mediating thermoregulation of ORF 2753.

Transcription of ORF 2755 prior to ORF 2754 and ORF 2753 may represent an inherent regulatory mechanism as described in other RM systems (Mruk et al., 2007). Regulatory proteins may delay the transcription of the restriction endonuclease so that the cognate methyltransferase has time to modify and thereby protect the host's own DNA (Knowle et al., 2005). Such a mechanism would be crucial during the establishment of the RM system in a new host.

*L. monocytogenes* has the ability to proliferate at low temperature and this trait is especially relevant in natural ecosystems as well as in food processing plants and foods themselves, where low temperatures often prevail. Listeriophages have been approved as biocontrol agents for foods (Mahony et al., 2011). However, it is important to keep in mind that *L.*

*monocytogenes* ECII strains (implicated in two multistate listeriosis outbreaks in the U.S.) may have a distinct fitness advantage in the presence of phage in such environments. Under low temperature conditions, use of phage as biocontrol may inadvertently select for ECII strains if these are present in a mixture with other listeriae.

In conclusion, we have described a temperature-regulated type-II RM system (LmoH7) responsible for the low temperature-dependent phage resistance of *L. monocytogenes* ECII strains. This phenotype of ECII strains and LmoH7 that mediates it should be considered in future listeriophage applications. Based on the findings, we propose the use of restriction endonuclease-deficient mutants such as J46C or J25FII-7 as phage propagating strains since this would mitigate the risk of selection for ECII in foods and the food processing plant environment. Further studies are needed to elucidate the molecular mechanism responsible for thermoregulation of the LmoH7 restriction and to understand the role of this RM system in the ecology and possibly virulence of *L. monocytogenes* ECII.

#### ACKNOWLEDGMENTS

This work was partially supported by a grant from the American Meat Institute Foundation and USDA grant 2006-35201-17377. We thank Dr. H. Marquis for the gift of pMC39. We are grateful to R. M. Siletsky for assistance and thankful to all other members of our laboratory for their support and encouragement.

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**Table 1.** Bacterial strains used in this study.

<b>Strains</b>	<b>Relevant genotype and features</b>	<b>Source/ Reference</b>
<i>L. monocytogenes</i>		
F2365	California outbreak (1985)	Nelson et al., 2004
H7550	ECII strain, hot dog outbreak (1998 – 1999)	Evans et al., 2004
H7550-Cd <sup>s</sup>	Cadmium-sensitive derivative of H7550 cured of pLM80	Elhanafi et al., 2010
DP-L862	Phage propagating strain	Kim et al., 2008
J1735	ECII strain, 2002 outbreak	Kathariou et al., 2006
J46C	ORF2753:: <i>mariner</i> based transposon mutant of H7550-Cd <sup>s</sup>	This study
J25FII-7	ORF2753 deletion mutant of H7550-Cd <sup>s</sup>	This study
J46C compØ	J46C with chromosomal insertion of pPLØ	This study
J25FII-7 compØ	J25FII-7 with chromosomal insertion of pPLØ	This study
SK2688	ECII strain, processing plant environment (2005)	E. Ryser
L0928	ECII strain, processing plant environment (2004)	Eifert et al., 2005
SK1106	ECII strain, processing plant environment (2003)	
34-6a	ECII strain, processing plant environment (2003)	Eifert et al., 2005
J3033	ECII strain, human listeriosis (2004)	Sperry et al., 2008
J1925	ECII strain, deli meat (2002)	Sperry et al., 2008
J3200	ECII strain, human listeriosis (2004)	Sperry et al., 2008
H6383	ECII strain, human listeriosis (1996)	Sperry et al., 2008
<b>Non-pathogenic</b>		
<i>Listeria</i>		
<i>L. innocua</i>		
CLIP11262		Glaser et al., 2001

Table 1. continued

<i>L. welshimeri</i>		J. Eifert
L1225		
CLIP11262pPLmet	CLIP11262 with chromosomal insertion of pPLmet	This study
L1225pPLmet	L1225 with chromosomal insertion of pPLmet	This study
<b><i>Escherichia coli</i></b>		
DH5 $\alpha$	F- $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>phoA</i> supE44 <i>thi-1 gyrA96 relA1</i> $\lambda$ -	Invitrogen
S17-1	Conjugation donor	Simon et al., 1983
DH5 $\alpha$ comp $\emptyset$	DH5 $\alpha$ with pPL $\emptyset$	This study
S17-1 comp $\emptyset$	S17-1 with pPL $\emptyset$	This study

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**Table 2.** Oligonucleotides used in this study

Name	Sequence (5' to 3')*
Delcon54-F:	GCTT <u>GAGCTC</u> TAATCTATATCCAAAGAATCATT
Delcon54-R:	GCTT <u>CTGCAG</u> TTATTCATGATAAGTCATAGTTTATTAT
Delcon52-F:	CATA <u>CTGCAG</u> TTAAGTATAAATGAGCGTATATATAC
Delcon52-R:	CATA <u>GGATCC</u> TAACAGAATTAGCACGAGAT
CompL2P_2753-F:	CTA <u>ACCCGGG</u> ATGACTTATCATGAAGTTC
CompL2p_2753-R:	GTAAG <u>GAGCTC</u> CTTAAATAGTAATATAGTTTGAAGC
Comp2752_2R:	GACT <u>GAGCTC</u> GGTTTATGTTACACAAGCAAAGTG
comp2755F:	GACT <u>CCCGGG</u> CTAGCCTGAAAGAAAGAGGCT
comp2753R:	GACT <u>GAGCTC</u> GTTACCTTCTACAA ACATCTG
2755_F:	TGGGCATCAGTTATAAACCCCTTA
2756_F:	CGA ACA AATTATTAG AGA AGCAGAGGAA
q2753_F:	AATCGTTTGTGTTAAATACTGTTC
q2753_R:	CCCAAGAAGGAAATAGAGAGAC
q2753_P:	AATTCAACACCTGCTGTAGCCATC (Modification:5' FAM and 3' BHQ1)
qspoVG_F:	GCAAGTAACAGATGTGAGATTAC
qspoVG_R:	GCGAATATCATGAACTACAAATTC
qspoVG_P:	CAATCGCTCTCATTCTTCCATCAGT (Modification:5'FAM and 3' BHQ1)

\* underlined sequences correspond to restriction enzyme sites: GAGCTC, SacI; CTGCAG, PstI; GGATCC, BamHI; CCCGGG, XmaI.

**Table 3.** Restoration of temperature-dependent phage resistance in genetically complemented ORF 2753 mutants

<b>Strain</b>	<b>EOP<sup>1</sup></b>
H7550-Cd <sup>s</sup>	$2.76 \times 10^{-5}$
J46C	0.92
J25FII7	1.24
J46C compØ	$1.43 \times 10^{-2}$
J25FII7 compØ	$1.18 \times 10^{-2}$

<sup>1</sup> EOP, Efficiency of plaquing, defined as described before (Kim et al., 2008) as the ratio of plaques obtained with the indicated strain grown at 25°C over plaques obtained with the same bacterial strain grown at 37°C. Bacteria were infected with listeriophage 20422-1 (propagated in *L. monocytogenes* DP-L862) as described before (Kim et al., 2008). Results represent average of duplicates from two independent experiments.

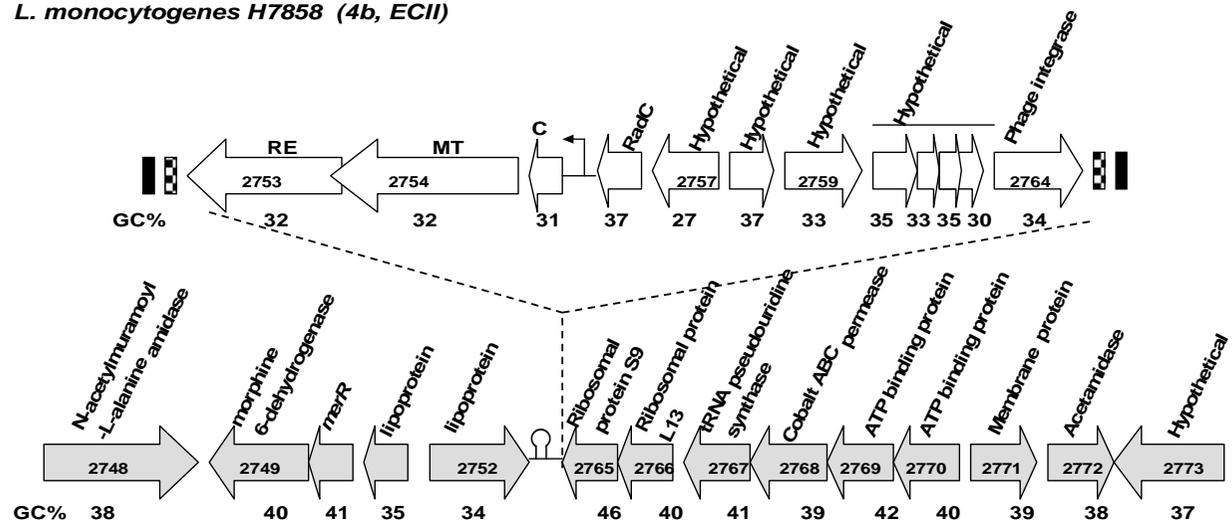
**Table 4.** Host-dependent modification of phage 20422-1 in ECII H7550-Cd<sup>s</sup> and mutant derivatives grown at different temperatures.

Propagating strain/ temperature	EOP <sup>1</sup>
DP-L862 / 37°C	$< 9.0 \times 10^{-6}$
H7550-Cd <sup>s</sup> / 37°C	$3.2 \times 10^{-1}$
J46C / 37°C	$7.7 \times 10^{-1}$
J25FII-7 / 37°C	1.6
J46C / 25°C	1.2
J25FII-7 / 25°C	2.7

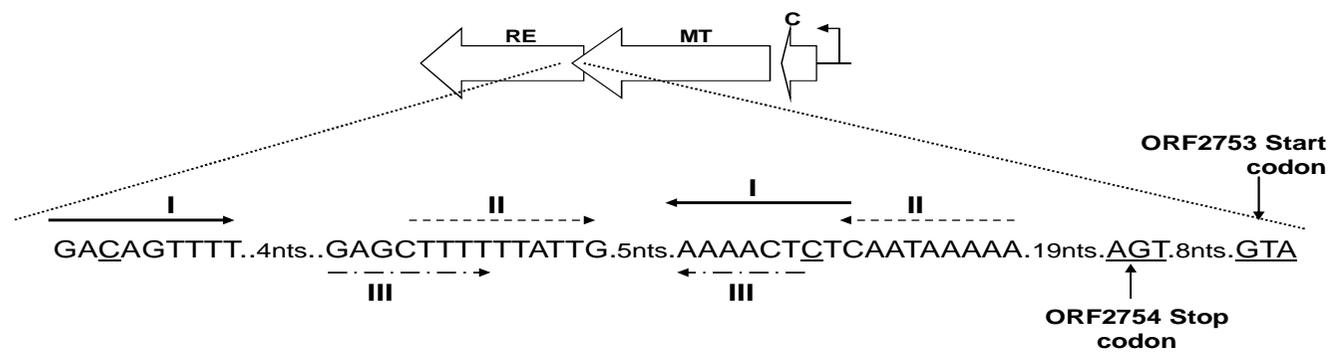
<sup>1</sup> EOP, Efficiency of plaquing, defined as ratio of plaques formed with *L. monocytogenes* ECII strain J1735 grown at 25°C over plaques formed with J1735 grown at 37°C. Results represent average of duplicates from one representative experiment.

**Figure 1. A.** Comparative genomic organization of the region harboring the ORF 2753-2755 RM system in *L. monocytogenes* strain H7858 (ECII, serotype 4b), F2365 (ECI, serotype 4b) and EGD-e (serotype 1/2a). Direction of arrows indicating direction of transcription of the corresponding ORF. Solid and mosaic rectangular boxes in H7858 refer to direct repeats with consensus sequences 5'TATATCAAC/TGTTT3' and 5'TTGAACCAAC/TTTGA/CACCAAAA3', respectively. **B.** Inverted repeats within the 5' portion of ORF 2753. Three sets of inverted repeats are designated I, II and III. RE, MT and C correspond to putative restriction endonuclease, methyltransferase and C-protein, respectively. Lollipop symbol between ORF 2752 and ORF 2753 indicates putative rho-independent terminator.

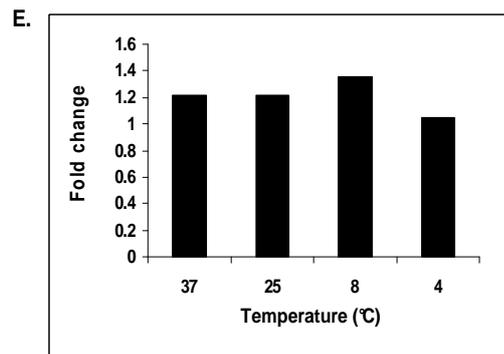
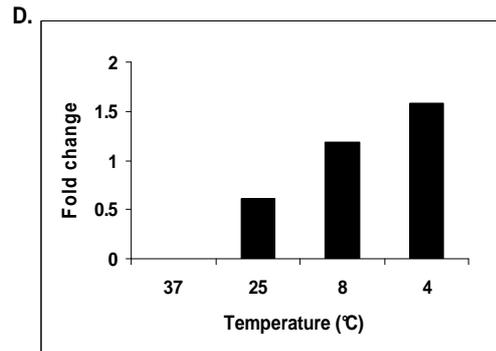
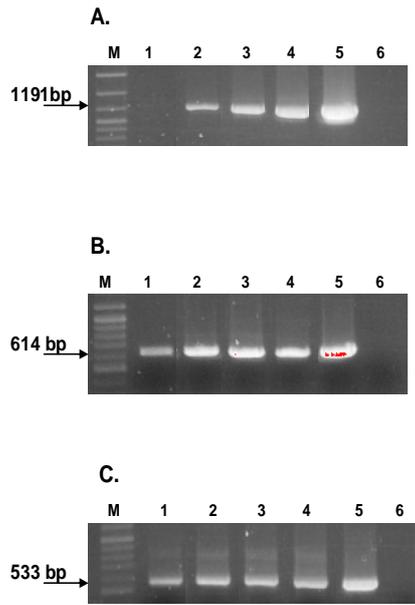
A.

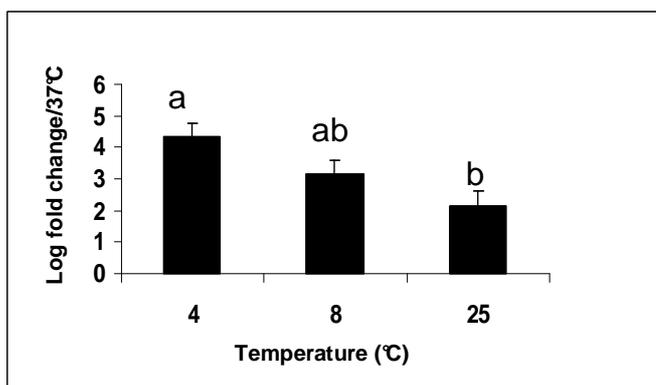
*L. monocytogenes* H7858 (4b, ECII)*L. monocytogenes* EGDe (1/2a)*L. monocytogenes* F2365 (4b, ECI)

B.

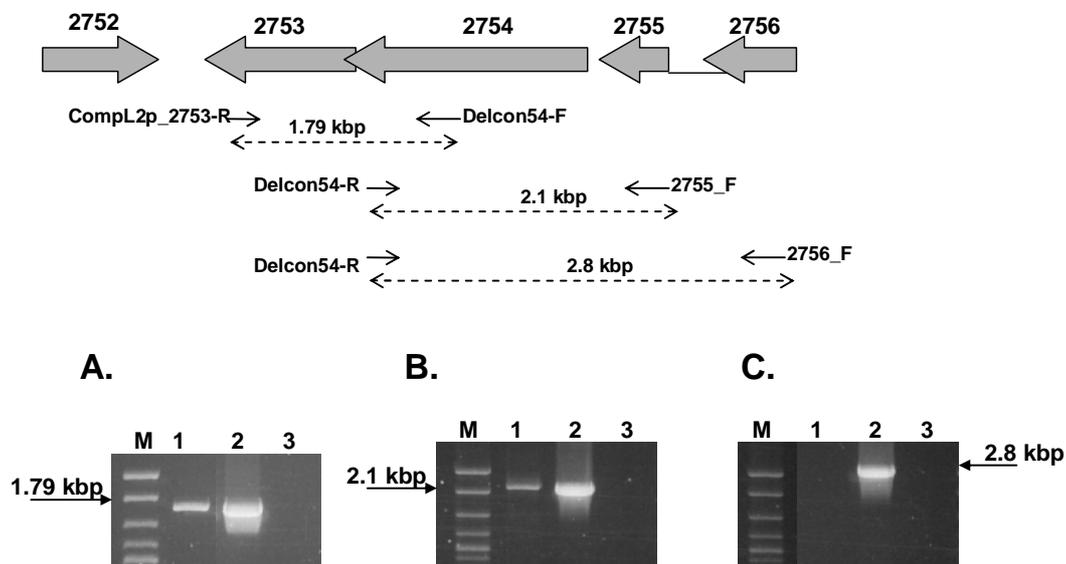


**Figure 2.** Effect of temperature on expression of ORF 2753 and ORF 2754 in *L. monocytogenes* strain H7550-Cd<sup>s</sup>. Total RNA was reverse transcribed into cDNA using primer Compl2p\_2753-R and PCR was performed using primers Compl2p\_2753-F and Compl2p\_2753-R to assess expression of ORF 2753 (**A**), and primers Delcon-F and Delcon-R for expression of ORF 2754 (**B**). For expression of *spoVG* (**C**), total RNA was reverse transcribed into cDNA using primer s2 and PCR was performed using primers s1 and s2. Arrows indicate expected amplicons. Lanes 1-4, RNA extracted from cells grown at 37, 25, 8, and 4°C, respectively. Lanes 5 and 6, H7550 genomic DNA and total RNA, respectively, used as positive and negative controls for RT-PCR. M, 100 bp - 2686 bp DNA molecular marker XIV (Roche, Indianapolis, IN). **D** and **E**, change in expression of ORF 2753 and ORF 2754, respectively, quantified by ImageJ. Fold change was calculated upon normalization with *spoVG* expression values at the respective temperatures as described in Materials and Methods. Data were from one of three independent trials.

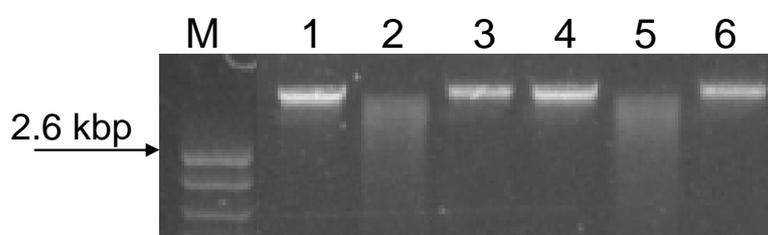




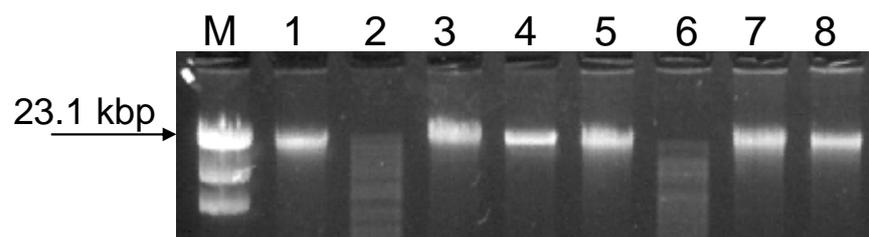
**Figure 3.** Effect of temperature on expression of ORF 2753, analyzed using qRT-PCR. Log fold change in ORF 2753 expression at 4, 8, and 25°C is indicated relative to 37°C. Different letters indicate significant differences ( $p < 0.05$ ).



**Figure 4.** Co-transcription of ORF 2753, ORF 2754 and ORF 2755 in *L. monocytogenes* H7550-Cd<sup>s</sup> grown at 8°C. Total RNA was reverse transcribed into cDNA using primer CompL2p\_2753-R and PCR was performed using primers Delcon54-F and CompL2p\_2753-R (A); 2755-F and Delcon54-R (B); 2756-F and Delcon54-R (C), Arrows indicated expected amplicons. Lanes: 1, H7550-Cd<sup>s</sup> grown at 8°C; 2 and 3, H7550-Cd<sup>s</sup> genomic DNA and total RNA, respectively, used as a positive and negative control, respectively. M, 100 bp - 2686 bp DNA molecular marker XIV (Roche).



**Figure 5.** Genomic DNA of *L. monocytogenes* H7550-Cd<sup>s</sup> is methylated at sites recognized by BfuI. Genomic DNA from *L. monocytogenes* strain H7550-Cd<sup>s</sup> grown at 25°C and 37°C without any digestion (lanes 1 and 4, respectively) following digestion with HindIII (lanes 2 and 5, respectively) and digested by BfuI (recognition site, at GTATCC (N6/5); lanes 3 and 6, respectively). M, 100 bp - 2686 bp DNA molecular marker XIV (Roche).



**Figure 6.** Heterologous expression of the putative methyltransferase in *L. innocua* and *L. welshimeri*. Lanes 1 and 2, uncut and BfuI-digested genomic DNA, respectively, from *L. welshimeri* strain L1225. Lanes 3 and 4, uncut and BfuI-digested genomic DNA, respectively, from L1225pPLmet. Lanes 5 and 6, uncut and BfuI-digested genomic DNA, respectively, from *L. innocua* CLIP11262. Lanes 7 and 8, uncut and BfuI-digested genomic DNA, respectively, from CLIP11262pPLmet. M, 564 bp - 23130 bp DNA molecular marker II (Roche).

**Chapter V: Tetrahymena-conditioned Medium Promotes Survival of *Listeria monocytogenes*.**

**Authors.**

Vikrant Dutta, and Sophia Kathariou

**Author's contribution.**

**VD:** Protozoa and *Listeria* co-culture trials, data analysis and manuscript writing; **SK:** mentor, data analysis, and manuscript writing

**Publication status.**

To be submitted to Food-borne Pathogens and Diseases.

## ABSTRACT

Environmental persistence of the ubiquitous food-borne pathogen *Listeria monocytogenes* is dependent on the interplay between various adaptive attributes. The comprehensive list of such attributes is evolving and we are only beginning to understand their nature. Free living protozoa have been hypothesized to serve as reservoirs for pathogenic bacteria. The underlying mechanisms governing protozoa-bacteria relationship are poorly understood. Existing reports describing *Listeria*-protozoa relationship have elucidated the role of intracellular niche within protozoa (*Tetrahymena* spp. and *Acanthamoeba* spp.) in protection of *L. monocytogenes*. In this study, several different strains of *L. monocytogenes* were indeed found to survive better in Osterhout's medium (a balanced salt solution) in the presence of the ciliate *Tetrahymena tropicalis* than in the absence of it. However, physical contact between *Tetrahymena* and *L. monocytogenes* was not required for the observed enhanced survival of the latter. Instead, *Tetrahymena*-conditioned medium was sufficient to confer enhanced survival to *L. monocytogenes*. The findings suggest that in the presence of *Tetrahymena tropicalis*, *L. monocytogenes* exhibits enhanced survival due to protective compound(s) produced by the ciliate and secreted into the medium. This mode of *Listeria* survival could work separately or in conjunction with previously hypothesized protective mechanisms associated with intracellular existence and replication of *Listeria* in the protozoa.

## INTRODUCTION

*Listeria monocytogenes* is a foodborne intracellular pathogen that has been associated with severe illness and death (mean mortality rate of 16%) in susceptible populations (Kathariou 2002, Painter and Slutsker 2007, Scallan et al 2011). *L. monocytogenes* is ubiquitous in

natural and man-made habitats (Norwood and Gilmour 1999, Kathariou 2002; Gandhi and Chikindas 2007, Freitag et al 2009). A number of physiological attributes, including cold tolerance, biofilm formation, disinfectant and phage resistance may contribute to the ability of *L. monocytogenes* to persist in the environment, including food processing plants (Kathariou 2002, Kornacki and Gurtler 2007; Gandhi and Chikindas 2007, Kim et al 2008; Elhanafi et al 2010). Nonetheless, our current understanding of the adaptations that may foster survival of *L. monocytogenes* in different environments remains limited.

Increasing evidence suggests a key role of protozoan hosts in the ecology and adaptations of bacterial pathogens (Molmeret et al 2005; Greub and Raoult, 2004). Studies with *Salmonella* showed that upon ingestion by protozoan hosts the bacteria were released in the form of excretory vesicles, and such microhabitats were speculated to protect bacteria from environmental stresses (Gourabathini et al., 2008). It has also been hypothesized that protozoa-bacterium interactions help bacterial pathogens in environments outside of animal hosts to maintain their virulence genes which may otherwise only be required for intracellular survival (Zhou et al., 2007; Cirillo et al., 1997).

*L. monocytogenes*-specific reservoirs have not been conclusively identified, and the organism is considered to fluctuate between a saprophytic lifestyle in natural environments and an intracellular lifestyle involving highly specialized adaptations related to pathogenesis (Fenlon 1999; Freitag et al 2009; Toledo-Arana et al 2009; Velge and Roche 2010).

Depending on the ambient environment, interaction of certain regulatory networks modulates *Listeria*'s ability to adapt to a pathogenic versus a saprophytic lifestyle (Toledo-Arana et al 2009). It is possible that *Listeria*-protozoa interactions contribute towards both the

intracellular and saprophytic life style of this pathogen. *L. monocytogenes* has been shown to survive ingestion by *Tetrahymena* and *Acanthamoeba* (Ly and Müller 1991; Akya et al 2007; Zhou et al 2007; Pushkareva and Elmoraeva 2010); however the survival reports of *L. monocytogenes* in *Acanthamoeba* have been ambiguous. On one hand reports by Akya et al have suggested that *L. monocytogenes* does not survive in *Acanthamoeba* and on the other hand Zhou et al has reported that *L. monocytogenes* can survive predation by *Acanthamoeba* (Akya et al 2007, Zhou et al 2007). Irrespective of the ambiguity and the host used, all but one study (Ly and Müller 1991) suggests that the protozoan intracellular environment is deleterious to survival of *Listeria* sp. (Gourabthini, et al 2008; Zhou et al 2007; Akya et al., 2007).

Even though previous findings have indicated a fostering affect of *Acanthamoeba*-extracellular medium on the survival of *L. monocytogenes*, similar effect of a ciliate *Tetrahymena* extracellular medium on *Listeria* survival has not been explained (Huws et al 2008; Akya et al 2010; Zhou et al 2007).

The ubiquity of protozoa in nature and in food-related environments (Gourabathini et al 2008; Vaerewijck et al 2008, 2010, 2011; Baré et al 2011) suggests the need for better understanding of the outcome of *Listeria*-protozoa interactions on the survival of *Listeria*. Such understanding can prove useful towards control of this pathogen in food production and processing. In this study, we have assessed survival of *L. monocytogenes* in the presence of *Tetrahymena tropicalis* and further investigated the impact of this protozoan in promoting survival of this pathogen.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table 1. Bacteria were grown at the indicated temperatures in brain heart infusion (BHI) broth (Becton, Dickinson & Co. , NJ) or on agar media (BHI with 1.2% Bacto-agar, Becton, Dickinson & Co.). *Salmonella enterica* serovar Heidelberg was grown on XLD agar (Becton, Dickinson & Co.). Enumerations (cfu/ml) were done by plating dilutions on BHI agar and incubating at 37°C for 36h, as described (Azizoglu and Kathariou 2010).

***Tetrahymena* isolate and growth conditions.** *Tetrahymena tropicalis* (THM) used in this study was isolated from biofilm in a cooling tower (Berk et al 2008). THM was cultured and maintained as described (Berk et al 2008). The THM cultures were routinely examined using a phase contrast microscope (Leica, Wetzlar, Germany).

**Co-culture experiments.** Osterhout's solution (OHS), a balanced salt solution, was used as the standard medium for THM-bacteria co-cultures (Osterhout 1906; Berk et al 2008). OHS contained NaCl (420 mg/l), KCl (9.2 mg/l), CaCl<sub>2</sub> (4 mg/l), MgSO<sub>4</sub>·7H<sub>2</sub>O (16 mg/l), MgCl<sub>2</sub>·6H<sub>2</sub>O (34 mg/l) and Tris base (pH 7.0; 121 mg/l). OHS was filter-sterilized using a 0.2 µm filter (Nalgene, Rochester, NY). For bacterial washes the overnight culture (1 ml) was washed at 8000g for 2 min with 100% OHS and then resuspended in 1ml of 100% OHS. Overnight cultures of THM propagated at 30°C in plate count broth (PCB) were sequentially centrifuged at 700g for 10 min with 30%, 60% and 100% OHS. After the final wash, THM were resuspended in 1ml of 100% OHS, as described (Berk et al 2008).

For co-cultures, washed THM (100 µl) and washed bacteria (100 µl of a suspension with 10<sup>4</sup> cfu/ml with MOI ca. protozoa: *Listeria*=2:1) were added into 1 ml of OHS in the wells of a

24-well tissue culture plate (Corning, NY). Such mixtures were designated as “THM+bacteria”. For controls, washed bacteria (100 µl of a suspension with  $10^4$  cfu/ml) were inoculated separately in 1 ml of OHS per well and the treatments were designated as “bacteria only”. After inoculations the 24-well plates were incubated at 30°C. Bacterial enumerations (cfu/ml) were conducted at different time points over 6-7 days.

**Assessment of the affect of THM-conditioned medium on bacterial survival.** The effect of THM-conditioned medium was assessed for two *L. monocytogenes* strains, H7550 and 4b1. After co-incubation of washed bacteria and washed THM in OHS at 30°C for 48 h, the medium was aspirated using a sterile 10 ml syringe (Beckton and Dickinson & Co.). The medium was then filtered through 0.2 µm nylon filter (Fisherbrand, UK) and the filtrate was used immediately as a conditioned medium for inoculation of freshly grown and washed bacterial cells in a 24-well plate (Corning). For control, similarly prepared conditioned medium was obtained from suspensions of washed bacteria incubated alone (without THM) in OHS at 30°C for 48 h, and from THM incubated alone (without bacteria) in OHS at 30°C for 48 h. Conditioned OHS (1ml/well of 24-well plates) was inoculated with freshly grown (37°C overnight) bacteria (100 µl of a suspension with  $10^4$  cfu/ml washed bacteria) and incubated at 30°C for 6-7 days as described above. As control, regular OHS was similarly inoculated with washed bacteria and processed in parallel. Inoculations were done in duplicate and the experiments were done in at least two independent trials.

**Statistics.** The log conversions of the data were analyzed using SAS version 9.1.3 mixed model (SAS, Cary, NC).

## RESULTS and DISCUSSION

**Enhanced survival of *L. monocytogenes* strains in the presence of THM.** *L. monocytogenes* strains i.e. F2365, H7550, and 4b1 were used as a representative of three major epidemic clonal groups, ECI, ECII, and ECIIa (ECIV), respectively. These clonal groups have been implicated in the food-related listeriosis outbreaks in the U.S. (Cheng et al., 2008). Strain *L. innocua* CLIP lacks most of the virulence determinants and hence was used as a non-pathogenic *Listeria* representative strain. The survival of *Listeria* strains in THM+bacteria treatment was significantly higher than in the absence of THM (bacteria only treatment) ( $p \leq 0.001$ ). In THM+bacteria treatment, strains F2365, H7550, 4b1, and CLIP provided an evidence of growth during first 48hrs (increase in total Lm counts by ca. two log cfu/ml) following which *Listeria* maintained their survival with recorded one log cfu/ml decrease to no decrease in the total bacterial count during the remainder of experiment (5-6 days). On the contrary, in bacteria only treatment, a consistent decrease in cell count (four log reduction over 5 days) was observed for all tested strains (Fig. 1A).

To assess the role of protection from oxidative damage to be the possible cause for better survival of *L. monocytogenes* in presence of THM, assay using catalase deficient *L. monocytogenes* strain F2365 $\Delta$ kat (ROA3) was performed (Azizoglu and Kathariou 2010). The survival of ROA3 was not different from its wild type strain F2365 in THM+bacteria or bacteria only treatments (data not shown). The role of *L. monocytogenes* surface composition on its survival upon co-incubation with THM was assessed by using strain M44 (lacking a surface antigen, Promadej et al 1999). The results were same as for other *L. monocytogenes*

strains where M44 survived better in THM+bacteria treatment than in bacteria only treatment (Fig 1A;  $p \leq 0.001$ ).

These findings were similar to the previous studies where *Listeria* sp. has shown to maintain survival in the presence of *Tetrahymena* sp. (Ly and Müller 1991; Pushkareva and Ermolaeva 2010). During the first 48 hrs of Tetrahymena-*Listeria* co-culture, the increase in the *Listeria* sp. counts were similar (ca. two log cfu/ml increase) to previous report by Pushkareva and Ermolaeva (2010). However the trend was different than reported by Ly and Müller where in first 48 hrs of co-culture *Listeria* cell count decreased by about one log cfu/ml. In the absence of *Tetrahymena*, during the first 48 hrs of co-culture studies by both Ly and Müller (1991) and Pushkareva and Ermolaeva (2010) reported increase in *Listeria* counts. The ability of non-pathogenic *L. innocua* CLIP strain to survive similar to the virulent *L. monocytogenes* was also intriguing as this will indicate that survival in the presence of protozoan hosts does not require virulence genes. This was also different from previous report where hemolysin (LLO) produced by *L. monocytogenes* was reported to be essential for growth and survival of *L. monocytogenes* in the presence of *Tetrahymena* (Pushkareva and Ermolaeva 2010). Other reports for *L. monocytogenes* and *Salmonella* interactions with amoebic hosts have indicated that virulence factors do not contribute to the survival of bacteria in the presence of protozoan hosts (Zhou et al 2007; Tezcan-Merdol et al 2004).

These discrepancies could be attributed to the difference in methodology, where in previous studies the co-culture medium used was either tap water (Ly and Müller 1991) or Luria-Bertani broth (Pushkareva and Ermolaeva 2010) instead of OHS (a balanced salt solution) used in this study. Similarly use of different host, *T. pyriformis* instead of *T. tropicalis* (used

in this study), could also contribute to the difference in results (Pushkareva and Ermolaeva 2010). Temperature of Tetrahymena-Listeria co-culture used in this study (30°C) was also different than previous studies, where Ly and Müller (1991) used 36°C, Pushkareva and Ermolaeva (2010) used 28°C.

Furthermore growth temperature (as tested at 25 and 37°C) of *L. monocytogenes* strain 4b had no impact on its survival in the presence or absence of THM where strain 4b1 survival was significantly ( $p \leq 0.001$ ) greater in the presence of THM at both temperatures than in bacteria only treatment (Fig. 1B).

Overall these findings indicated no strain-specific tropism related to the survival of *Listeria* sp. in the presence of THM. This was particularly surprising for F2365 $\Delta$ kat which was a catalase deficient mutant and was expected to survive worse than its wild type strain F2365 because of its reduced ability to sustain oxidative damage (Azizoglu and Kathariou 2010). These findings could prove crucial to understand ecology of *Listeria* in the environment where protozoa-*Listeria* interactions are ubiquitous (Gourabathini et al 2008; Vaerewijck et al 2008, 2010, 2011; Baré et al 2011).

**Protective effect of THM was also seen for *Salmonella enterica* serovar Heidelberg.** SEH also provided an evidence for growth (increase by ca. 2 log cfu/ml) in THM+bacteria treatment during the first 48 hrs of co-incubation. After the initial increase, the total bacterial count was maintained around  $10^6$  cfu/ml over the duration of co-incubation (six days) and was significantly ( $p \leq 0.001$ ) higher from bacteria only treatment where total bacterial counts declined. In bacteria only treatment, over the duration of experiment total SEH count decreased by one log and at the end of the co-culture experiment, the bacterial cell count was

maintained ca.  $10^4$  cfu/ml (Fig 1C). It was interesting to note that the decline trend for SEH was less pronounced than *Listeria* spp. for same treatment (bacteria only). At the end of the assay (144 hrs) detectable numbers of SEH (ca.  $10^4$  cfu/ml) were observed compared to *Listeria* spp. where bacterial counts dropped to non-detectable levels.

***Tetrahymena* conditioned medium was enough to sustain *L. monocytogenes* survival.** *L. monocytogenes* strains H7550 and 4b1 survive similarly in the presence of THM conditioned medium, irrespective of whether the medium was obtained from just THM+bacteria, or THM alone treatments. The total bacterial counts for strains H7550 and 4b1 in conditioned medium obtained from THM only or THM+bacteria were significantly different from the treatments without THM conditioned filtrate (obtained from bacteria alone conditioned medium, or bacteria only treatment) ( $p \leq 0.001$ ). For strain H7550, in the presence of THM conditioned filtrate (obtained from THM only or THM+bacteria) bacterial counts were increased (by one log cfu/ml) during the first 48 hrs of incubation. For the rest of the experiment, cell counts were maintained at ca.  $10^5$  cfu/ml over the period of one week, whereas in the absence of THM conditioned filtrate, a consistent decline in the total bacterial counts was observed until bacterial count dropped below detectable levels in 120h (Fig 1 D). Similar results were obtained for strain 4b1 where the survival of bacteria was significantly higher in the presence THM conditioned media than in the absence of it (Fig 1E;  $p \leq 0.001$ ). Unlike H7550, after initial increase in the cell count (by one log cfu/ml), during first 48 hrs, reduction (by one log cfu/ml) in total bacterial count was recorded for strain 4b1 in the presence of THM conditioned media over one week such that the bacterial count at the end of experiment were same as at the beginning (ca. 4 log cfu/ml; Fig 1E). For both strains (H7550 and 4b1),

survival of bacteria in the presence of bacteria only treatment was significantly higher than in the conditioned filtrate obtained from the bacteria only treatment (Fig 1D and Fig 1E;  $p \leq 0.001$ ).

As per our knowledge this is a first report describing the fostering effect of extracellular medium obtained from *Tetrahymena* upon the survival of *L. monocytogenes*. Previously *Acanthamoeba* extracellular medium has shown to support *Listeria* survival (Huws et al 2008; Zhou et al 2007; Akya et al 2010). The findings from Huws et al (2008) were similar to our findings where the protective effect of *A. polyphaga*+ *L. monocytogenes* strain NCTC 11994 extracellular medium on strain NCTC 11994 cell count over the duration of 50 hrs was reported. However unlike our findings the effect of just *A. polyphaga* conditioned medium was not protective and was similar to the bacteria only treatment. Zhou et al., reported that upon using *A. castelleni* as the protozoan host, the intracellular *L. monocytogenes* count decreased (ca. one log cfu/ml) in first 48 hrs whereas total bacterial count was maintained, indicating that the extracellular medium was supporting the *Listeria* growth during first 48 hrs. Similar findings were also reported by Akya et al (2010) upon co-culture of *L. monocytogenes* strain DRDC8 with *A. polyphaga*, the total bacterial counts of bacteria were detectable by the end of the 5h experiment whereas intracellular bacterial count were reduced to non-detectable levels both at higher (37°C) and lower (22°C) co-culture incubation temperature. As mentioned above the duration of experiment was only 5 hrs compared to 96 hrs in our experiment and from the previous report by Zhou et al (2007). Akya et al (2010) also monitored the protective effect of *A. polyphaga* conditioned medium (ACM) on the survival of *L. monocytogenes* strain DRDC8 over the duration of 96 hrs but at 15°C co-culture

incubation temperature; furthermore ACM used in this study was supplemented with killed *E. coli*. Given that *L. monocytogenes* is a psychrotrophic saprophyte; this aspect of the methodology would alter the outcome.

The factors and conditions underlying better survival of *L. monocytogenes* in protozoan extracellular medium are currently unknown. It has been hypothesized that either the products or metabolites released/excreted by amoeba can contribute towards sustained survival of *Listeria* more work will be needed to understand the details. Given that both *L. monocytogenes* and fresh water ciliate protozoa are ubiquitous in the environment, including the food associated environments, the fostering role of protozoa can prove crucial determinant for persistence of *L. monocytogenes* in the environment. Based on the current and previous findings, it can be stated that medium extracellular to protozoan can promote the survival or perhaps contribute towards growth of *L. monocytogenes*. Currently the contribution of protozoan extracellular medium towards overall survival of *L. monocytogenes* due to Listeria-protozoa relationship is unknown. Detailed understanding of this relationship will provide more information about the ecology of *L. monocytogenes* thereby adding a crucial determinant in future *L. monocytogenes*-based risk assessments which in turn would prove beneficial in designing more informed disinfection protocols.

#### **ACKNOWLEDGEMENT**

We are thankful to all other members of our laboratory for their support and encouragement. We would also like to thank the contribution of Reaching Incoming Students Enrichment (RISE) program funded by Howard Hughes Medical Institute (HHMI). We are also appreciative of the *Tetrahymena* related insights provided by Rafael Emanuel Garduño.

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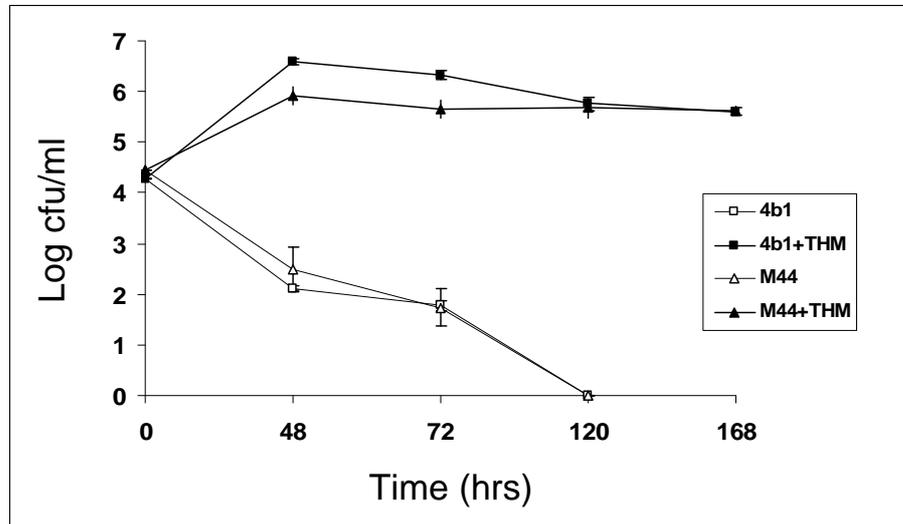
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**Table 1.** Bacterial strains used in this study.

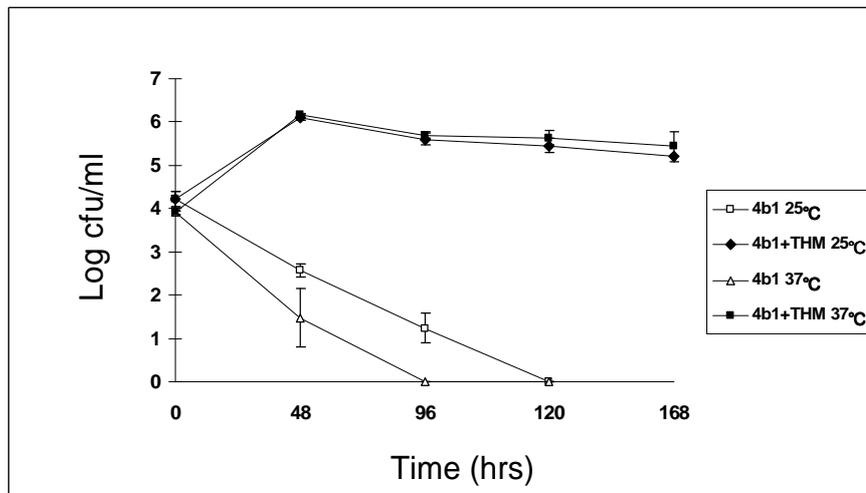
Bacterial strain	Reference
<i>L. monocytogenes</i> strains:	
H7550- 1998-1999 hot dog outbreak strain, epidemic clone II	(Evans et al., 2004)
F2365- 1985 California outbreak strain, epidemic clone I	(Nelson et al., 2004)
4b1- sporadic serotype 4b, member of epidemic clone Ia	(Promadej et al 1999; Cheng et al 2008)
M44- teichoic acid glycosylation-deficient mutant of 4b1 with transposon insertion in <i>gtcA</i>	(Promadej et al 1999)
ROA3- Katalase-deficient mutant of F2365 with a transposon insertion in <i>kat</i>	(Azizoglu and Kathariou, 2010)
<i>L. innocua</i> CLIP 11262	(Glaser et al., 2001)
<i>Salmonella enterica</i> serovar Heidelberg	Eric Fletcher

**Figure 1.** Survival of *L. monocytogenes* strains in the presence of *Tetrahymena* sp. and its conditioned medium. **A.** Survival of *L. monocytogenes* strains 4b1 and M44 in the presence and absence of *Tetrahymena* sp. at 30°C. **B.** Survival of 25 and 37°C grown *L. monocytogenes* strain 4b1 in the presence and absence of *Tetrahymena* sp. **C.** Survival of *Salmonella enterica* serovar Heidelberg in the presence and absence of *Tetrahymena* sp. at 30°C. **D.** *L. monocytogenes* strain H7550 in the filtrate obtained from co-culture of *Tetrahymena* + 4b1, *Tetrahymena* only, 4b1 only, and OHS only. **E.** *L. monocytogenes* strain 4b1 in the filtrate obtained from co-culture of *Tetrahymena* + 4b1, *Tetrahymena* only, 4b1 only, and OHS only.

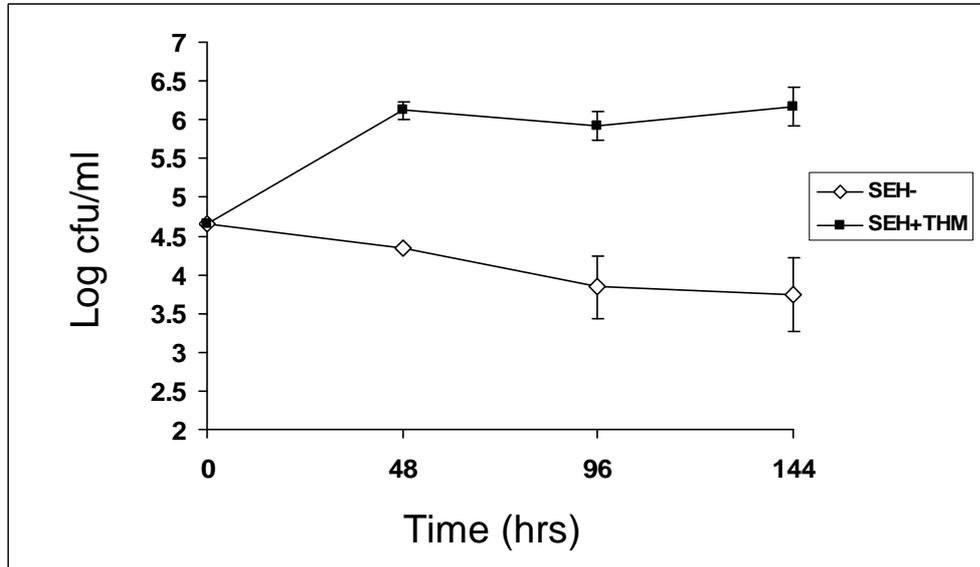
A.



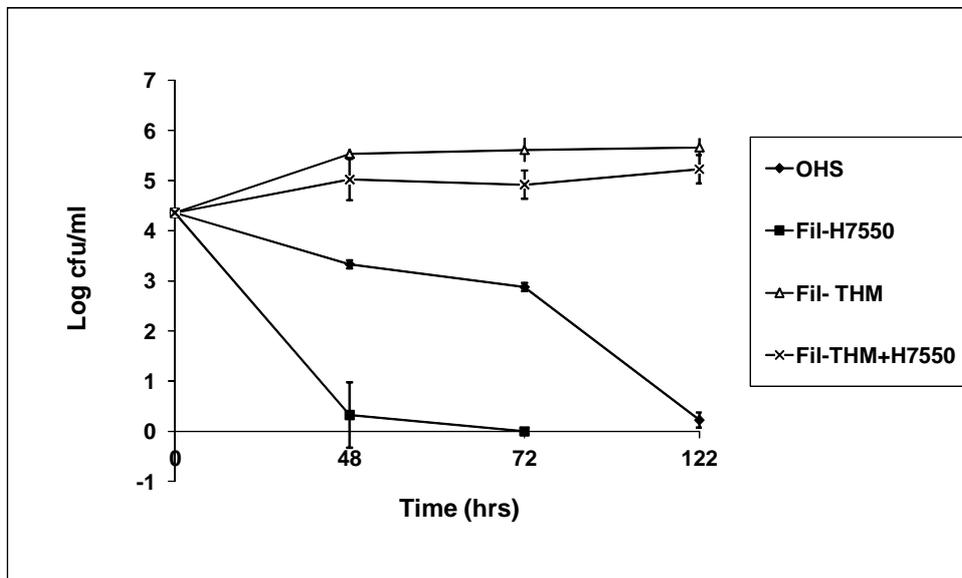
B.



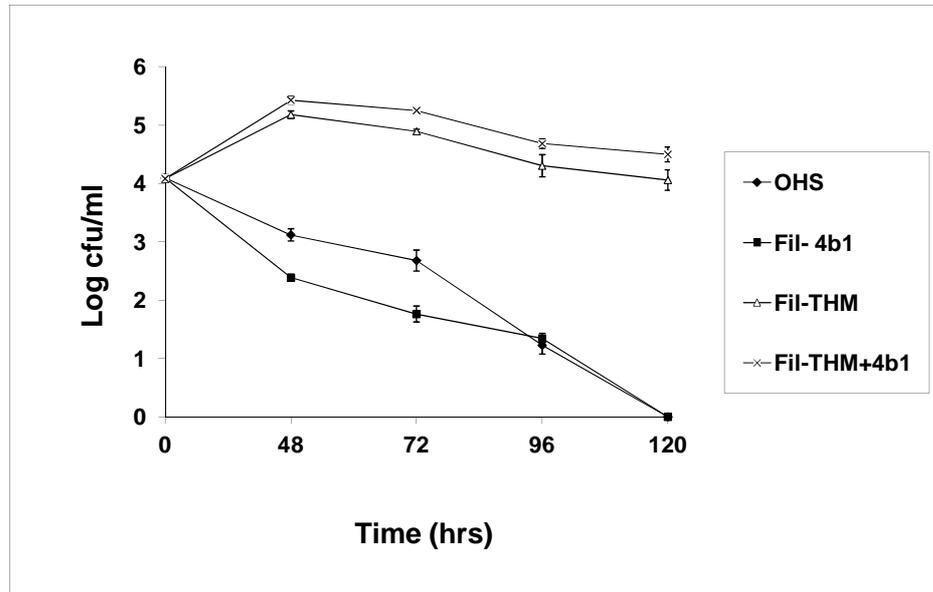
C.



D.



E.



**Chapter VI: Genetic Characterization of Plasmid-Associated Triphenylmethane Reductase in *Listeria monocytogenes*.**

**Authors.**

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**Author's contribution.**

**VD:** Recombinant plasmid constructs, RT-PCR experiments, phenotypic characterization experiments, data analysis and manuscript writing; **RS:** Strain analysis, phenotypic characterization, data analysis, and manuscript writing; **SK:** mentor, data analysis, and manuscript writing

**Publication status.**

To be submitted to Applied and Environmental Microbiology.

## ABSTRACT

Triphenylmethane dyes such as crystal violet and malachite green may be encountered in industrial effluents, especially from textile plants. Such dyes are highly toxic to bacteria. Given that *Listeria monocytogenes* is ubiquitous in the environment, an adaptation to survive in the presence of triphenylmethane dyes would prove crucial to its persistence in certain ecosystems polluted with these dyes. In this study we have characterized a putative triphenylmethane reductase gene (*tmr*) in *L. monocytogenes* strain H7550. *tmr* was located downstream of a gene cassette (*bcrABC*) mediating resistance to benzalkonium chloride (BC) on a large plasmid (pLM80). Our findings suggest that *tmr* mediates decolorization and detoxification of crystal violet (CV) and that its expression is induced by sublethal levels of the dye. Previously we have shown that *bcrABC* was co transcribed with *tmr*. Here using sub-cloning experiments we have confirmed that indeed *tmr* was encoded by a canonical promoter upstream to *bcrA* (P1) and that a second promoter (P2) immediately upstream to *tmr* also contributed to *tmr* transcription. The transcriptional association between *bcrABC* and *tmr* was further confirmed by the finding that besides BC, CV also increases the *bcrABC* transcript levels. Using qRT PCR analysis from this study and based on previous analysis we have shown that the regulation of *bcrABC* and hence *tmr* transcription was multifaceted. Promoters P1 and P2, and sequence flanking them were playing a crucial role in this transcriptional regulation. Screening of a panel of *L. monocytogenes* strains of different serotypes and sources revealed that *tmr* was harbored by all crystal violet-resistant strains. All *tmr*-positive strains also harbored *bcrABC*. Furthermore, with few exceptions, strains harboring *tmr* also

harbored a specific cadmium resistance determinant, *cadA2C2* (alone, or in combination with *cadA1C1*).

## INTRODUCTION

*Listeria monocytogenes* is a food-borne pathogen that has been associated with severe illness in susceptible individuals (Kathariou, 2002; Kornacki and Gurtler, 2007; Scallan et al., 2011). *L. monocytogenes* appears to spend more time in the environment than as an intracellular pathogen, suggesting a major influence of environmental conditions on the overall ecology of this pathogen (Lawrence and Gilmour 1995; Ojeinyi et al., 1996; Soumet et al., 2005; Thevenot et al., 2005). It has been hypothesized that adaptive attributes such as biofilm formation, resistance to disinfectants, resistance to *Listeria*-specific viruses, and ability to replicate at low temperatures are among the major attributes that contribute to the prevalence and persistence of *L. monocytogenes* in food-related environments (Norwood and Gilmour, 1999; Gandhi and Chikindas, 2007; Kim and Kathariou 2008; Mullapudi et al., 2008; Freitag et al., 2009; Toledo-Arana et al., 2009). The current list of adaptive attributes of *L. monocytogenes* is not exhaustive. A constant effort has been directed towards the identification of new adaptations and niches that might lead to persistence of *L. monocytogenes* in the environment.

Triphenylmethane dyes such as crystal violet (CV) and malachite green have a wide industrial application. Their extensive use is accompanied with release into the environment as industrial effluents (Gregory 1993; Hessel et al., 2007). Due to increasing interest in finding bioremediation tools for these dyes, a number of studies have highlighted the ability of microbes, including fungi and bacteria, to enzymatically detoxify the dyes (Azmi et al., 1998).

Triphenylmethane reductase (TMR) is one such enzyme which has shown to be encoded by Gram-negative bacteria such as *Citrobacter*, *Aeromonas* and *Pseudomonas* spp (Jang et al., 2005; Schlüter et al., 2007; Huan et al., 2011). The structure and putative mechanism of CV decolorization by TMR has been characterized in a heterologous host (*Escherichia coli*) (Jang et al., 2005, 2007; Kim et al., 2008). No information is currently available regarding the mechanism, function and ecological role and importance of TMR in its native host.

Recently we have described functional benzalkonium chloride (BC) resistance genes (*bcrABC*) on plasmid (pLM80) of *L. monocytogenes* strain H7550 which was isolated from 1998-1999 listeriosis outbreak in the United States (Elhanafi et al., 2010). The *bcrABC* cassette (3.8 kb) was transcribed by a canonical promoter (P1) and was part of a larger, 3.8 kb transcript. Interestingly, the last gene in this operon was annotated as a putative TMR (Elhanafi et al., 2010). In this study we have characterized this gene (*tmr*) in *L. monocytogenes* H7550 and assessed its prevalence among *L. monocytogenes* isolates of different serotypes and origin.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table 1 and 3. *L. monocytogenes* was grown either in brain heart infusion (BHI) broth (Becton, Dickinson & Co., Sparks, MD) or on agar media where BHI was supplemented with 1.2% Bacto-agar (Becton, Dickinson & Co.). *Escherichia coli* strains were grown at 30°C in Luria Bertani (LB) broth (Becton, Dickinson & Co.) or in LB broth supplemented with 1.2% Bacto-agar. For long-term storage all bacterial strains were kept at -80°C in BHI

supplemented with 20% glycerol (Fischer scientific, Fair Lawn, NJ). All antibiotics used in this study were purchased from Sigma- Aldrich (St. Louis, MO).

**BC, crystal violet (CV) and cadmium susceptibility.** BC susceptibility of *L. monocytogenes* was assessed as described (Mullapudi et al., 2008; Elhanafi et al., 2010). Briefly, a single colony from a blood agar plate (Remel, Lenexa, KS) was suspended in 100  $\mu$ l of Mueller Hinton broth (Becton, Dickinson & Co.) and 5  $\mu$ l of the suspension was spotted in duplicate on Mueller Hinton agar (Becton, Dickinson & Co.; 1.2% Bacto-agar) supplemented with 1.2% sheep defibrinated blood, and 20  $\mu$ g/ml BC. For CV and cadmium susceptibility, a single colony from a blood agar plate (Remel) was inoculated in 100  $\mu$ l of BHI broth and 5  $\mu$ l was then spotted on either BHI agar supplemented with 15  $\mu$ g/ml CV (Fisher, Fair Lawn, NJ) or Iso-sensitest agar (Oxoid Ltd, Hampshire, England) supplemented with 70  $\mu$ g/ml of cadmium chloride. The plates were incubated at 37°C for 48 h. Strains H7550 and its plasmid-cured derivative H7550-Cd<sup>S</sup> were used as resistant and susceptible controls, respectively (Elhanafi et al., 2010).

**CV-MIC determination.** For CV MIC determinations, a single colony from BHI agar was suspended in 100  $\mu$ l of Mueller Hinton broth (Becton, Dickinson & Co.) and 5  $\mu$ l of the suspension was spotted in duplicate on BHI agar plates supplemented with variable concentrations of CV (0, 10, 20, 25, 30, 35, 40, 50, 60, 65, 70, 75  $\mu$ g/ml) and the plates were incubated at 37°C for 48 h. MIC was defined as the lowest assessed concentration of CV that prevented growth.

**Recombinant plasmid constructs.** Primers used to construct the recombinant plasmids are listed in Table 2, and their locations are indicated in Fig. 1. PCR employed the Takara Ex

Taq kit (Takara, Madison, WI) and a T1 thermal cycler (Biometra, Goettingen, Germany). Primers BcF and 66R were used to amplify 4158 bp PCR fragment extending from 105 nt upstream of *bcrA* along with the canonical promoter (P1), up to 281 nt downstream of the *tmr* stop codon. The BcF- 66R PCR fragment was restricted with XmaI (New England Biolabs, Beverly, MA), and was ligated with T4 DNA ligase (Promega, Madison, WI) into similarly restricted pPL2 (Lauer et al., 2002) to produce pPL81. Primers BcF3 and 66R2 were employed to amplify 4953 bp fragment. In addition to the BcF- 66R fragment, this fragment harbored 795 nts of the upstream sequence, including intergenic region upstream of *bcrA*. This BcF3- 66R2 PCR fragment was restricted with KpnI and was ligated into similarly digested pPL2 resulting in pPL83.

Primers TMR\_F and 66R2 harbored all of the intergenic space (426 bp) upstream of the start codon of *tmr* (including P2 located 175 nts upstream of *tmr* start codon) along with *tmr* and 281 nt downstream of the *tmr* stop codon to yield a 1571 bp fragment. This fragment was restricted with KpnI (New England Biolabs) and then ligated into similarly digested pPL2 resulting in pPL82. Primers pairs TMR2\_F-66R2 and TMR3\_F- 66R2 were used to amplify 1358 bp and 1245 bp fragments, respectively. These fragments varied in the region upstream to the *tmr* start codon where TMR2\_F-66R2 harbored 213 bp (including P2) and TMR3\_F-66R2 harbored 100 bp (excluding P2), respectively. These fragments were also restricted with KpnI (New England Biolabs) and ligated into similarly digested pPL2 resulting in pPL84 and pPL82A, respectively.

All the recombinant plasmids were electroporated first into *E. coli* DH5a followed by selection on LB supplemented with 15µg/ml CV for 24-48hrs, until the medium was

decolorized. The plasmids preps from *E. coli* DH5 $\alpha$  were confirmed with PCR and restriction analysis followed by electroporation into *E. coli* S17-1. The transformed *E. coli* S17-1 colonies were also selected on LB with 15  $\mu$ g/ml CV for 24 hrs at 30°C. Recombinant plasmids and the empty vector (pPL2) were then mobilized into H7550-Cd<sup>S</sup> via conjugation, as described (Lauer et al., 2002; Elhanafi et al., 2010). Transconjugants were selected on BHI agar plates supplemented with chloramphenicol (6  $\mu$ g/ml) and nalidixic acid (20  $\mu$ g/ml) at 30°C for 2-3 days, and confirmed using PCR as described before (Lauer et al., 2002).

#### **Phenotypic characterization of the recombinant strains.**

Decolorization curves: Overnight cultures (37°C in BHI) of *L. monocytogenes* strains H7550, H7550-Cd<sup>S</sup> harboring pPL2 (Cd<sup>S</sup>-pPL2), J0161 and derivatives of H7550-Cd<sup>S</sup> i.e. pPL81, pPL82, pPL83, and pPL84 were diluted 1:20 in BHI supplemented with 15  $\mu$ g/ml of CV in separate wells of a 24-well plate (Corning, NY). Strain J0161 (serotype 1/2a) harbors an identical copy of IS1216 harboring *tmr* on pJ0161 (Kuenne et al., 2010). The inoculated plate was incubated at 37°C and the decrease in absorbance ( $\lambda = 590$  nm) of the cultures was measured using a spectrophotometer (SmartSpec 3000; Bio-Rad, Hercules, CA). The readings were recorded until the CV was completely decolorized. The findings from this assay were confirmed in at least two independent trials. For growth curves, overnight cultures (37°C in BHI) of strains H7550, Cds-pPL2, J0161 and recombinant derivatives of H7550-Cd<sup>S</sup> were diluted 1:20 in BHI followed by incubation at 37°C. OD<sub>600</sub> was recorded using a spectrophotometer (SmartSpec 3000) at hourly intervals up to eight hours.

Susceptibility to Leuco-CV: Overnight cultures of strain H7550 and Cds-pPL2 grown at 37°C in BHI were diluted 1:20 in BHI supplemented with 7.5  $\mu$ g/ml of CV. Plate counts were

performed at this stage and the time point was labeled as  $t=0$ . After incubation of the cultures at  $37^{\circ}\text{C}$  for ca. 4.5 hrs (until CV was completely decolorized), another plate count was performed ( $t=4.5$ ). Two more plate counts was performed at  $t=24$  hrs and  $t = 48$  h. The assay was performed in two independent trials.

**RNA isolation and reverse transcription PCR (RT-PCR).** *L. monocytogenes* H7550 and the derivatives of H7550-Cd<sup>S</sup> (pPL81 and pPL83) were grown in BHI at  $37^{\circ}\text{C}$  until mid to late logarithmic phase ( $\text{OD}_{600}$  value~ 0.7-0.9, measured using a spectrophotometer [SmartSpec 3000]). Cultures were then divided into two portions; one part was mixed with 15  $\mu\text{g}/\text{ml}$  of CV while the other was left untreated. Both parts were incubated at  $37^{\circ}\text{C}$  for 30 min and total RNA was isolated from CV treated and untreated cultures using the SV Total RNA Isolation System (Promega, Madison, WI). After isolation, total RNA was subjected to DNase treatment using Turbo DNA free (Ambion, Austin, TX). The concentration and quality of RNA was determined by measuring the absorbance at 260 nm (Nanodrop, Wilmington, DE). For each temperature, RNA was isolated in three independent trials.

Total RNA was reverse-transcribed to produce cDNA using 200 ng of total RNA and ImProm-II<sup>TM</sup> Reverse Transcription System according to the manufacturer's protocol (Promega). RNA was stored at  $-80^{\circ}\text{C}$  as and when necessary. The gene-specific cDNA was reverse transcribed using 0067-R for TMR, and s2 for the housekeeping gene *spoVG* (Liu and Ream, 2008). Reverse transcription for *bcrABC* was performed as described before (Elhanafi et al., 2010).

For band quantifications we used image processing software ImageJ (<http://rsbweb.nih.gov/ij/>). In order to calculate the fold increase in transcript levels of genes

in response to the presence of BC or CV, gel density values obtained from the RT-PCR gel images using ImageJ were normalized to those of the reference gene *spoVG* from the same treatment. The ratio of the normalized *bcrABC* levels to those of the control (culture without BC or CV) corresponded to fold increase (Elhanafi et al., 2010).

**Quantitative real-time PCR.** The mRNA expression levels of the selected genes were analyzed with quantitative real-time PCR using Applied Biosystems 7500 Fast Real-Time PCR system as per manufacturer's instructions (Applied Biosystems, Foster City, CA). Gene specific TaqMan probe and primer sets were designed using AlleleID 7.0 software (Premier Biosoft, Palo Alto, CA). Briefly, for gene expression mix, 18 $\mu$ M of forward (qTMR\_F and qspoVG2\_F) and reverse (qTMR\_R and qspoVG2\_R) primers and 5 $\mu$ M of Taqman probe (qTMR\_P and qspoVG\_P) were used (Table 2). For each reaction (total volume of 20  $\mu$ L per reaction) 1  $\mu$ L of gene expression mix was added to 10 $\mu$ L of Taqman Fast Universal PCR Master Mix (2X), No AmpErase UNG (Applied Biosystem, Foster City, CA), and 1  $\mu$ L of cDNA was amplified. The following thermocycling parameters were used: 95°C for 20 sec; 40 cycles of 95°C for 3 sec, and 60°C for 30 sec. Fluorescence measurements were collected every cycle during the extension step (60°C). Cycle threshold (Ct) values were calculated for each sample corresponding to the cycle where amplification rate was maximal. Within a single instrument run, three replicates per cDNA samples were quantified.

**Screening of *L. monocytogenes* strains for CV decolorization ability and presence of *tmr*.**

A total of 235 *L. monocytogenes* strains with different serotypes, pulsed field gel electrophoresis (PFGE) types, and origins (147 clinical and 98 environmental [turkey processing plant]) were tested for CV-15 decolorization as described above. Only 51 strains

decolorized CV-15 and a subset of these strains (n=34; 13 clinical and 21 environmental) along with those that did not decolorize CV-15 (n= 9 all environmental) were PCR screened with *tmr*-specific primers (0067F-0067R) (Fig 1; Table 2, 3; Elhanafi et al., 2010).

**Calculations and Statistics.** Gene expression levels for *tmr* were reported relative to those of housekeeping gene *spoVG* which was used as an internal control (Liu and Ream, 2008). The quantification of *tmr* transcript levels for each strain and treatment (with or without CV exposure) was done by normalizing the *tmr* Ct values obtained for a specific strain to that of housekeeping gene for the same strain and treatment. The resulting values were termed as  $\Delta\text{Ct}$ . Equation ( $2^{-\Delta\text{Ct}}$ ) was used to express the fold change between the *tmr* and housekeeping gene *spoVG* transcript levels, assuming gene amplification efficiency for both *tmr* and *spoVG* as 2 (Pfaffl, 2001).

For statistics, a mixed linear model (SAS, Cary, NC) was fit to  $\Delta\text{Ct}$  measurements in order to test hypotheses regarding factorial effects of strain and the crystal violet treatment. In particular, random effects were included for trial and trial-by-treatment interaction so that appropriate error terms were used in tests for these hypotheses. Residual diagnostics did not reveal any violation of the assumptions of normally distributed  $\Delta\text{Ct}$  measurements and homogeneity of variance necessary for statistical inference. To test for equality of gene expression across strains separately with or without the CV treatment, a nested model was fit in which the strain effect was nested within the CV factor.

## RESULTS

*In silico* analysis suggested that the putative *tmr* (864 bp) annotated as LMOh7858\_plm80\_0067 (Refseq No.NZ\_AADR00000000), had unusually high GC content

(45%; normal GC content for *Listeria* is 38%). Homologs with 99-100% nt identity and 100% query coverage were found in *Citrobacter* sp. strain KCTC 18061P isolated from the soil of a textile and dyeing industry effluent treatment plant (Jang et al., 2005), *Pseudomonas* sp. K9 isolated from the sludge of a waste-water treatment system of a chemical plant (Huan et al., 2011), *Pseudomonas* sp. MG-1 isolated from water of an aquatic hatchery (Li et al., 2009), IncP-1-beta plasmid pGNB1 from an unknown bacterium isolated from waste water treatment plant (Schlüter et al., 2007), *Pseudomonas* sp. JT-1 (acc. no. HQ116525; Wu et al., 2011), *Pseudomonas* sp. MDB-1, and *Aeromonas hydrophila* subsp. Decolorationis (acc. no. EF010984). Schlüter et al (2007) also reported the presence of hypothetical protein (LMOh7858\_plm80\_0066) homolog (100% nt identity and 100% query coverage) downstream to *tmr* on pGNB1 from an unknown bacterium. Interestingly upstream to *tmr* a Mer family transcriptional family regulator was found in strain *Pseudomonas* sp. K9. More homologs with 86% query coverage and 98-99% nt identity were also found in *Geobacillus* sp. Y4.1MC1 and *Geobacillus thermoglucosidasius* strain C56-YS93. Among *Listeria* spp. homologs with 99-100% nt identity were found with *L. monocytogenes* J0161, and *L. seeligeri* strain N1-067. Strain J0161 was a serotype 1/2a strain isolated from sliced turkey outbreak in the US (2000) and *L. seeligeri* strain N1-067 was isolated from fish processing plant and was recently sequenced as a representative non-pathogenic typical hemolytic strain (Olsen et al., 2005; den Bakker et al., 2010).

Protein BLAST indicated homologs with 100% aa identity and 100% query coverage with *Citrobacter* sp. strain MY-5, *Aeromonas hydrophila* subsp. Decolorationis, and *L. seeligeri* strain N1-067. Homologs with *Geobacillus* sp. Y4.1MC1 and *Geobacillus*

*thermoglucoasidarius* strain C56-YS93 with 86% query coverage and 98-99% aa identity were also found. It is possible that other homologs from the nt BLAST were not found with protein BLAST due to submission of just nucleotide sequences or incomplete annotations in the NCBI database. Beside the *tmr* homolog in *L. seeligeri* strain N1-067, protein BLAST against just *Listeria* genomes indicated other homologs with 36% aa identity and 99% query coverage to putative nucleoside-diphosphate-sugar epimerase in a number of *Listeria* genomes.

Seven nt upstream to *tmr* start codon a Shine-Dalgarno (SD) sequence (AGGAGA) and 146 nt upstream to *tmr* start codon a putative promoter (P2: -10 TATAAT; -35 TTGATT) were identified (Fig 1A). Upon performing BLAST with 395 nts intergenic sequence upstream to *tmr*, identical sequences with different query coverage were found for strains *Pseudomonas* sp. K9 (query coverage 53%), *Pseudomonas* sp. MDB-1 (query coverage 60%), IncP-1-beta plasmid pGNB1 (query coverage 81%), and *Citrobacter* sp. strain KCTC 18061P (query coverage 81%).

Promoter P2 (175 nts upstream to *tmr* start codon) was found in all these strains upstream to their respective *tmr* homologs (Jang et al., 2005; Schlüter et al., 2007; Huan et al., 2011). As described before, a putative Rho- independent transcriptional terminator ( $\Delta G = -18.7$  kcal/mol) was found 110 nts downstream to *tmr* stop codon (Elhanafi et al., 2010). Previously we have also shown that *tmr* was a part of a putative composite IS1216 transposon that also harbors *cadA2C2* along with *bcrABC* (Elhanafi et al., 2010).

***tmr* confers CV-decolorization phenotype to a plasmid-cured derivative of *L. monocytogenes* H7550.** Preliminary association between *tmr* and CV decolorization was

assessed by comparing the CV decolorization ability of *L. monocytogenes* strain H7550 and its plasmid (pLM80)-cured derivative H7550-Cd<sup>S</sup>. Indeed, H7550 was able to grow and decolorize crystal violet when incubated at 37°C for few minutes in BHI supplemented with 15 µg/ml CV (CV-15), while no decolorization was observed with H7550-Cd<sup>S</sup> (data not shown). On agar media (BHI supplemented with CV-15; BHI+CV-15), no growth was seen for H7550-Cd<sup>S</sup>, whereas H7550 growth was confluent and CV decolorization was seen around the colonies after overnight incubation at 37°C. CV-MIC for H7550 as tested on BHI agar plates (supplemented with different concentrations of CV) was 75µg/ml, whereas for plasmid cured strain H7550-Cd<sup>S</sup> was 5 µg/ml. We have also tested the susceptibility of these strains with other triphenylmethane dye where BHI agar was supplemented with malachite green (15 µg/ml; MG-15). Unlike BHI+CV-15 agar plates, both H7550 and H7550-Cd<sup>S</sup> were able to grow on BHI+MG-15 agar plates, however H7550 growth was confluent as compared to H7550-Cd<sup>S</sup> where growth was stunted (data not shown). We suspected that other chromosome-based mechanisms were interfering with malachite green decolorization phenotype, and because of the relatively distinct phenotype (growth vs. no growth) with CV, we used latter as a substrate triphenylmethane dye throughout the study.

We have previously shown that *tmr* was part of a 3.8 kbp long transcript which also contains message for a drug efflux system *bcrABC*. This transcript was initiated by a canonical promoter (P1) upstream to *bcrA*. We have also demonstrated that the possible transcript termination occurs 110 nt downstream to *tmr* with a Rho-independent terminator. As described above beside P1 we also found P2, 175 nts upstream to *tmr* start codon and we suspected the role of this promoter in *tmr* transcription.

Cloning of *tmr* with different portions of the upstream and downstream region resulted in the plasmids shown in Fig. 1B. Plasmid pPL81 harbored 4158 bp fragment which included 105 nts upstream to *bcrA* start codon (harboring P1), *bcrABC*, and the downstream region including *tmr* and 281 nts downstream to *tmr* stop codon (harboring putative Rho-independent terminator). Plasmid pPL83 harbored a 4953 bp fragment which included 795 nts long sequence upstream to pPL81 fragment and the pPL81 fragment (4158 bp). Plasmid pPL82 harbored 1571 bp fragment including 402 bp sequence upstream to *tmr* start codon (including P2), *tmr* and 281 nt downstream to *tmr* stop codon. Unlike pPL82, pPL84 harbored a shorter (1358 bp) fragment including 213 nt sequence upstream to *tmr* start codon (including P2), *tmr* and 281 nts downstream to *tmr*. Introducing pPL81, pPL82, pPL83 and pPL84 into H7550-Cd<sup>S</sup> resulted into Cds-pPL81, Cds-pPL82, Cds-pPL83 and Cds-pPL84, respectively. All these derivative strains restored the CV-15 decolorization phenotype in the parent strain H7550-Cd<sup>S</sup>; whereas H7550-Cd<sup>S</sup> transformed with just pPL2 (Cds-pPL2) remained unable to decolorize CV-15 (Table 1; Fig 1B). Furthermore, introducing pPL82-A into H7550-Cd<sup>S</sup> (harboring a 1245 bp fragment including only 100 nts upstream to *tmr* start codon [excluding P2], *tmr*, and the intergenic space downstream to *tmr*) did not render the strain to decolorize CV-15 (Fig 1B). As expected only strains Cds-pPL81 and Cds-pPL83 acquired BC resistance (BC 20µg/ml) whereas strains Cds-pPL82 and Cds-pPL84 remained BC susceptible (Fig 1B). Strains harboring just P1 and the downstream *bcrABC* (pDS195, pDS202; Elhanafi et al., 2010) did not decolorize CV-15 even though they restored resistance to BC-20 µg/ml. From these data it can be concluded that even though both P1 and P2 contribute towards the *tmr* transcription, minimally P2 would be enough to express *tmr*.

***tmr* transformants with different upstream regions decolorize CV with different efficiencies.** The CV decolorization efficiency of the wild type strain H7550 was not fully complemented by any of the derivative strains (Fig 2). No differences were found in the growth rates of strains H7550, H7550-Cd<sup>S</sup>, or the H7550-Cd<sup>S</sup> derivatives (data not shown). Interestingly decolorization efficiency of strain J0161, which also harbors a pLM80-like plasmid (pJ0161) based copy of *IS1216* transposon, was not better than H7550 (Fig 2; Elhanafi et al., 2010; Kuenne et al., 2010). CV MIC for strain J0161 was 40 µg/ml compared to H7550 with CV MIC of 75µg/ml (Table 1). However based on the decolorization curves, J0161 CV decolorization efficiency was better than those of the H7550-Cd<sup>S</sup> derivative strains (Fig 2). Among the derivative strains, decolorization efficiency of Cds-pPL81 was consistently better than Cds-pPL83, even though the CV-MIC for both strains was 75µg/ml (Table 1; Fig 2). Decolorization efficiency for strains Cds-pPL82 and Cds-pPL84 although identical was least efficient among the derivative strains and was accompanied by the lowest CV-MIC (40 µg/ml) among other derivatives (Table 1; Fig 2). It is possible that *tmr* transcription was more efficient due to both promoters (P1 and P2) than transcription due to one promoter (P2).

**Transcript levels of *tmr* increased in the presence of CV-15.** qRT PCR analysis suggested that upon exposing strain H7550 and its derivative (Cds-pPL81, Cds-pPL83) cells to CV, *tmr* expression were increased significantly for each tested strains, where for strain H7550 fold change in  $\Delta C_t$  were 2.6 fold (p-value= 0.012), for strain Cds-pPL81 fold increase was 4.3 fold (p-value= 0.003), and for strain Cds-pPL83 fold increase was ca. 3 fold (p-value= 0.005) (Fig.

3). Due to the suboptimal quality and quantity of total RNA obtained from the cultures of Cds-pPL82 and Cds-pPL84 upon exposure to CV-15, the impact of CV-15 on *tmr* induction for these strains could not be assessed. We suspect that increased cell death due to CV exposure even during the 30 min exposure led to poor yield and quality of RNA.

Baseline levels for *tmr* expression were recorded for all strains, including Cds-pPL82 and Cds-pPL84. Pairwise comparisons of  $\Delta C_t$  values indicated that baseline *tmr* expression levels were significantly different between Cds-pPL81 and Cds-pPL83 ( $p=0.01$ ) as well as between Cds-pPL81 and Cds-pPL84 ( $p=0.002$ ). Similarly, Cds-pPL82 *tmr* expression levels were significantly different than Cds-pPL83 ( $p=0.03$ ) and Cds-pPL84 ( $p=0.0048$ ). The baseline *tmr* levels between H7550 and any of its derivatives were not significantly different (Fig 3).

Since *bcrABC* and *tmr* were co-transcribed (Elhanafi et al., 2010) and each mediated resistance to different toxic agents (BC and CV, respectively), we assessed whether CV could also induce expression of *bcrABC*. Indeed, expression of *bcrABC* increased by approx. 1.5 fold in the presence of CV, with a 2 fold increase observed in the presence of 10  $\mu\text{g/ml}$  BC (Fig 4).

#### **Decolorized (leuco-version) CV is not deleterious to *L. monocytogenes* strain H7550.**

Following a 4.5 h exposure of *L. monocytogenes* H7550 and Cds-pPL2 to CV both strains exhibited a decrease in viability (ca. 2  $\log_{10}$  reduction in cfu/ml). During this period *L. monocytogenes* H7550 completely decolorized CV and resumed growth while no decolorization was detected for Cds-pPL2, and survival of this strain continued to decline, with an additional ca. 2.5  $\log_{10}$  reduction by 20 h (Fig. 5). Assessing CFU/ml at 48 h indicated

further reductions (ca. 2 log<sub>10</sub> reduction in cfu/ml) in Cds-pPL2 but not in H7550 (data not shown). Thus, decolorization of CV results in detoxification of this dye for *L. monocytogenes*.

**Presence of *tmr* is correlated with CV decolorization in *L. monocytogenes* strains of diverse serotypes and origin.** All 34 CV-decolorizing isolates yielded the expected amplicon for *tmr*, whereas all 9 isolates that did not decolorize CV did not yield any amplicon with *tmr* primers. Among the 21 environmental isolates six were 1/2a or 3a, 11 were 1/2b or 3b, three were 1/2c or 3c, and one were 4b serotype. Among the 13 clinical isolates 10 were 4b, and three were 1/2a or 3a (Table 3).

Further PCR analysis of the strains revealed that all *tmr*-positive and CV-decolorizing strains were also PCR-positive for *bcrABC*, and were resistant to BC. With a single exception (the serotype 4b clinical strain J5202), all *tmr*-positive strains were also resistant to cadmium. Of the remaining 33 strains the majority (n = 28) harbored a specific cadmium resistance cassette (*cadA2C2*) either alone or together with another cadmium resistance cassette (Tn5422-associated *cadA1C1*; Table 3). Most (8/10) cadmium-resistant strains that harbored only *cadA1C1* lacked *tmr* and *bcrABC*, and were susceptible to CV and BC (Table 3).

## DISCUSSION

In this study we have identified and characterized *tmr* of *L. monocytogenes*, encoding a putative triphenylmethane reductase and mediating decolorization of the triphenylmethane dye, crystal violet (CV). It was intriguing that the *L. monocytogenes tmr* had unusually high GC content (45%) and that it was highly conserved (99-100% identity at the DNA sequence level) with *tmr* in several Gram-negative bacteria, including *Aeromonas*, *Citrobacter* and

*Pseudomonas* spp. (Li et al., 2009; Huan et al., 2011). Interestingly as per our knowledge *Listeria* spp. is the only Gram positive bacteria harboring *tmr*. Also unlike Gram-negative bacteria where *tmr* has been associated with bacterial isolates from industrial effluent, in *Listeria* spp. *tmr* positive isolates were either isolated from the listeriosis outbreak (H7550, J0161), or from the fish processing plant environment (*L. seeligeri* N1-067). The possible correlation to *tmr* prevalence in *Listeria* spp. may be attributed to the widespread use of triphenylmethane dyes as an antifungal agent in fish farming industry in many parts of world (Alderman et al., 1985; Schnick, 1988). Association of *tmr* with mobile elements such as IS*Ppu12*, IncP-1-beta plasmid pGNB1, and IS*I216* in *L. monocytogenes* (Schlüter et al., 2007; Elhanafi et al., 2010; Huan et al., 2011) and presence of *tmr* in wide diversity of bacteria provides a tentative evidence for *tmr* translocation by horizontal gene transfer.

Analysis of sequenced *Listeria* spp. genomes has revealed only three (*L. monocytogenes* strains H7858 and J0161 and *L. seeligeri* N1-067) that harbored *tmr*. In all three cases, *tmr* was harbored by a putative IS*I216* composite transposon which in case of two *L. monocytogenes* strains (H7858 and J0161) was located on large plasmids such as pLM80 (Nelson et al., 2004; Elhanafi et al., 2010; Kuenne et al., 2010). Our analysis of a panel of diverse *L. monocytogenes* strains revealed that all those harboring *tmr* also harbored *bcrABC* and exhibited both corresponding phenotypes (resistance to BC and decolorization of CV). These findings were suggestive of acquisition of *tmr* by plasmid-associated IS*I216* and subsequent dissemination via the plasmid among different *L. monocytogenes* strains. It is worthy of note that in case of all three above-mentioned sequenced genomes, that harbored *tmr* along with *bcrABC*, the determinants were on a putative IS*I216* composite transposon

that also harbored a specific cadmium resistance determinant, *cadA2C2*. In fact, this multi-resistance combination (*cadA2C2-bcrABC-tmr*) was encountered in the majority (28/32, approx. 88%) of the *L. monocytogenes* strains that decolorized CV and were resistant to this toxic dye.

Our earlier transcriptional analysis of the *bcrABC* region indicated that *bcrABC* and *tmr* were part of the same transcript suggesting transcription from the canonical promoter upstream of *bcrA* (P1). Integration of a plasmid harboring the entire fragment containing P1, *bcrABC* and *tmr* into plasmid-cured H7550-Cd<sup>S</sup> (Cds-pPL81 and Cds-pPL83) resulted in restoration of both BC resistance and CV decolorization, whereas strains harboring only P1 and *bcrABC* restored resistance to BC only (data not shown; Elhanafi et al., 2010). Similarly, recombinant strains such as Cds-pPL82 and Cds-pPL84 that harbored just *tmr* and upstream promoter (P2) restored CV decolorization but not BC resistance (Fig. 1).

The differentiating factor between strains encoding *tmr* under P1 and P2 (Cds-pPL81, Cds-pPL83) and the strains encoding *tmr* under just P2 (Cds-pPL82 and Cds-pPL84) was their CV decolorization efficiency. CV decolorization by strains Cds-pPL82 and Cds-pPL84 was identical and least efficient (CV MIC 40 µg/ml) among other tested strains (H7550, Cds-pPL81, and Cds-pPL83; CV MIC 75 µg/ml). Furthermore we were not able to assess the effect of CV exposure upon the *tmr* transcription, primarily due to low quality of RNA obtained upon exposing these strains to CV-15. We suspect this was directly linked to excessive cell death upon CV treatment. qRT PCR analysis did suggest that constitutive

baseline levels exist for both strains Cds-pPL82 and Cds-pPL84, suggesting that the *tmr* transcription from P2 was constitutive.

*In silico* analysis have revealed that P2 was conserved upstream of *tmr* in available *tmr* sequences from the strain *Pseudomonas* sp. K9, IncP-1-beta plasmid pGNB1 and *Citrobacter* sp. strain MY-5, while all these strains lacked *bcrABC*. These findings will indicate that in nature *tmr* is primarily encoded by P2 and these strains are equipped with only the baseline *tmr* expression levels to combat the stress due to triphenylmethane dye exposure. Furthermore *tmr* transcript levels for strain Cds-pPL82 were significantly lower than Cds-pPL84, suggesting repressive effect of the sequence upstream to P2 upon the *tmr* transcription. This transcriptional difference could not be detected at the phenotypic level since Cds-pPL82 did not differ from Cds-pPL84 in efficiency of CV decolorization. It is possible that the transcriptional differences between Cds-pPL82 and Cds-pPL84 were not enough to result in a change at the phenotypic level. Together these data suggest an advantage for the observed *tmr* gene arrangement in *L. monocytogenes* strain H7550, which places expression of the gene under both its own promoter (P2) and the canonical promoter upstream of *bcrA* (P1). Further studies are needed to identify conditions under which CV exposure leads to expression of *tmr* alone, from its own promoter, in strains such as H7550. Furthermore, molecular mechanisms that have led to *tmr* being in its unique genomic arrangement as a part of the *bcrABC* transcriptional unit remain to be elucidated.

The inverted sequence identified between the -10 and -35 sequences for P1, which also overlaps with the -10 sequence, was hypothesized to be a binding site for BcrA (Elhanafi et

al., 2010). *In silico* analysis suggests that *bcrA* belongs to a TetR family of transcriptional repressors. Members of this family have shown to control transcription of multidrug efflux systems (Ramos et al., 2005). TetR represses transcription by binding to the operator sequence (usually of dyad symmetry) overlapping the promoter sequences. A similar mechanism of regulation has been proposed for the QacAR system in *Staphylococcus aureus* (Grkovic et al., 1996, Paulsen et al., 1996).

Previously we have shown that *bcrABC* was transcribed even in the absence of BC, suggesting that transcriptional repression due to *bcrA* was weak (Elhanafi et al., 2010). This could also provide an explanation for the observed expression of *tmr* even in the absence of CV in all tested strains harboring P1. However, baseline *tmr* expression levels for Cds-pPL81 were significantly lower than those for strain Cds-pPL83, and lower but not significantly than H7550. This further suggests the putative role of *bcrA* as a transcriptional repressor and also suggests a potential transcriptional de-repressive role of the sequences upstream of P1.

Upon CV exposure the increase in *tmr* transcript levels for Cds-pPL81 was higher (ca. 4 fold) than for Cds-pPL83 (ca. 2 fold). The ability of Cds-pPL81 to transcribe *tmr* more efficiently (than Cds-pPL83) would explain its faster rate of CV decolorization than Cds-pPL83. However, this would not explain the faster CV decolorization observed for H7550, strain where fold-increase in *tmr* transcript levels was 2.6 fold. In strain H7550, *tmr* is located on a plasmid (pLM80) and thus possibly in multiple copies, whereas Cds-pPL81 and Cds-pPL83 harbor a single chromosomally integrated copy of the gene. This difference in copy number may underlie the observed differences. Furthermore, we cannot exclude other currently

unidentified plasmid-based factors that may contribute to the observed CV decolorization ability of H7550. Such additional determinants may also explain the finding that strain J0161 (serotype 1/2a) decolorized CV slower than the wild type strain H7550, even though it harbored *tmr* on a *IS1216* transposon that was identical in sequence to that in pLM80 (Elhanafi et al., 2010; Kuenne et al., 2010); however, J0161 and H7550 are of different serotypes and have a number of differences in their chromosome (Nelson et al., 2004; Orsi et al., 2008) which may also contribute to the observed differences in their ability to decolorize CV.

Since decolorization of CV resulted in detoxification of the dye, one may speculate that in triphenylmethane dye-rich environments there would be a selective advantage for *L. monocytogenes* strains bearing *tmr*. This could prove crucial in understanding the environmental fitness of *L. monocytogenes* strains such as H7550 and J0161 which harbor *tmr* on highly related large plasmids. Given that *tmr* was co-transcribed with *bcrABC* and transcription of the latter can be increased by either QACs or CV, only one substrate would be enough to sustain both *tmr* and *bcrABC*.

Since no strains harboring *tmr* but lacking *bcrABC* were found, it can be speculated that *tmr* has become established only as a part of the *bcrABC* cassette-associated operon in *L. monocytogenes*. Strains harboring just *tmr* may be unable to sustain themselves in dye-rich environments and would also lack resistance to BC and other QACs. One explanation would be the loss of the canonical promoter which would place such strains at disadvantage in triphenylmethane dye-rich environments. Alternatively, *tmr* acquisition into a plasmid-borne

IS1216-associated *bcrABC* region may have occurred recently and resulting plasmids have been acquired by different strains (such as the H7550 and J0161, which have different serotype and belong to different lineages of *L. monocytogenes*, but harbor closely related plasmids with *cadA2C2-bcrABC-tmr*). In either case, the putative composite transposon IS1216 may play a key role in intra-*Listeria* spp. transmission of *tmr* along with *bcrABC*. This is also supported by the observed correlation between the presence of *tmr*, *bcrABC* and *cadA2C2* among the majority of *tmr*-harboring strains of *L. monocytogenes*. Further studies are needed to elucidate the mechanisms underlying dissemination of *tmr* among *L. monocytogenes* and other *Listeria* spp.

#### ACKNOWLEDGEMENTS

This work was partially supported by a grant from the American Meat Institute Foundation and USDA grant 2006-35201-17377. We are thankful to all the members of our laboratory for their support and encouragement.

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**Table 1.** *Listeria monocytogenes* strain H7550 and its derivatives used in this study

Strain	BC-20*	CV-15*	CV MIC* (µg/ml)	Source (Reference)
H7550	+	+	75	CDC, 1999
H7550-Cd <sup>s</sup>	-	-	5	Elhanafi et al., 2010
Cd <sup>s</sup> -pPL81	+	+	75	This study
Cd <sup>s</sup> -pPL82	-	+	40	This study
Cd <sup>s</sup> - pPL83	+	+	75	This study
Cd <sup>s</sup> -pPL84	-	+	40	This study
Cd <sup>s</sup> -pPL2	-	-	5	This study

\*Resistance to CV, BC, and CV-MIC were determined as described in Materials and Methods. “+” refers to the confluent growth, and “ - ” refers to no growth on respective agar media.

**Table 2.** Primers used in this study.

Primer	Sequence (5'-3')
BCF:	GACT <u>CCCGGG</u> GATTCTGGAACATCCCTATC
66R:	GGT <u>CCCGGG</u> GCAATGCAATTGTCTATATCAAC
BcF_3:	GACT <u>GGTACC</u> TTCAATTAGATCGAGGCACG
66R2:	GACT <u>GGTACC</u> GCAATGCAATTGTCTATATCAAC
TMR_F:	GACT <u>GGTACC</u> CAATCAATTACGCATTTGTA CTTG
TMR2_F:	GACT <u>GGTACC</u> TCCAGTCTTCAATTGCGGCC
TMR3_F:	GACT <u>GGTACC</u> GAGGCATTCACCTTGTTTAG
qspoVG_F:	GCAAGTAACAGATGTGAGATTAC
qspoVG_R:	GCGAATATCATGAACTACAAATTC
qspoVG_P:	CAATCGCTCTCATTCTTCCATCAGT (Modification: 5' FAM and 3' BHQ1)
qTMR_F :	GAGTCTGGAGCAATTGTTAC
qTMR_R:	CAAGGTTATACGTTTTGTTTTTCATG
qTMR_P:	TTCTCTGTAAGAACCGTTGCG (Modification: 5' FAM and 3' BHQ1)
s1	ATTACAGATGTGAGATTACGACG
s2	ACGTTTACTTGGCATAGCTAC
LtrC_F	CAGAGTGAACGTGATCAGCATC
p1	CATTAGAAGCAGTCGCAAAGCA
p2	GTTTTCGTGTCAGCAGATCTTTGA
0067R	CTGAAATTACGGCAGCGATCTATGAC
0067F	GTCAAATCATTGCCATCGTGCGTA
0065R	CTTCACTCGTTCTACCGTGTTGG

\*underlined sequences correspond to restriction enzyme sites: CCCGGG, XmaI; GGTACC, KpnI.

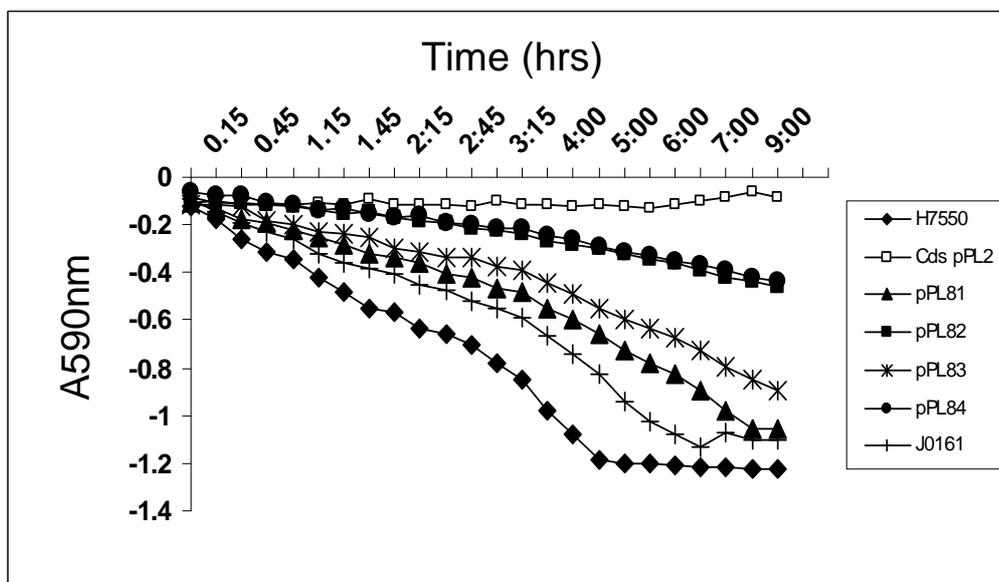
**Table 3.** *L. monocytogenes* strains for which *tmr* presence and CV decolorization was assessed.

Strain	Isolation date	Source	Serotype	Cad determinant	<i>bcrABC</i> PCR	<i>tmr</i> PCR	CV decolorization
L1624a	5/18/2005	Processing Plant	1/2a, 3a	A1	+	-	-
#265E	10/21/04	Processing Plant	1/2a, 3a	A1	+	-	-
101b-4	12/3/2003	Processing Plant	1/2a, 3a	A1	+	-	-
139a-5	2/10/2004	Processing Plant	1/2a, 3a	A1	+	-	-
26-1a	9/24/2003	Processing Plant	1/2a, 3a	A1	+	-	-
339b-5	12/14/04	Processing Plant	1/2a, 3a	A1	+	-	-
367b-1	4/5/05	Processing Plant	1/2a, 3a	A1	+	-	-
494b-1	3/29/2006	Processing Plant	1/2a, 3a	A1	+	-	-
258a-1	10/21/04	Processing Plant	1/2b, 3b	A2	+	-	-
J0161		CDC	1/2a, 3a	A2	+	+	+
F6825	1/1/1989	CDC	1/2a, 3a	A2	+	+	+
F6972	1/24/1989	CDC	1/2a, 3a	A2	+	+	+
#176A	4/13/2004	Processing Plant	1/2a, 3a	A1, A2	+	+	+
#192A	6/15/2004	Processing Plant	1/2a, 3a	A1, A2	+	+	+
#201A	6/15/2004	Processing Plant	1/2a, 3a	A1, A2	+	+	+
#210A	6/15/2004	Processing Plant	1/2a, 3a	A2	+	+	+
231b-1	8/23/2004	Processing Plant	1/2a, 3a	A1, A2	+	+	+
L1712a	6/6/2005	Processing Plant	1/2a, 3a	A2	+	+	+
#171D	4/13/2004	Processing Plant	1/2b, 3b	A1, A2	+	+	+

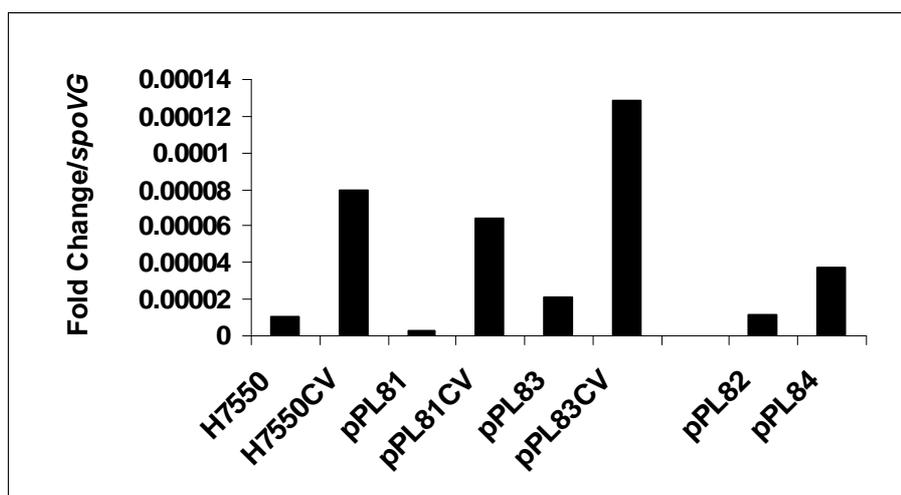
Table 3 continued

#201B	6/15/2004	Processing Plant	1/2b, 3b	A1, A2	+	+	+
#4 MI 16	12/16/04	Processing Plant	1/2b, 3b	A2	+	+	+
128b-2	2/10/2004	Processing Plant	1/2b, 3b	A1, A2	+	+	+
223a-1	8/23/2004	Processing Plant	1/2b, 3b	A1, A2	+	+	+
326b-5	12/14/04	Processing Plant	1/2b, 3b	A1, A2	+	+	+
34-2a	9/24/03	Processing Plant	1/2b, 3b	A1, A2	+	+	+
360b-1	4/5/05	Processing Plant	1/2b, 3b	A1, A2	+	+	+
414a-1	10/13/05	Processing Plant	1/2b, 3b	A1, A2	+	+	+
506b-1	3/29/2006	Processing Plant	1/2b, 3b	Unknown	+	+	+
91a-1	12/3/2003	Processing Plant	1/2b, 3b	A1, A2	+	+	+
#2 MI 40	7/27/2004	Processing Plant	1/2c, 3c	A2	+	+	+
283a-3	10/21/04	Processing Plant	1/2c, 3c	A1	+	+	+
516b-1	3/29/2006	Processing Plant	1/2c, 3c	A1	+	+	+
H7550		CDC	4b	A2	+	+	+
H7738		CDC	4b	A2	+	+	+
H7762		CDC	4b	A2	+	+	+
H7858		CDC	4b	A2	+	+	+
H7962		CDC	4b		+	+	+
J2446	1/1/2003	CDC	4b	A1, A2	+	+	+
J3916	1/1/2006	CDC	4b	Unknown	+	+	+
J5202	9/5/2008	CDC	4b	Unknown	+	+	+
34-6a	9/24/03	Processing Plant	4b	A2	+	+	+
14LA				A1, A2	+	+	+
26LA				A1, A2	+	+	+



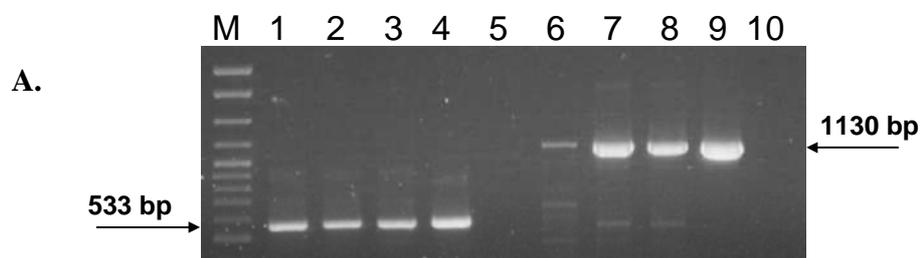


**Figure 2.** *Listeria monocytogenes* strain H7550 and the derivatives of H7550-Cd<sup>S</sup> harboring different *tmr* segments decolorize CV (15 µg/ml) at different rates. The experimental details are provided in Materials and Methods. This graph is a representation of one experiment from three independent trials.

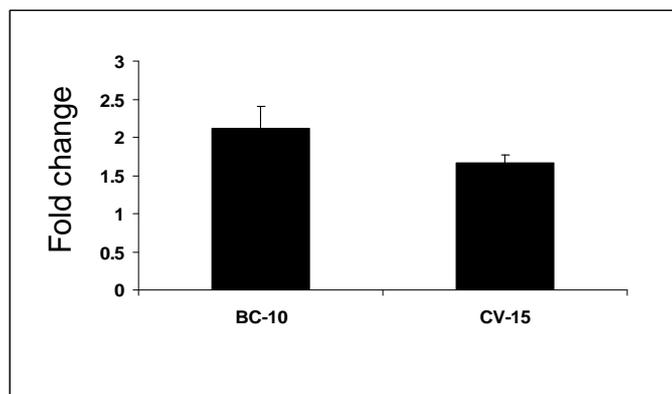


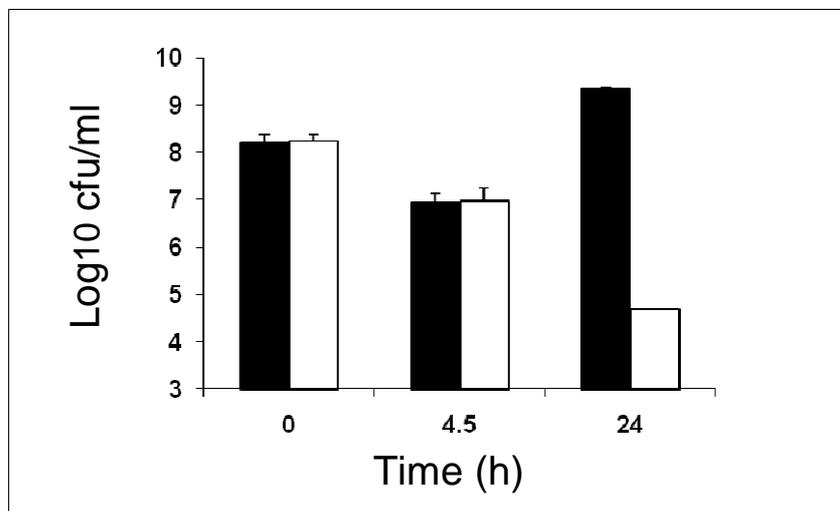
**Figure 3.** *tmr* transcription was constitutive and the transcript levels were increased upon exposure to CV (15  $\mu\text{g/ml}$ ). Quantification of *tmr* was performed using qRT-PCR as described in Materials and Methods. The ratio represents *tmr* transcript levels relative to those of internal control *spoVG* and was calculated by  $\Delta\text{Ct}$  method described before (Pfaffl, 2001).

**Figure 4.** Transcription of *bcrABC* was increased in the presence of CV (15  $\mu\text{g/ml}$ ). **A.** RT-PCR of *spoVG* and *bcrABC*. Lanes 1-5 RT-PCR of *spoVG* using primer s2 (for cDNA) and primers s1,s2 for PCR. Lanes: 1, H7550 in the absence of BC or CV; 2, H7550 exposed to BC-10  $\mu\text{g/ml}$ ; 3, H7550 exposed to CV-15  $\mu\text{g/ml}$ ; 4 and 5, H7550 genomic DNA and total RNA, respectively, used as a positive and negative control for RT-PCR. Arrow points to the expected *spoVG* PCR product of 533 bp. Lanes 6-10 RT-PCR of *bcrABC* using primer p2 (for cDNA) and primers p1,p2 for PCR. Lanes: 6, H7550 in the absence of BC or CV; 7, H7550 exposed to BC-10  $\mu\text{g/ml}$ ; 8, H7550 exposed to CV-15  $\mu\text{g/ml}$ ; 9 and 10, H7550 genomic DNA and total RNA, respectively, used as a positive and negative control for RT-PCR. Arrow points to the expected *bcrABC* PCR product of 1130 bp. M, is a 100 bp to 2686 bp DNA molecular marker XIV (Roche). CV and BC exposures were for 30 min as described in Materials and Methods. **B.** Fold change in the transcript levels of *bcrABC* after exposure to BC and CV. Fold change was determined as described in Materials and Methods using Image J software and the data are averages from two independent trials.



**B.**





**Figure 5.** Leuco crystal violet was not toxic to *L. monocytogenes* strain H7550. Black bars represent colony counts for H7550 upon exposure to CV-15 µg/ml whereas white bars represent colony counts for H7550 upon exposure to CV-15 µg/ml. The data presented here is based on two independent trials.

**Chapter VII: Cadmium resistance determinants of *Listeria* sp.: a mini review.**

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**Author's contribution.**

**VD:** Bioinformatics analysis, data analysis and manuscript writing; **SK:** mentor, data analysis, and manuscript writing

**Publication status.**

To be submitted to Applied and Environmental Microbiology as a Minireview.

## ABSTRACT

The role of bacterial efflux systems as an effective mechanism for resistance to heavy metals including cadmium is widely recognized. The increasing availability of sequenced *Listeria* genomes along with genome-wide transcriptome assessments has provided new insights into the diversity of cadmium resistance determinants in this organism. Three distinct efflux proteins of the P-type ATPase transporter family (designated CadA1, CadA2 and CadA3) have been associated with cadmium resistance in *L. monocytogenes*. All three transporter proteins were accompanied by an upstream transcriptional regulator protein (designated CadC1, CadC2 and CadC3, respectively). *In silico* analysis reveals the presence of invariable *cadA1C1* and *cadA2C2* cassettes in several non-pathogenic *Listeria* spp. and in different serotypes and lineages of *L. monocytogenes*, typically on plasmids. In addition, genomes of several lactic acid bacteria harbored *cadA2C2* cassettes with 100% identity at the DNA sequence level to their counterparts in *Listeria* spp. In *L. monocytogenes*, *cadA2C2* cassettes were significantly more common in strains of lineage I than those of lineage II and were also markedly more common among strains resistant to the quaternary ammonium disinfectant benzalkonium chloride (BC) and harboring the BC-resistance cassette *bcrABC* than among BC-susceptible strains. In contrast to *cadA1C1* and *cadA2C2* which appeared to be plasmid-associated, *cadA3C3* was identified as a component of a conjugative integrative element (ICE) and was only detected in *L. monocytogenes* EGD-e. Interestingly, *cadC3* was found to be strongly expressed in the spleens of mice infected by *L. monocytogenes* EGD-e. The multiple types and complex distribution of cadmium resistance cassettes in *Listeria* spp.

warrant further analysis to elucidate their evolution and contributions to the organism's adaptations and ecology.

## INTRODUCTION

Heavy metals are a common constituent of industrial effluents, biosolids, and compounds for agriculture and medical applications (Smith, 2009). The majority of heavy metals are transition elements which can form complex compounds with redox activities (Nies, 1999; Valls and de Lorenzo, 2002). Structural and valence similarities between heavy metals and other elements assist their translocation into the cytosol via non-specific transport systems (Nies, 1999). Upon entry into the cytoplasm, formation of intracellular non-specific complexes leads either to increased oxidative stress or interference with the normal functioning of enzymes (Nies, 1999; Gong et al., 2000).

Like most other heavy metals, cadmium ions are highly toxic to bacteria. It has been hypothesized that oxidative damage and binding to essential respiratory proteins are the underlying cause for cadmium toxicity (Vallee and Ulmer, 1972; Stohs and Bagchi, 1995). Both Gram-positive and Gram-negative bacteria have shown to use energy-based efflux systems to avoid the toxic effects of cadmium (Nucifora et al., 1989; Silver and Phung, 1996; Lee et al., 2001; Nies, 2003). The cadmium-transporting ATPase, a P-type ATPase that is a member of the cation-transporting ATPase transporter family, remains one of the best characterized mechanisms for both intrinsic and acquired cadmium resistance in bacteria (Nies, 2003).

*L. monocytogenes* is a food-borne pathogen associated with severe illness and death in susceptible individuals (Kathariou, 2002; Kornacki and Gurtler, 2007; Painter and Slutsker,

2007; Scallan et al., 2011). This bacterium is ubiquitous in the environment, and its reservoirs remain unidentified. A number of environmental adaptations such as biofilm formation, cold tolerance, disinfectant resistance and resistance to phage assist the environmental persistence of this pathogen (Gandhi and Chikindas, 2007; Kornaki and Gurtler, 2007; Kim and Kathariou 2009; Elhanafi et al., 2010). Cadmium resistance has also been frequently detected in *L. monocytogenes* to the extent that it has been employed as a strain typing tool (Harvey and Gilmour, 2001).

The first cadmium efflux transporter system identified in *L. monocytogenes* (CadA1C1) was associated with the transposon Tn5422 of the Tn3 family (Lebrun et al., 1994a, 1994b; Fig 1A). A distinct cadmium efflux system (CadA2C2) was found on large plasmids of *L. monocytogenes* and *L. innocua* CLIP (pLI100) (Francis and Thomas, 1997; Glaser et al., 2001; Nelson et al; 2004; Kuenne et al., 2010; Fig 1B). A third cadmium resistance system (CadA3C3) was identified in *L. monocytogenes* strain EGD-e (serotype 1/2a) where the cassette was associated with a Tn916-like conjugative integrative chromosomal element (Glaser et al., 2001; Fig 1C).

In all three cadmium resistance systems CadA was annotated as efflux pump belonging to the E1-E2 ATPase superfamily (PF00122) where the N-terminal has a heavy metal associated domain (PF00403) and the C-terminal harbors haloacid dehalogenase-like hydrolases (acc. number cl11391). At the nucleotide sequence level most identity was seen in between *cadA1* and *cadA3* (ca 70%) whereas *cadA2* exhibited 54% and 51% identity to *cadA1* and *cadA3*, respectively. The GC content for *cadA1*, *cadA2* and *cadA3* was 40%, 37% and 34%, respectively, suggesting that only *cadA3* was noticeably lower in GC content than the genome

average for *L. monocytogenes* genome (ca. 38%). At the aa level CadA1 exhibited 68 and 70% identity with CadA2 and CadA3 respectively, whereas identity between CadA2 and CadA3 was 73%.

In all three cassettes CadC harbored a winged helix-turn-helix DNA-binding domain typical of the GntR family of transcriptional regulators (acc. number cl00088), more specifically functions as a transcriptional repressor (Endo and Silver, 1995; Busenlehner et al., 2001). At the nt level most identity was seen in between *cadC1* and *cadC2* (ca 63%) whereas *cadC3* had 52-53% identity to *cadC1* and *cadC2*. The GC content for *cadC1*, *cadC2* and *cadC3* were 35%, 31% and 28%, respectively. At the aa level CadC1 exhibited 50 and 57% identity to CadC2 and CadC3 respectively, whereas identity between CadC2 and CadC3 was 46%. These findings clearly indicated substantial sequence diversification among these cassettes.

**Prevalence of cadmium resistance in *L. monocytogenes* and co-relation of the cadmium resistance to the known cadmium resistance determinants.** Lebrun et al (1994a) reported that among the *L. monocytogenes* strains ca. 36% were cadmium-resistant with increasing predilection for the food and environmental isolates being cadmium-resistant (Lebrun et al., 1992). Cadmium resistance tended to be plasmid associated, with only a small portion (ca. 12.8%) of cadmium -resistant strains lacking plasmids indicating a chromosomal origin of cadmium resistance (Lebrun et al., 1992). These findings were later confirmed by Mullapudi et al (2008) where more cadmium resistant strains were associated with the environmentally prevalent serotypes 1/2a (or 3a) (83%) and 1/2b (or 3b) (74%), than with clinically prevalent serotype 4b complex (19%) (Fig. 2 A, B). It was hypothesized that the higher predilection of serogroup 1/2 strains (35%) to harbor plasmids than serogroup 4 (15%) might be responsible

for the aforementioned association of cadmium resistance to the food and environmental isolates, which tend to be of serogroup 1/2 (Lebrun et al., 1992; McLauchlin et al., 1997; Harvey and Gilmour, 2001). These findings would imply the crucial role of cadmium resistance mechanisms in environmental prevalence of *L. monocytogenes*, however the effect of harboring cadmium resistance determinants upon the virulence of this pathogen cannot be ignored. Upregulation and significant effect of CadC3 upon virulence of *L. monocytogenes* has been documented (Camejo et al., 2009).

Distribution of *cadA1*, *cadA2* and *cadA3* varied among cadmium-resistant *L. monocytogenes* strains from the environment of turkey slaughter and processing plants in the United States. Prevalence of strains harboring just *cadA1*, just *cadA2*, and both *cadA1* and *cadA2* was 46, 20, and 30%, respectively (Fig. 2B) (Mullapudi et al., 2010). *cadA1C1* was more prevalent among cadmium-resistant serotype 1/2a (or 3a) strains (68%) than among those of serotype 1/2b (or 3b) (17%). In contrast, *cadA2C2* was more common among cadmium-resistant strains of serotype 1/2b (or 3b) (ca. 35%) than among those of serotype 1/2a (or 3a) (7%) (Fig.2B). It is worthy of note that a substantial fraction (30 %) of these cadmium-resistant serogroup 1/2 strains harbored both *cadA1C1* and *cadA2C2* (Fig. 2B) (Mullapudi et al., 2010). Among sequenced genomes of *Listeria*, only one strain (*L. seeligeri* N1-067) was found to harbor both *cadA1C1* and *cadA2C2* (Table 1).

Among the environmental isolates of *L. monocytogenes*, all strains that were resistant to disinfectant BC were also resistant to cadmium (Mullapudi et al., 2008). Incidence of joint cadmium and BC resistance was seen more among strains of serotype 1/2a (or 3a) (60%) and 1/2b (or 3b) (51%) than those of serotype 4b (<10%) (Fig. 2 A, C). Furthermore, the

presence of *cadA2*, alone or together with *cadA1*, was significantly correlated to BC resistance (Fig 2C; Mullapudi et al., 2010). BC resistance has been found to be conferred by *bcrABC*, a gene cassette identified adjacent to *cadA2C2* on pLM80 and closely related large plasmids of *L. monocytogenes* H7858 and J0161, as well as in the genome of *L. seeligeri* N1-067 (den Bakker et al., 2010; Elhanafi et al., 2010) and this genetic linkage between *cadA2C2* and *bcrABC* has been detected in numerous cadmium-resistant, BC-resistant isolates of *L. monocytogenes*, of clinical, food or environmental origin (VD and SK, unpublished findings). The association of *cadA2C2* along with *bcrABC* on a transposon and/or a plasmid would suggest a venue for co-dissemination among other strains. In the presence of this genetic organization, either cadmium or BC would be expected to select for both of the determinants. Certain cadmium-resistant strains, primarily of serotype 4b, did not harbor any of the known cadmium determinants (*cadA1C1*, *cadA2C2*, or *cadA3C3*) (Mullapudi et al., 2010). For these strains it is possible that enhanced expression of one or more of the other E1-E2 ATPase superfamily homologs that we identified (Table 2) may mediate resistance to cadmium, or novel yet unidentified determinants unique to these strains are involved. Genome sequence and transcriptomic characterization of such strains would elucidate this issue.

***In silico analysis.*** *cadA1C1* cassette was identified based on DNA sequence homology with *cadA* sequences in *S. aureus* plasmid pI258 (Fig 1A; Lebrun et. al., 1994a; Lebrun et. al., 1994b; Silver and Walderhaug, 1992). In same study, sub-cloning and transcriptional analysis of *cadA1C1* confirmed the role of this system in cadmium resistance; however unlike the *cadAC* from pI258, *cadA1C1* did not confer resistance to zinc (Yoon and Silver, 1991; Lebrun et al., 1994a). Later *cadA1C1* were associated to a Tn3 family transposon Tn5422, which was

closely related to a previously described Tn917 transposon from *E. faecalis*. Other than *cadA1C1* this transposon also harbored transposase (TnpA) and resolvase (TnpR). Evidence for functional Tn5422 was provided, where Tn5422 was suggested to be involved with intramolecular replicative transposition (Lebrun et al., 1994b). These findings provided an indication pertaining to mode of dissemination for *cadA1C1* genes. This was substantiated by the finding that 95% of the cadmium resistant *L. monocytogenes* isolates harbored *cadA1C1* cassette. *In silico* analysis also suggested that DNA sequences with 100% identity at the DNA sequence level over the entire length of *cadA* and *cadC* were identified on plasmids from several *L. monocytogenes* strains of serotype 1/2a (F2-515, 08-5578), 1/2b (J1-194, R2-503, SLCC2755, LM1), 1/2c (SLCC2372) and serogroup 7 (SLCC2482) (Kuenne et al., 2010; Canchaya et al., 2010, <http://www.broadinstitute.org>; Table 1). Two *L. seeligeri* strains (N1-017 and S4-171) also harbored *cadA1C1*, although their location (chromosomal vs. plasmid) could not be determined due to the unfinished annotation of their genomes (Table 1, den Bakker et al., 2010).

It was not until recently that *cadA1C1* was found to be in close proximity to a copper efflux system (multicopper oxidase; CopA and copper translocating P-type ATPase; CopB) (Fig 1A). Our analysis based on dot blot hybridization assays using a large panel of *L. monocytogenes* strains have suggested that *cadA1C1* were always found in close proximity to *copAB* (SR, VD, SK unpublished). This was later confirmed by Kuenne et al (2010) where the mutual prevalence of *cadA1C1* and *copAB* was reported on plasmids from several strains (pJ1-194, pLM1-2bUG1, pLM7UG1, pLM1-2cUG1, pR2-503, p5578, and pN1-017). Our preliminary reverse transcription PCR analysis have suggested that in *L. monocytogenes* strain

J1-194 the transcription of both *copA* and *copB* was increased upon exposure to sublethal copper sulfate (10mM) (V. Dutta and S. Kathariou, unpublished) indicating the putative role of *copAB* in copper efflux.

In comparison to *cadA1C1*, *cadA2C2* has been much less commonly identified among sequenced genomes of *L. monocytogenes*, having been detected in large plasmids of three different *L. monocytogenes* strains (Francis and Thomas, 1997; Kuenne et al., 2010). Interestingly, two were from *L. monocytogenes* associated with major outbreaks in the United States (the serotype 4b epidemic clone II strain implicated in the hotdog outbreak of 1998-1999 strain H7858 and the serotype 1/2a strain from a turkey deli meats outbreak in 2000 strain J0161 [CDC, 1999, 2002; Olsen et al., 2005; Nelson et al., 2004; Kuenne et al., 2010]). However, genome sequence analysis of non-pathogenic *Listeria* spp. revealed *cadA2C2* on a large plasmid (pLI100) of *L. innocua* CLIP11262 as well as in the genome of *L. seeligeri* N1-067 (which also harbored *cadA1C1*) (Glaser et al., 2001; den Bakker et al., 2010; <http://www.broadinstitute.org>). These sequences exhibited 100% identity at the DNA sequence level with those in *L. monocytogenes*. Of further interest was the identification of *cadA2C2* on a number of plasmids of *Lactococcus lactis* e.g. pGdh442, pAH82, pVF21, pIL5, pAG6, and pNP40, as well as in several other lactic acid bacteria such as *S. dysgalactiae* strain NS3396, *S. thermophilus*, *S. pseudoporcinus* strains LQ 940-04 and SPIN 20026, and *Enterococcus italicus* strain DSM 15952 (Table 1). *E. italicus* and *S. pseudoporcinus* sequences were obtained as a part of a metagenomic Human Microbiome Project with *E. italicus* derived from the human oral cavity.

In *L. monocytogenes* H7550 (1998-1999 outbreak, epidemic clone II) *cadA2C2* are harbored on a large (ca. 80 kb) plasmid, pLM80 (Nelson et al., 2004). Plasmid-cured derivatives were susceptible to cadmium (Elhanafi et al., 2010). In addition, genetic complementation of plasmid-cured derivative (H7550-Cd<sup>s</sup>, cadmium MIC <10 µg/ml) with *cadA2C2*, integrated in the chromosome, rendered the strain resistant to cadmium (MIC, <140 µg/ml compared to the wild type strain Cd MIC >160 µg/ml) (VD, SK unpublished). Transcription of *cadA2* increased approx. 4 fold in the presence of sublethal amounts of cadmium (10µg/ml) (Elhanafi et al., 2010).

In pLM80 *cadA2C2* was present in close proximity to *bcrABC* and *tmr* (triphenylmethane reductase), associated with resistance to benzalkonium chloride (BC) and with the ability to reduce and thus detoxify triphenylmethane dyes, respectively (Fig. 1B; Elhanafi et al., 2010; VD, SK unpublished). Transcription of *cadA2* was induced in the presence of cadmium but not of BC (and inversely, transcription of *bcrABC* was induced in the presence of BC, but not cadmium) (Elhanafi et al., 2010). All three determinants (*cadA2C2*, *bcrABC* and *tmr*) were present on a putative *IS1216* composite transposon, which was also detected on a large plasmid (closely related to pLM80) harbored by the serotype 1/2a strain J0161 (Elhanafi et al., 2010; Kuenne et al., 2010). Thus, even though the determinants and mechanisms for resistance to cadmium, BC and toxic dyes are distinct and independent, their genetic linkage on the plasmid-harbored transposon would facilitate their concurrent dissemination upon selective pressure exerted by any of the toxic compounds (cadmium, disinfectant, or dyes).

Interestingly, the *cadA2C2-bcrABC-tmr* clustering observed in these large plasmids of epidemic-associated *L. monocytogenes* was also observed in the non-pathogenic *L. seeligeri*

N1-067, suggesting dissemination of these mobile elements between pathogenic and non-pathogenic listeriae. Upstream to *cadA2C2* in pLM80 a metal ion ATPase was found. Nucleotide BLAST suggested that identical copy of this ATPase was also found in pLI100, pJ0161, and the genome of *L. seeligeri* strain N1-067 and the ATPase was found upstream to *cadA2C2* in all these genomes. Interestingly truncated copy (99% ID; 79% query) of this gene was found in pDRDC8 (which harbors *cadA2C2*), and the gene was located upstream to *cadA2C2*. ATPase was also found in lactic acid bacteria, *L. lactis* strain KF147 (95% identity), *S. dysgalactiae* strain spp. equisimilis strain NS3396, and *S. pseudoporcinus* strains LQ940-04 and SPIN20026 (97% identity and 89% query coverage each). Except *L. lactis* strain KF147 in all other lactic acid bacteria, metal ion ATPase was found upstream to *cadA2C2* homolog.

Relatively limited information is currently available about the *cadA3C3*, which has to date been detected only in strain EGD-e (Glaser et al., 2001). The cassette is located in the chromosome of EGD-e, as a component of a Tn916-like integrative conjugative element (ICE), and EGD-e is resistant to high levels of cadmium (MIC>140 µg/ml) as are strains harboring *cadA1C1* or *cadA2C2*. The ICE-harboring genomic region in EGD-e substitutes the virulence-associated listeriolysin S cluster (*Listeria* pathogenicity island 3 [LPI-3]) in certain strains of 4b and 1/2b serotype (lineage I) (Fig 1C; Cotter et al., 2008; Cheng et al., 2010). Even though *cadA3* was unique to EGD-e, the 3' end of the gene was similar (75% identity) to its counterpart in *cadA1* (Table 1). Analysis of numerous cadmium-resistant isolates of *L. monocytogenes*, from environmental and other sources, also failed to identify any that harbored *cadA3* (Mullapudi et al., 2010; SR and SK, unpublished findings). However, a

sequence highly homologous to *cadA3* (100 % identity over 91% of the gene) in the integrated conjugative element CIME19258 of *Streptococcus thermophilus* (Pavlovic et al., 2004); less conserved *cadA3* homologs (75% identity over 81% of the gene) were detected in the genomes of *Enterococcus faecalis* strains TX0411, TX2134 and TX0635 from a metagenomic Human Microbiome Project (Table 1). However, *cadA3* in these genomes of *S. thermophilus* and *E. faecalis* was accompanied by putative regulatory genes (putative *cadC*) with pronounced divergence (40-58 % identity at the nucleotide sequence level) from *cadC3* of EGD-e, suggesting a unique *cadC* sequence in the latter strain. This is of interest in the context of the finding that in mice infected intravenously with EGD-e, *cadC3* expression in spleen was significantly increased and strain deficient in *cadC* had an impaired virulence compared to the wild type indicated the crucial role played by *cadC3* in *L. monocytogenes* virulence. The details pertaining to *CadC3 in vivo* function remain limited and need further investigation (Camejo et al., 2009).

**Other *cadA* determinants in *L. monocytogenes*.** Sequencing of the genome of *L. monocytogenes* 08-5578, implicated in large listeriosis outbreak in Canada (2008) revealed that this strain harbored *cadA1C1* on a plasmid as well as a novel *cadA* determinant (*cadA4*) on the same plasmid (Table 1; Gilmour et al., 2010). The novel *cadA4* exhibited 55-57% identity at the nucleotide sequence level to *cadA1*, *cadA2* and *cadA3* (30-32% identity at the deduced polypeptide level). The GC content of this *cadA4* was unusually high (45%), and unlike the cadmium resistance systems discussed above, this *cadA* was not accompanied by a transcriptional regulator. Putative transposases present at the 5' and 3' of *cadA4* suggest transposon-mediated transmission of this determinant. Conserved (100% identity over the

entire gene) homologs of this *cadA* were detected on plasmids of several *L. monocytogenes* strains, mostly of serotype 1/2a or 1/2b (Table 1). These plasmids also harbored *cadA1C1*, except for pJ0161 (from the strain implicated in the 2000 turkey deli meats outbreak; Olsen et al., 2005) which harbored *cadA2C2*. Furthermore, *cadA4* was detected in pLI100 which harbored *cadA2C2*, and in *L. seeligeri* N1-067, which in addition, harbored both *cadA1C1* and *cadA2C2* (Table 1). Experimental evidence for the role of *cadA4* in cadmium resistance remains to be obtained.

CadA homologs were identified via protein BLAST analysis of the completely sequenced genomes of *L. monocytogenes* strain EGD-e (serotype 1/2a) and F2365 (serotype 1/2b). Six homologs were identified (lmo1100, lmo1853, lmo0818, lmo2681, lmo0841, and lmo2689), belonging to the E1-E2 ATPase superfamily (Table 2). Three of these transporter proteins (lmo1100, lmo1853 and lmo2689) harbored a transcriptional regulator upstream. The deduced polypeptide of lmo0064, annotated as *cadA*, exhibited the highest similarity to the CadA1, CadA2, CadA3, and the novel *cadA4* of the Canadian outbreak strain encoded by pLM5578\_p74 (Table 2). Further studies are needed to assess the involvement of these ORFs in cadmium tolerance of *L. monocytogenes*. These chromosomal putative *cadA* sequences were highly conserved among different sequenced *L. monocytogenes* genomes along with *L. innocua* CLIP 11262 (data not shown).

### **Transcriptome analysis of the cadmium transporters and their associated genes.**

Genome-wide transcriptome analysis has shed some light on the putative alternative role, other than the anticipated cadmium/heavy metal efflux, played by the cadmium resistance systems and their homologs (Camejo et al., 2009; Toledo-Arana et al., 2009; Chatterjee et al.,

2006; Table 1). Cadmium resistance systems and their homologs showed differential transcriptional patterns under *in vitro* conditions such as low oxygen environment and low temperatures; however, what is even more intriguing but remains poorly characterized is their expression *in vivo* (Chatterjee et al., 2006; Camejo et al., 2009; Toledo-Arana et al., 2009). In *L. monocytogenes*, multidrug efflux systems (*mdrM* and *mdrT*, of the MFS family) were found to play critical roles for activation of the host cytosolic surveillance pathway and IFN- $\beta$  production in infected macrophages (Crimmins et al., 2008). These transporters were later found to efflux c-di AMP into the cytoplasm which in turn led to IFN- $\beta$  production (Woodward et al., 2010).

Chatterjee et al. (2006) first looked at global gene expression of *L. monocytogenes* EGD-e levels in the vacuolar and cytosolic environment using murine macrophage-like cell lines. Toledo-Arana et al (2009) described the complete operon map for *L. monocytogenes* EGD-e and analyzed global transcriptional patterns under selected *in-vivo*, *ex-vivo* and *in-vitro* conditions. Camejo et al. (2009) used a mouse infection model to characterize transcription of *L. monocytogenes* EGD-e *in vivo* (spleen) to *in vitro* (exponential and stationary growth phase at 37°C) conditions.

Of the six CadA homologs identified in the chromosome of *L. monocytogenes* (Table 2), three (lmo0818, lmo1852 and lmo02689) were part of multicistronic units (operons-128, 328 and 483, respectively) (Table 3). Operon 128 consisted of four ORFs and the transporter protein lmo0818 was up-regulated inside macrophages. In operon-328, lmo1852 (the putative *cadA*) was up-regulated in the intestine (Camejo et al., 2009; Toledo-Arana, 2009; Table 3). For operon-483 the TetR family regulator located immediately upstream to putative *cadA*

(lmo2689) was up-regulated in the intestine, blood, and within macrophages; however, lmo2689 expression was not found to be different from the control housekeeping genes (Chatterjee et al., 2006; Toledo-Arana et al., 2009; Table 3).

Putative *cadA* transporters proteins that were monocistronic were lmo0641, lmo0841, lmo1100, and lmo2681. ORFs with winged helix-turn-helix (W-HTH) DNA binding domain from GntR superfamily of transcriptional regulators were found upstream of lmo0841 and lmo1100. lmo0641 was down-regulated in the intestine, blood, and in the intracellular environment; but up-regulated in the spleen. lmo0841 and the putative transcriptional regulator upstream (lmo0840) were up-regulated within macrophages and only lmo0841 was up-regulated in the spleen, but down-regulated in the intestine and blood (Table 3). Transcription of lmo1100 (*cadA3*) was up-regulated within macrophages whereas lmo1102 (*cadC3*) was strongly down-regulated in the spleen (Table 3). lmo2681 was up-regulated in the intestine, blood and macrophages, but down-regulated in the spleen (Table 3).

The lack of strong correlation between the findings from different studies may be attributed to different model systems used for the transcriptome analysis. These transcriptional data clearly indicate the differential role played by these transporters in the intracellular survival of *L. monocytogenes* in various host tissues. More work is needed to identify the details regarding the overall contribution of these transporters in the environmental persistence and perhaps virulence of *L. monocytogenes*.

**Conclusion.** In this review we have provided details about putative *L. monocytogenes* cadmium efflux systems while highlighting their putative role/s in the environmental persistence of this pathogen. Our knowledge about the effect of harboring these systems on

listerial pathogenesis is limited. Existing data provide enough rationale to further investigate the role of cadmium resistance systems and their homologs on host-pathogen interactions. Such information would not only broaden our knowledge about bacterial ecology, but will also help us to devise better control and treatment strategies for *Listeria*.

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**Table 1.** Nucleotide homologs for known *L. monocytogenes* cadmium resistance systems in *Listeria* and other bacterial genomes.

	<b>Orf#</b>	<b>Genus</b>	<b>Species</b>	<b>Strain</b>	<b>Serotype</b>	<b>Identity (Query coverage)</b>	<b>Plasmid/ Genome</b>	<b>Reference</b>
<b>cadA1 (2136 bp)</b>	p0039	Listeria	monocytogenes	SLCC27 55	1/2b	100 (100)	pLM1- 2bUG1	Kuenne et al., 2010
	p0031	Listeria	monocytogenes	SLCC24 82	7	100 (100)	pLM7UG 1	Kuenne et al., 2010
	p0030	Listeria	monocytogenes	SLCC23 72	1/2c	100 (100)	pLM1- 2cUG1	Kuenne et al., 2010
	p0010	Listeria	monocytogenes	Lm1	1/2b	100 (100)	pLM33	Canchaya et al., 2010
	p0064	Listeria	monocytogenes	08-5578	1/2a	100 (100)	pLM5578	Gilmour et al., 2010
	p0048	Listeria	monocytogenes	R2-503	1/2b	100 (100)	pR2-503	Kuenne et al., 2010
	p0044	Listeria	monocytogenes	J1-194	1/2b	100 (100)	pJ1-194	Kuenne et al., 2010
	p0031	Listeria	monocytogenes	F2-515	1/2a	100 (100)	pF2-515	Kuenne et al., 2010
	p0037	Listeria	monocytogenes	N1-017*	4b or 1/2b	100 (100)	pN1-017	Kuenne et al., 2010
	NT04LS_1 626	Listeria	seeligeri	S4-171	-	100 (100)	Genome	den Bakker et al., 2010
	NT03LS_3 049	Listeria	seeligeri	N1-067	-	100 (100)	Genome	den Bakker et al., 2010
	lmo1100	Listeria	monocytogenes	EGD-e	1/2a	75 (27)	No known plasmid	Glaser et al., 2001

Table 1 Continued

	cadA	Streptococcus	thermophilus	-	-	76 (27)	Genome	Pavlovic et al., 2004
<b>cadC1 (360 bp)</b>	p0038	Listeria	monocytogenes	SLCC27 55	1/2b	100 (100)	pLM1- 2bUG1	Kuenne et al., 2010
	p0030	Listeria	monocytogenes	SLCC24 82	7	100 (100)	pLM7UG 1	Kuenne et al., 2010
	p0029	Listeria	monocytogenes	SLCC23 72	1/2c	100 (100)	pLM1- 2cUG1	Kuenne et al., 2010
	p0009	Listeria	monocytogenes	Lm1	1/2b	100 (100)	pLM33	Canchaya et al., 2010
	p0063	Listeria	monocytogenes	08-5578	1/2a	100 (100)	pLM5578	Gilmour et al., 2010
	p0047	Listeria	monocytogenes	R2-503	1/2b	100 (100)	pR2-503	Kuenne et al., 2010
	p0043	Listeria	monocytogenes	J1-194	1/2b	100 (100)	pJ1-194	Kuenne et al., 2010
	p0030	Listeria	monocytogenes	F2-515	1/2a	100 (100)	pF2-515	Kuenne et al., 2010
	p0036	Listeria	monocytogenes	N1-017*	4b or 1/2b	100 (100)	pN1-017	Kuenne et al., 2010
	NT04LS_1 627	Listeria	seeligeri	S4-171	-	100 (100)	Genome	den Bakker et al., 2010
NT03LS_3 050	Listeria	seeligeri	N1-067	-	100 (100)	Genome	den Bakker et al., 2010	
Table 1 Continued <b>cadA2 (2118 bp)</b>	p0026/pCT 008	Listeria	monocytogenes	DRDC8	4b	100 (100)	pDRDC8	Kuenne et al., 2010; Francis

p0082	Listeria	monocytogenes	J0161	1/2a	100 (100)	pJ0161	and Thomas, 1997
p0080/ pLM80_0083	Listeria	monocytogenes	H7858	4b	100 (100)	pLM80	Kuenne et al., 2010
pli0061	Listeria	innocua	CLIP 11262	-	100 (100)	pLI100	Kuenne et al. 2010; Nelson et al., 2004
cadA	Listeria	seeligeri	N1-067	-	100 (100)	Genome	Glaser et al., 2001
ICESde33 96_29	Streptococcus	dysgalactiae	NS3396	-	100 (100)	Genome	den Bakker et al., 2010
cadA	Lactococcus	lactis	-	-	100 (100)	pGdh442	Davies et al., 2009
cadA	Lactococcus	lactis	-	-	100 (100)	pNP40	Tanous et al., 2005
cadA	Lactococcus	lactis spp. lactis	-	-	100 (100)	pAH82	O'Driscoll et al., 2004
pIL5_5	Lactococcus	lactis spp. lactis	IL594	-	100 (100)	pIL5	O'Sullivan et al., 2000
PVF_pVF 21p13	Lactococcus	lactis			100 (100)	pVF21	-
Table 1 Continued							
cadA	Lactococcus	lactis spp. cremoris	-	-	100 (100)	pAG6	-
cadA	Streptococcus	thermophilus	-	-	100 (100)	Genome	

	cadA	Streptococcus	pseudoporcinus	LQ 940-04 SPIN	-	100 (100)	Genome	-
	cadA_2	Streptococcus	pseudoporcinus	20026 DSM	-	100 (100)	Genome	-
	cadA	Enterococcus	italicus	15952	-	100 (100)	Genome	-
<b>cadC2 (360 bp)</b>	p0025/pCT007	Listeria	monocytogenes	DRDC8	4b	100 (100)	pDRDC8	Kuenne et al., 2010; Francis and Thomas, 1997
	p0081	Listeria	monocytogenes	J0161	1/2a	100 (100)	pJ0161	Kuenne et al., 2010
	p0079/pLM80_0082	Listeria	monocytogenes	H7858 CLIP	4b	100 (100)	pLM80	Nelson et al., 2004
	pli0060	Listeria	innocua	11262	-	100 (100)	pLI100	Glaser et al., 2001
	cadC	Listeria	seeligeri	N1-067	-	100 (100)	Genome	den Bakker et al., 2010
	ICESde339_6_28	Streptococcus	dysgalactiae	NS3396	-	100 (100)	Genome	Davies et al., 2009
	cadC	Lactococcus	lactis	-	-	100 (100)	pGdh442	Tanous et al., 2005
Table 1 Continued								
	cadC	Lactococcus	lactis	-	-	100 (100)	pNP40	O'Driscoll et al., 2004
	cadC	Lactococcus	lactis spp. lactis	-	-	100 (100)	pAH82	O'Sullivan et al., 2000
	pIL5_4	Lactococcus	lactis spp. lactis	IL594	-	100 (100)	pIL5	-
	cadC	Lactococcus	lactis spp.	-	-	100 (100)	pAG6	-

<b>cadA3 (2124 bp)</b>	cadC	Streptococcus	cremoris thermophilus	-	-	100 (100)	Genome	-	
	cadC	Streptococcus	pseudoporcinus	LQ 940- 04	-	100 (100)	Genome	-	
	cadC_2	Streptococcus	pseudoporcinus	SPIN 20026	-	100 (100)	Genome	-	
	cadC	Enterococcus	italicus	DSM 15952	-	100 (100)	Genome	-	
	lmo1100	Listeria	monocytogenes	EGD-e	1/2a	100 (100)	No known plasmid	Glaser et al., 2001 Pavlovic et al., 2004	
	cadA	Streptococcus	thermophilus	-	-	100 (91)	Genome	-	
	cadA	Enterococcus	faecalis	TX0411	-	75 (81)	Genome	-	
	cadA	Enterococcus	faecalis	TX2134	-	75 (81)	Genome	-	
	cadA	Enterococcus	faecalis	TX0635	-	75 (81)	Genome	-	
	p0039	Listeria	monocytogenes	SLCC27 55	1/2b	75 (28)	pLM1- 2bUG1	Kuenne et al., 2010	
	p0031	Listeria	monocytogenes	SLCC24 82	7	75 (28)	pLM7UG 1	Kuenne et al., 2010	
	Table 1 Continued								
	p0030	Listeria	monocytogenes	SLCC23 72	1/2c	75 (28)	pLM1- 2cUG1	Kuenne et al., 2010	
p0010	Listeria	monocytogenes	Lm1	1/2b	75 (28)	pLM33	Canchaya et al., 2010		
p0064	Listeria	monocytogenes	08-5578	1/2a	75 (28)	pLM5578	Gilmour et al., 2010		
p0048	Listeria	monocytogenes	R2-503	1/2b	75 (28)	pR2-503	Kuenne et al., 2010		
p0037	Listeria	monocytogenes	N1-017*	4b or	75 (28)	pN1-017	Kuenne et al.,		

					1/2b				2010
	p0044	Listeria	monocytogenes	J1-194	1/2b	75 (28)	pJ1-194		Kuenne et al., 2010
	p0031	Listeria	monocytogenes	F2-515	1/2a	75 (28)	pF2-515		Kuenne et al., 2010
	NT04LS_1 626	Listeria	seeligeri	S4-171	-	75 (28)	Genome		den Bakker et al., 2010
	NT03LS_3 049	Listeria	seeligeri	N1-067	-	75 (28)			den Bakker et al., 2010
<b>cadC3</b> <b>(336 bp)</b>	lmo1100	Listeria	monocytogenes	EGD-e 1/2a	-	100 (100)	No known plasmid		Glaser et al., 2001
	pli0048	Listeria	innocua	CLIP 11262	-	100 (100)	pLI100		Glaser et al., 2001
<b>pLM5578</b> <b>_p74</b> <b>(cadA4)</b> <b>(1884 bp)</b>									
	p0028	Listeria	monocytogenes	SLCC27 55	1/2b	100 (100)	pLM1- 2bUG1		Kuenne et al., 2010
Table 1 Continued									
	p0026	Listeria	monocytogenes	N1-017*	4b or 1/2b	100 (100)	pN1-017		Kuenne et al., 2010
	p0030	Listeria	monocytogenes	R2-503	1/2b	100 (100)	pR2-503		Kuenne et al., 2010
	-	Listeria	monocytogenes	J0161	1/2a	100 (100)	pJ0161		Kuenne et al., 2010
	NT03LS_3 501	Listeria	seeligeri	N1-067	-	100 (100)	Genome		den Bakker et al., 2010

\*Strain N10017 was listed as serotype 4b, but multiplex PCR (Doumith et al., 2004) provided a profile typical for serotype 1/2b (R. M. Silezky and S. Kathariou, unpublished findings).

Table 2. CadA homologs within *L. monocytogenes* strain EGD-e.

<b>EGD-e homolog</b>	<b>cadA1</b>	<b>cadA2</b>	<b>cadA3</b>	<b>pLM5578_p74 (cadA4)</b>
lmo1100	70 (99)	73(99)	100 (99)	34 (95)
lmo0641				
cadA	37(88)	35 (86)	37 (87)	44 (91)
lmo1853	30 (98)	28(99)	29 (99)	32 (92)
lmo0818	23(75)	22 (83)	29 (69)	29 (80)
lmo2681	27(66)	27 (63)	24 (75)	29 (63)
lmo0841	25(75)	24 (78)	31 (58)	28 (70)
lmo2689	23 (73)	22 (76)	23 (67)	23 (77)

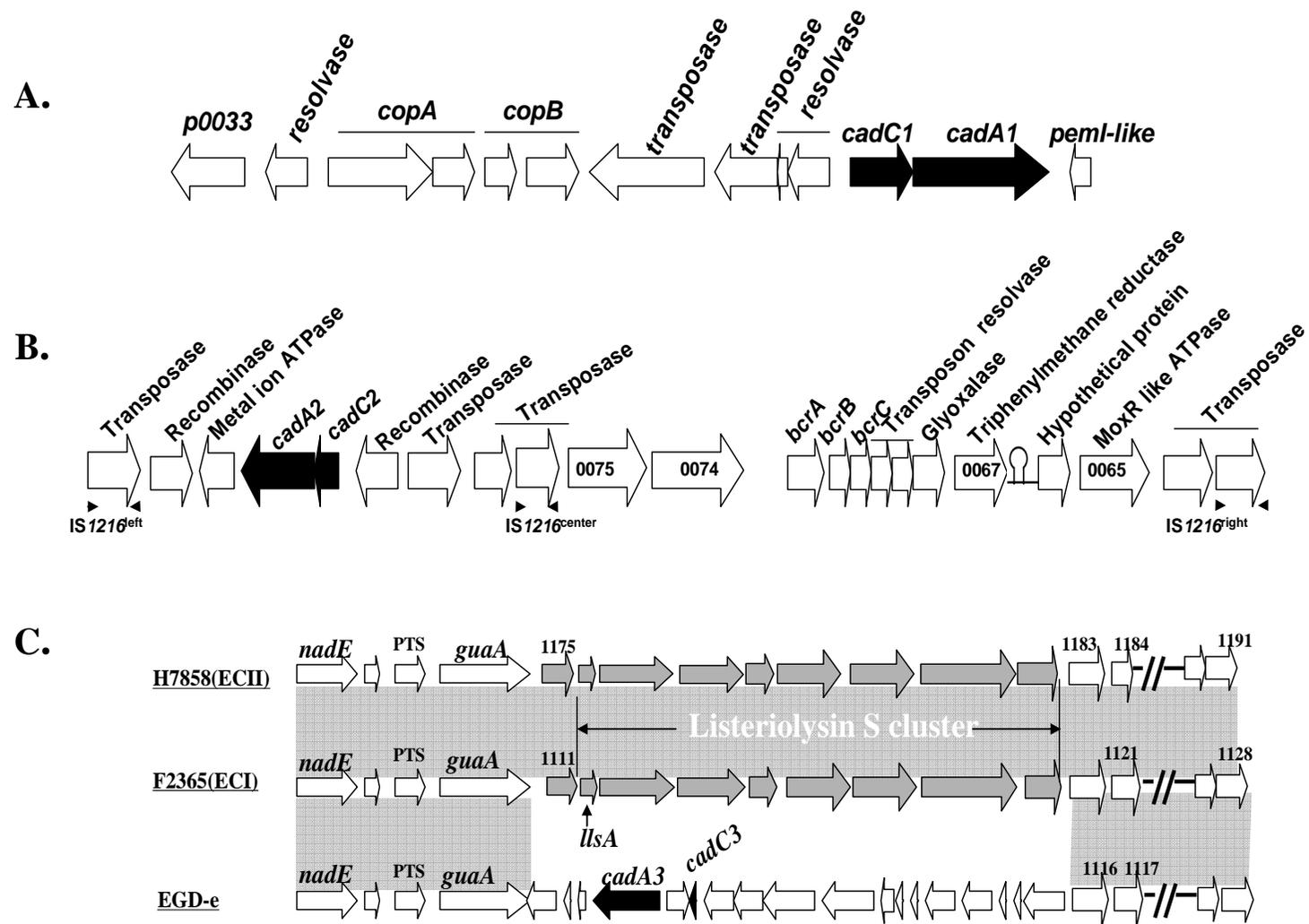
**Table 3.** Summary of transcriptome analysis of the cadmium resistance genes and their homologs in *L. monocytogenes* EGDe based on three different studies (Chatterjee et al., 2006; Camejo et al., 2009; Toledo-Arana et al., 2009).

EGD-e homolog	Conserved Domain	GC%	Operon number	Toledo-Arana et al., 2009	Chatterjee et al., 2006	Camejo et al., 2009	
						Comparative to log phase	Comparative to stationary phase
<b>lmo0641 cadA</b>	cadmium-translocating P-type ATPase	41	Not on an operon	Downregulated in blood and intestine	downregulated	no data	upregulated
<b>lmo0841</b>	E1-E2 ATPase superfamily	42	Not on an operon	Downregulated in blood and intestine	no data	upregulated	
lmo0840	WHTH-DNA binding domain GntR superfamily	33	Not on an operon	no data	no data	upregulated	no data
<b>lmo0818</b>	E1-E2 ATPase superfamily	42	operon 128	Upregulated in blood and intestine	upregulated	no data	no data
lmo0819	no known conserved domain	37	operon 128	Upregulated in intestine	upregulated	no data	no data
lmo0820	N-Acyltransferase superfamily	39	operon 128	Downregulated in intestine	upregulated	no data	no data
lmo0821	no known conserved domain	34	operon 128	Downregulated in intestine	upregulated	no data	no data
<b>lmo1100</b>	E1-E2 ATPase superfamily	34	Not on an operon	no data	upregulated	no data	no data

Table 3 Continued

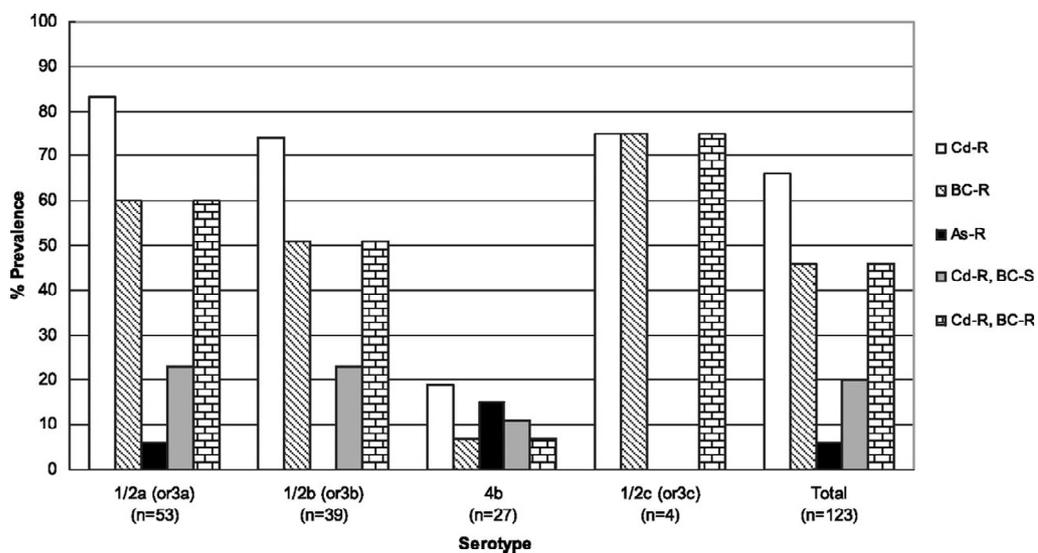
lmo1102	WHTH-DNA binding domain GntR superfamily	28	Not on an operon	N/A	no data	upregulated	no data
lmo1852	Heavy-metal-associated domain	38	operon 328	Upregulated in intestine	no data	no data	no data
<b>lmo1853</b>	Cation transport ATPase/ E1-E2 ATPase	40	operon 328	Downregulated in intestine	no data	no data	no data
lmo1854	Transcriptional regulators CsoR (copper-sensitive operon repressor)	38	operon 328	Downregulated in blood and intestine	no data	no data	downregulated
<b>lmo2681/kdpB</b>	E1-E2 ATPase superfamily- type-I	44	Not on an operon	Upregulated in blood and intestine	upregulated	downregulated	no data
lmo2686	no known conserved domain	32	operon 483	Downregulated in blood	no data	no data	no data
lmo2687		42	operon 483	N/A	no data	no data	no data
lmo2688	FTSW_RODA_SPOVE FTSW_RODA_SPOVE	42	operon 483	no data	no data	no data	no data
<b>lmo2689</b>	Magnesium-translocating P-type ATPase	41	operon 483	N/A	no data	no data	no data
lmo2690	TetR family transcriptional regulator	36	Not on an operon	Upregulated in blood and intestine	upregulated	no data	no data

**Figure 1. A.** Genetic organization of *cadA1C1* cassette on Tn5422 transposon as described (Kuenne et al., 2010.) **B.** Genetic organization of *cadA2C2* cassette on putative IS1216 transposon on pLM80 in *L. monocytogenes* H7858. Three copies of IS1216 are indicated as IS1216<sup>left</sup>, IS1216<sup>center</sup>, and IS1216<sup>right</sup> with solid arrows indicating the orientation of IS1216 inverted repeats (Modified from Elhanafi et al., 2010). **C.** Genomic organization of *cadA3C3* in region 1168 in *L. monocytogenes* EGD-e strain and comparing the region to other *L. monocytogenes* strains H7858 (ECII serotype 4b), F2365 (ECI serotype 1/2a). Homologous regions are linked by shading. The listeriolysin S cluster (*Listeria* pathogenicity island 3 [LIPI-3]) is indicated. (Modified from Cheng et al., 2010).



**Figure 2. A.** Prevalence of resistance to cadmium, BC, and arsenic among isolates of *L. monocytogenes* of different serotypes from the environment of turkey-processing plants. Resistance and susceptibility were determined as described in Materials and Methods. Cd-R, cadmium resistant; BC-R, BC resistant; As-R, arsenic resistant; Cd-R, BC-S, resistant to cadmium but susceptible to BC; Cd-R, BC-R, resistant to both cadmium and BC (Mullapudi et al., 2008) **B.** Prevalence of cadmium resistance determinants among *L. monocytogenes* isolates of different serotypes from turkey processing plant environments. Cadmium resistance determinants *cadA1* and *cadA2* were detected by Southern blot analyses using probes derived from *cadA* of Tn5422 and pLM80, respectively. The total includes three strains of serotype 1/2c (or 3c) and five of the serotype 4b complex (Mullapudi et al., 2010). **C.** Prevalence of cadmium resistance determinants among *L. monocytogenes* strains differing in susceptibility to BC. Cd<sup>r</sup> BC<sup>s</sup>, resistant to cadmium but susceptible to BC; Cd<sup>r</sup> BC<sup>r</sup>, resistant to both cadmium and BC (Mullapudi et al., 2010).

**A.**



**B.**

