ABSTRACT

AZIZOGLU, REHA ONUR. "Temperature-Dependent Freeze-Thaw Tolerance and Genetic Characterization of Stress Response Mechanisms of *Listeria* spp." (Under the direction of Dr. Sophia Kathariou.)

Listeria monocytogenes is a gram-positive foodborne pathogen that can cause severe illness (listeriosis) with high fatality rate. *L. monocytogenes* is ubiquitous in the environment and encounters a number of different stress conditions, including freeze-thaw stress. The growth temperature of the bacteria plays an important role in expression of several key virulence factors. However, our understanding of the impact of growth temperature on *Listeria*'s stress responses remains limited. In this study, we investigated the impact of growth temperature on the freeze-thaw tolerance of *Listeria* spp. *Listeria* cells grown at 37^{o}C showed significantly higher tolerance against repeated freezing and thawing then cells grown at 4^{o}C or 25^{o}C ($p<0.05$). In order to address if this phenomenon is seen in other psychrotrophic foodborne pathogens, we investigated the impact of growth temperature on the freeze-thaw tolerance of *Yersinia enterocolitica*. However, impact of growth temperature on the cryotolerance of *Y. enterocolitica* was opposite of that observed with *Listeria* spp: *Yersinia* cells grown at 4^{o}C were markedly more tolerant to the damaging effects of repeated freezing and thawing than 37^{o}C-grown bacteria.

To identify genes responsible for the observed temperature-dependent cryotolerance of *L. monocytogenes* we constructed mutant libraries of two *L. monocytogenes* strains, F2365 (serotype 4b) and 10403S (serotype 1/2a). The mutant libraries were constructed with a *mariner*-based transposition system and high efficiency of transposon insertion was achieved. However, screening directly for impaired cryotolerance following repeated freezing and thawing in a 96-well plate format did not

allow consistent differentiation between 37^{o}C-grown and 4^{o}C-grown cultures. Therefore, our subsequent studies focused on screening for the loss of tolerance to the stresses expected to take place during repeated freeze-thaw treatments. The stresses included oxidative stresses, cold stress, and osmotic stress. One mutant in a putative oxidative stress gene was identified based on its susceptibility to paraquat and was found to have markedly impaired freeze-thaw tolerance. Thermoregulated control of cryotolerance resembles that of key virulence genes but is exhibited by both pathogenic and non-pathogenic species of *Listeria.* Growth temperature-dependent cryotolerance may represent an adaptation of ancestral *Listeria* lineages that preceded the emergence of non-pathogenic clades, and may involve thermoregulated attributes at the transcriptional or proteomic levels. The impact of growth temperature on cryotolerance of *Listeria* was unexpected, and underlying mechanisms remain to be identified.

Temperature-Dependent Freeze-Thaw Tolerance and Genetic Characterization of Stress Response Mechanisms of *Listeria* spp.

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

Food Science

Raleigh, North Carolina

2009

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BIOGRAPHY

Reha Onur Azizoglu was born on February 20th, 1979 in Yigilca, Turkey. He grew up in Ankara, the capital of Turkey, with his parents, Sunduz and Halit, and elder brother Suha. He proudly attended TED Ankara Private High School. After graduation he pursued his Bachelor of Science degree in the Department of Food Engineering at Middle East Technical University. He graduated in June 2002, and in Fall 2002 he was awarded a fellowship by the Turkish Republic Ministry of National Education to pursue his graduate studies in the United States. In summer 2003, he began his graduate studies in Department of Food Science at North Carolina State University under the mentorship of Dr. MaryAnne Drake. During his graduate studies he married to Eda Azizoglu. Upon completion of his Masters degree, he joined Dr. Sophia Kathariou's research group to pursue doctoral degree at North Carolina State University in Food Science with a minor in Biotechnology.

ACKNOWLEDGEMENTS

I am thankful to my mentor, Dr. Sophia Kathariou, for all of her support, understanding, and encouragement throughout my study. Her scientific insight stimulated my interest in broadening my knowledge of the basic principles in every aspect of this research. I am grateful to her for giving me the opportunity to improve myself in Microbial Genetics. I would also like to acknowledge the members of my committee, Dr. Ilenys Diaz-Perez, Dr. MaryAnne Drake, and Dr. Hosni Hassan for their guidance to attain my degree.

I would like to thank Robin for all her technical assistance and friendship throughout my research. I would like to thank Driss, Suleyman, Ying, and Jae-Won for their help during the setup of my research. I also would like to acknowledge the past and current members of Kathariou lab: Joo-Sung, Sandra, Lili, Carol, Ertugrul, Sangmi, Sheea, Vikrant, Judy, and Mohammed, for their friendship.

My parents and my brother always supported and encouraged me throughout my life. Without them I would never be able to achieve my degree.

Finally, I would like to thank my lovely wife, Eda. She always supported and encouraged me, without her love I wouldn't be able to complete this degree. It is a great feeling to now that she is always there for me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: Literature Review

1.1. *Listeria monocytogenes* and Listeriosis

Listeria monocytogenes was first described by Murray et al. in 1926. It was called *Bacterium monocytogenes*, because of its monocytosis characteristic seen in laboratory rabbits and guinea pigs (Farber & Peterkin, 1991). In 1927, Pirie isolated an identical bacterium from gerbils (African jumping mouse) and named it *Listeria hepatolytica*. Pirie suggested the genus name *Listeria* in 1940 in honor of Lord Lister, one of the pioneers in the field of antiseptics and disinfection. *L. monocytogenes* was first isolated from humans by Nyfeldt in 1929. He isolated the bacterium from 3 patients that had infectious mononucleosis-like disease (Gray & Killenger, 1966).

The *Listeria* genus contains six species: *L. monocytogenes, L. seeligeri, L. ivanovii, L. innocua, L. welshimeri* and *L. grayi*. Among these six species *L. monocytogenes* and rarely *L. ivanovii* are potentially pathogenic to humans (Vazquez-Boland et al. 2001). *L. monocytogenes* is a gram-positive, non-sporeforming, facultative anaerobic, facultative intracellular rod. It has the ability to grow between 1 and 45^{o}C (Farber & Peterkin, 1991), has peritrichous flagella and motile at temperatures between 20^{o}C and 28^{o}C (Allerberger, 2003). *L. monocytogenes* is catalase positive, oxidase negative and produces clear zones on blood agar, because of its β-hemolysin activity (Farber & Peterkin, 1991). It has the ability to survive over a wide range of pH-values from 4.3 to 9.0, and salt concentrations up to 10%. In addition *Listeria* has better growth under reduced oxygen conditions compared to anaerobic or strictly aerobic conditions, and can therefore be designated as a facultatively microaerophilic organism (Allerberger, 2003).

The genus *Listeria* has low GC content (36-42%). Based on the somatic (O) and flagellar (H) antigens *Listeria* strains are divided into 13 serotypes; 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Allerberger, 2003).

Listeria spp. are ubiquitously distributed in the environment and isolated from different environmental sources such as soil, water, effluents, large variety of foods, and the feces of humans and animals. The natural habitat for *Listeria* is the decomposing plant materials, in which they live as saprophytes (Vazquez-Boland et al. 2001). Three main routes of transmission of *L. monocytogenes* to humans are foods, cross-infection of neonates in hospital, and (primarily for skin infections) contact with animals (Bell & Kyriakides, 2005, pg 16).

First reported transmission of *L. monocytogenes* to humans by contaminated food occurred in 1981 in Canada, which was traced back to contaminated coleslaw (Sclech et al. 1983). Since then with the advancements in the subtyping tools, the understanding of the transmission through foods to humans has improved. Coleslaw, unpasteurized cheese, pasteurized milk, seafood, processed meat products, and leafy vegetables are some food products that have been contaminated with *L. monocytogenes* (Bell and Kyriakides, 2005, pg 17-19). Especially at risk are cold stored ready-to-eat (RTE) foods, because of *Listeria'*s ability to grow at refrigeration temperatures.

L. monocytogenes has the ability to cause severe illness (listeriosis). People at high risk for the infection with *L. monocytogenes* are neonates, pregnant women, elderly and immunocompromised people (Farber et al., 1991, Schlech, 2000). Listeriosis is a systemic disease, which has severe complications such as; meningitis, liver failure,

septicemia, abortion, and central nervous system infections. Reported *L. monocytogenes* cases indicated that it can cause both sporadic cases and outbreaks of listeriosis. Mead et al. (1999) reported that approximately 2500 human cases of *L. monocytogenes* per year occur in the United States with a 28% death rate, which makes it a big concern for food safety. In a recent report in MMWR (CDC, 2007) a significant decline was reported in the laboratory-diagnosed cases of *L. monocytogenes* compared to 1996-1998 period. On the other hand, the lowest point of cases was seen in 2002, and in 2006 the incidence of *L. monocytogenes* infections were higher than this lowest point (CDC, 2007), which indicates *L. monocytogenes* still possesses a high risk for food safety.

1. 1. 1. Outbreaks of *L. monocytogenes*

On average 2500 cases of listeriosis and about 500 deaths are reported per year in the United States (Gellin et al. 1991). More than 95% of human listeriosis cases are caused by strains of serotype 1/2a, 1/2b, and 4b. Among these serotypes strains of serotype 4b are more frequently responsible for foodborne outbreaks (Kathariou 2002, McLauchlin 1990). *L. monocytogenes* has been isolated from variety of foods such as; uncooked meats and vegetables, ready to eat foods, unpasteurized milk, soft cheeses, and cold cuts at deli counters (Bula et al. 1995, Linnan et al. 1988, Schwartz et al. 1989). Some of the major outbreaks of listeriosis are listed in Table 1.

Table 1. 1: Major Foodborne outbreaks of listeriosis (Chen et al. 2005, Kathariou, 2003)

Year/Location	Source	Serotype	No. Case (deaths)
1979/Boston, USA	Vegetable	4b	20 (5)
1981/Nova Scotia, Canada	Coleslaw	4b	41 (18)
1983/Boston, USA	Pasteurized milk	4b	49 (14)
1983/Boston, USA	Vegetable	4b	
1983-1987/Switzerland	Soft Cheese	4b	122 (34)
1985/California, USA	Mexican-style cheese	4b	142 (48)
1985-1987/Denmark	NA	4b	
1988/USA	Turkey franks	1/2a	
1989/UK	Patè	4b	
1992/France	Jellied pork tongue	4b	279 (85)
1998-1999/Multistate, USA	Hot dog	4b	105 (21)
2000/Multistate, USA	Turkey deli meat	1/2a	
2001/North Carolina, USA	Cheese	4b	12
2002/Multistate, USA	Turkey deli meat	4b	54

1. 1. 2. Molecular virulence determinants of *L. monocytogenes*

Among the species of *Listeria* genus *L. monocytogenes* is the only one that has the ability to cause serious invasive illness (listeriosis) in both humans and animals. As a facultative

intracellular organism, *L. monocytogenes* can invade and replicate in the epithelial cells and macrophages. The studies of the virulence of *L. monocytogenes* in mouse and guinea pig models indicated that *L. monocytogenes* is taken up by enterocytes or M cells near Peyer's patches in the small intestine and then multiplies in phagocytic cells (MacDonald & Carter, 1980). Later, the bacteria lyse the vacuole and escape into the cytoplasm by listeriolysin O (LLO) encoded by *hly*. The secretion of hemolysin is crucial for the intracellular growth of the organism and recognition of T-cell of the host (Farber & Peterkin, 1991). In the cytoplasm, *L. monocytogenes* can replicate and move by the actin-based motility. The bacteria continue its intracellular life cycle by the formation of double-membrane protruding pseudopods that are taken by the adjacent cells. *L. monocytogenes* lyses these membranes and follows the same intracellular life cycle.

1. 1. 2. 1. Cellular adhesion and entry to epithelial cells

L. monocytogenes cause the infection by invading the intestinal epithelial cells upon ingestion of the contaminated foods. Since the intestinal epithelial cells are not phagocytic, the bacteria need to adhere to the surface receptors of the host cells by their internalins to enter these cells (Dramsi et al. 1993, Dramsi et al. 1997, Gaillard et al. 1991, Gaillard et al. 1996). The adhesion of *L. monocytogenes* to the target cell plays an important role in its intracellular life cycle. Currently, it is known that two internalins, InlA and InlB, play the major role for *L. monocytogenes* adhesion and the entry to epithelial cells. Among these internalins, InlA, mediates the adherence of *L. monocytogenes* to mammalian cells. It is previously reported that the mutants of *inlA* gene shows less binding to Caco-2 epithelial cells (Bergmann et al. 2002, Reglier-Poupet

et al. 2003). Structurally, the InlA of *L. monocytogenes* is characterized by leucine-rich repeats (LRR) and they are covalently linked to peptidoglycan by LPXTG motif at the carboxyl terminus. Other member of the internalin family, InlB, involves in the entry to broad range of cell lines, including hepatocytes and nonepithelial cells.

1. 1. 2. 2. Intracellular life cycle

Upon entry to cells, *L. monocytogenes* lyses the vacuole by listeriolysin O (LLO) encoded by *hly*, along with the phospholipases (PLCs). Hemolytic activity of *L. monocytogenes* is the result of the action of LLO, and it is the major virulence determinant (Kuhn & Goedel, 2007). LLO belongs to the family of thiol-activated, cholesterol-dependent, pore-forming toxins (Palmer, 2001). LLO is essential for the escape from the primary and secondary intracellular vacuoles. Previous studies indicated that all virulent *L. monocytogenes* strains have the hemolytic activity; on the other hand, the strains lacking the hemolytic activity are avirulent (Cossart et al. 1989).

Two listerial phospholipases, phosphatidylinositol (PI)-PLC and phosphatidylcholine (PC)-PLC play roles in the LLO-independent escape of *L. monocytogenes* from primary vacuoles in human epithelial cells (Marquis et al. 1995). It was also reported that these enzymes act synergistically with LLO in the lyses of primary and secondary vacuoles (Camilli et al. 1993, Gedde et al. 2000).

Following its escape from the phagocytic vacuole, *L. monocytogenes* starts replicating in the cytoplasm of the host cell. The movement of the cells in the cytoplasm is crucial for their cell-to-cell spread. A surface protein, ActA, promotes the movement of

L. monocytogenes in the cytoplasm of the host. ActA, induces the polymerization of actin filaments (Dussurget et al. 2004).

1. 1. 2. 3. Virulence factors

Genes involved in the pathogenicity of *L. monocytogenes* are clustered together on a pathogenicity island, and they are regulated by a transcriptional regulator encoded by *prfA* (Fig. 1). The virulence genes present in this island include *prfA, plcA, hly, mpl, actA,* and *plcB*. These genes encode PrfA, PI-PLC, LLO, metalloprotease, ActA, and PC-PLC, respectively (Vazquez-Boland et al. 2001). In addition to these six well-characterized genes, *inlA* and *inlB* are involved in the virulence of *L. monocytogenes*. Although, these two genes are present in a different location on the chromosome, they are under partial control of *prfA*. The expression of these genes is regulated by a 14-bp palindromic sequence, called PrfA box (Sheehan et al. 1996). The expression of the *prfA* gene is regulated by temperature and the expression is high at 37^{o}C, and impaired below 30^{o}C. Johansson et al. (2002) explained this by the formation of secondary structure in untranslated mRNA preceding the *prfA,* which blocks the ribosome binding region. Other identified virulence factors in *L. monocytogenes* include *bsh* (bile salt hydrolase) (Dussurget et al. 2002), and p60 (involved in cell division) (Bubert et al. 1992).

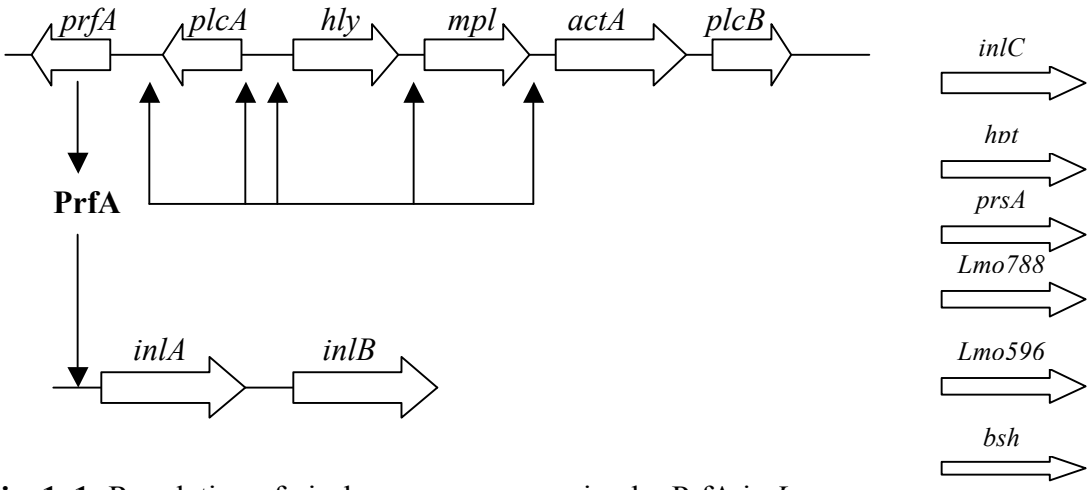

Fig. 1. 1: Regulation of virulence gene expression by PrfA in *L. monocytogenes.* Modified from Sheenan et al. (1994).

1. 1. 3. Genetics of *L. monocytogenes*

Following the recognition of *L. monocytogenes* as a foodborne pathogen, and the development of genetic tools, our understanding about this pathogen greatly improved. *L. monocytogenes* serotype 1/2a strain EGD-e and *L. innocua* were sequenced first. It was previously reported that *L. monocytogenes* serotype 4b strain F2365 has single circular chromosome, and average GC content is 38% (Nelson et al., 2004). The comparison of whole genome sequence of three *L. monocytogenes* strains indicated that genomic differences are results of phage insertions, transposable elements and single nucleotide polymorphisms (Nelson et al., 2004). *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b are predominant cause of listeriosis, even though 13 serotypes of *L. monocytogenes* have the ability to cause human listeriosis (Farber et al., 1991). By using different molecular typing methods, such as ribotyping, pulsed field gel electrophoresis (PFGE), resriction fragment length polymorphism (RFLP) etc., the evolution and the population genetics of

L. monocytogenes is explored. By using these tools *L. monocytogenes* is 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, and 4c (Piffaretti et al. 1989, Call et al. 2003, Rasmussen et al. 1995, Wiedmann et al. 1997).

It was also reported that the epidemic isolates of 4b strains show distinct genetic characteristics than other strains of the same serotype. It is suggested that these strains are members of distinct lineages and designated as epidemic clone (EC) I, ECII, ECIII, ECIV or ECV (Kathariou, 2002).

1. 3. 1. 1. Epidemic Clone I (ECI)

It was previously reported that the majority of the *L. monocytogenes* outbreaks in Europe and North America was caused by ECI strains (Kathariou, 2002). *L. monocytogenes* strains associated with the following outbreaks are characterized as ECI; Nova Scotia, Canada (coleslaw, 1981), California (coleslaw, 1981), California (Mexican-style cheese, 1985), and France (pork tongue in aspic, 1992). The ECI strains share unique characteristics. Zheng and Kathariou (1997) reported that the genomic DNA of ECI strains were resistant against digestion by the restriction enzyme *Sau3*AI, by the methylation of the cytosine at GATC site. ECI strains have unique RFLPs, which differentiate them from the other serotype 4b strains (Zheng and Kathariou; 1997;Tran et al. 2002). In addition, it was found that ECI strains harbor a number of genomic fragments and gene clusters that are not present in other serotype 4b strains (Chen and Knabel 2007, Yildirim et al. 2004). Herd and Kocks (2001) reported several genomic fragments or gene clusters, DNA fragment 85, specific to ECI strains. Later Yildirim et

al. (2004) indicated that this region was internal to a gene with putative involvement in cytosine methylation at GATC site. This fragment was part of a gene cassette consisting of three genes; 85R (5-methyl cytosine restriction), 85M (methylase), and 85S (endonuclease *Sau3*AI) (Yildirim et al. 2004). This suggests that ECI-specific cassette has the GATC-specific restriction-modification system responsible for the observed resistance against digestion with restriction enzyme *Sau3*AI.

In addition, the genomic comparison of a fully sequenced ECI strain F2635 with other sequenced strains revealed four unique genomic island including Lmof2365_2798 to Lmof2365_2800, Lmof2365_2701 to Lmof2365_2707, Lmof2365_2347 to Lmof2365_2348, and Lmof2365_0687 (Chen and Knabel 2007). Among these regions Lmof2365_2798 corresponds to 17B fragment, and it was previously identified as ECI-specific region by macroarray analysis (Herd and Kocks, 2001). Therefore, 17B fragment along with the 85M is used as ECI-specific markers.

Above all, multilocus enzyme electrophoresis (MLEE) and ribotyping results identified strains from two listeriosis outbreaks as another serotype 4b epidemic clones. These strains were involved in the pasteurized milk outbreak in Massachusetts in 1983, and the Boston outbreak (source was not identified) in 1985 (Piffaretti et al. 1989). Although these strains were closely related to ECI, some genomic regions specific to ECI, including the cytosine methylation at GATC sites, were absent (Zheng and Kathariou 1997). Recent multilocus genotyping (MLGT) indicated that ECIa strains were closer to ECII than ECI (De Cesare et al. 2001, Ducey et al. 2007). Further analysis is required to have a full understanding of this clonal group.

1. 1. 3. 2. Epidemic Clone II (ECII)

The multistate outbreak of listeriosis between August 1998 and March 1999 was associated with contaminated hot dog and 101 human cases were reported with 21 deaths (CDC 1998, and CDC 1999). The strains associated with this outbreak have unique genetic fingerprints determined by ribotyping and PFGE. Therefore, these strains were designated as Epidemic Clone II (ECII). Another multistate outbreak of listeriosis in the United States 2002 was associated with contaminated turkey deli meat. The PFGE and the ribotyping of the strains isolated from this outbreak revealed that they were closely related to the strains associated with 1998-1999 outbreak (CDC, 2002).

It was previously reported that one serotype 4b-specific genomic region, designated "genomic region 18" was diversified in ECII strains (Evans et al. 2004). This region is used to differentiate ECII strains from other serotype 4b strains, by using PCR or Southern blots. The absence of a PCR product or signal in Southern blot indicates that the strain is a ECII strain (Evans et al. 2004; Cheng et al. 2007). The genome of ECII strain H7858 (hot dog isolate of 1998-1999 multistate outbreak in the USA) was sequenced (Nelson et al. 2004). The genomic comparison of ECI and ECII strains revealed a number of genes and gene clusters unique to ECII strains.

1. 1. 3. 3. Epidemic Clone III (ECIII)

The multistate listeriosis outbreak in 2000 in the United States, resulted in 29 cases in 10 states and it was associated with contaminated turkey deli meat (CDC, 2000). Unlike other outbreaks, the associated strain in this outbreak belongs to serotype 1/2a. The genotype analysis of this strain revealed that it had the same genotype with an early

human isolate associated with the consumption of contaminated turkey franks in 1988 (CDC, 1989). Surprisingly, the turkey deli meat implicated in the outbreak in 2000 was from the same processing plant as the turkey franks implicated in the previous outbreak in 1998. This indicates that the bacteria survived over several years without any detectable genotypic change in the facility (Kathariou, 2002). The ECIII strain F6854 was sequenced and as a result of the sequence analysis nine ECIII-specific genomic loci were identified.

1. 1. 3. 4. Epidemic Clone IV (ECIV)

Two isolates from the paté outbreak in the UK in 1989 and the vegetable outbreak in Boston in 1979 showed the same ribotype and multi-virulence-locus sequence typing (MVLST) results. These isolates were designated as ECIV (Chen et al. 2005). ECIV isolates have not been fully characterized yet. The MVLST data indicated that the phylogenetically ECIV strains are closely related to the ECIa strains.

1. 1. 3. 5. Epidemic Clone V (ECV)

The *L. monocytogenes* isolate associated with the Mexican style cheese outbreak was designated as ECV (MacDonald et al. 2005, Cheng et al. 2008). It was reported that these strains are highly similar to genes in the ECII strain specific region 18. On the other hand, other ECII specific regions are absent. Further characterization of this clonal group is necessary to fully understand the specific regions of this group.

1. 2. Stress Response

Stress can be defined as any deleterious factor or condition that adversely affects the growth or survival of microorganisms. Foodborne bacteria are subjected to number of stresses both in the environment, and during food preservation and processing. Stress factors may be chemical, physical, or biological (Vorob'eva, 2003). In order to survive during these stress conditions bacteria should adapt to these changing environments. As a result of stress, microorganisms may initiate one of the following response mechanisms (Yousef et al., 2002);

- Production of proteins that repair damage, maintain the cell, or eliminate the cell, or stress agent.
- Transient increase in resistance or tolerance to deleterious factors.
- Cell transformation to a dormant state, i.e., spore formation.
- Evasion of host organism defenses.
- Adaptive mutations.

The response of the bacteria against the stress conditions can be sudden such as response to shock, or long-term adaptation (Hengge-Aronis, 1999). Previously, it was reported that the stationary growth phase, and prior sub-lethal stress conditions induced the production of protective proteins and improve the resistance of the cell against subsequent stress conditions (Arnold et al., 1995). A number of studies indicated that the general stress factor, Sigma B, whose activity is stimulated by stationary phase, plays an important role in the adaptation of cells to the stress conditions (Becker et al. 2000, Wemekamp-Kamphuis et al. 2004, Chaturongakul et al. 2006). Becker et al. (2000)

reported the importance of sigmaB for adaptation of stationary phase cells against low temperature by coordinating the accumulation of betaine and carnitine as cryoprotectants.

1. 2. 1. Cold stress

As the ability of *L. monocytogenes* to grow at low temperatures possesses a big concern for the food industry numerous studies were conducted to understand the response mechanisms of the organism at low temperatures. So far low temperature response mechanisms described include changes in the cell membrane fatty acid composition (Annous et al. 1997), uptake of compatible solutes such as glycine betaine etc. (Smith, 1996, Verhaul et al., 1997, Ko et al. 1999, Smiddy et al., 2004), and production of cold stress proteins (Csps), and cold acclimation proteins (Caps) (Wemekamp-Kamphius et al. 2002, Liu et al. 2002, Chan et al. 2007, Tasara et al., 2006).

1. 2. 1. 1. Change in cell membrane fatty acid composition

In order to grow at low temperatures, it is important for bacteria to maintain optimum membrane fluidity. As the temperature drops, the state of the membrane changes from liquid-crystalline to gel-phase. Therefore, cellular changes occur to maintain the liquid-crystalline state. Altering the cell membrane fatty acid composition is the main determinant of providing the necessary liquid-crystalline state for the bacteria by lowering the liquid to solid phase transition temperatures. The main molecular changes occurring in the cell-membrane lipids to adapt low temperatures include decrease in fatty acid chain length, increase in fatty acid unsaturation, and change in branching of fatty acids (Tasara et al., 2006). The cell membrane of *L. monocytogenes* is

composed high concentrations of iso- and anteiso-, odd-numbered, and branched fatty acids (Julak et al., 1989, Raines et al,. 1968).

Previously, Annous et al. (1997) investigated the cell membrane fatty acid composition change in the cells grown at different temperatures (5^{o}C, 10^{o}C, 20^{o}C, 30^{o}C, 37^{o}C, 42^{o}C, and 45^{o}C). They reported two modes of cell membrane fatty acid concentration change during the adaptation to low growth temperatures; fatty acid chain length shortening and change in branching of the fatty acids from iso to anteiso (Annous et al. 1997). They reported that major fatty acids in 37^{o}C-grown cells of *L. monocytogenes* strains 10403S, and SLCC 53 were anteiso-$C_{15:0}$ (41%), anteiso-$C_{17:0}$ (32%), and iso-$C_{15:0}$ (13%) and in cells grown at 5^{o}C they showed that the composition of anteiso-$C_{17:0}$ fatty acids decreased and iso-$C_{15:0}$ fatty acids increased, indicating the shortening and the change in the branching of the fatty acid chain length as the growth temperature decrease. Between the cells grown at 20^{o}C and 5^{o}C the branching of the fatty acids changed from iso- to anteiso-.

1. 2. 1. 2. Accumulation of compatible solutes

Another cold adaptation mechanism of *L. monocytogenes* is the accumulation of osmolytes and short peptides (compatible solutes). Osmolytes are low-molecular-weight organic compounds that have minimal impact on normal cellular functions in bacteria and can be accumulated to high intracellular concentrations with minimal effect on normal functions in bacteria (Sleator et al. 2001). *L. monocytogenes* does not have the capability to synthesize compatible solutes intracellularly. However, the presence of them in the growth media, such as glycine betaine, and carnitine, enhanced the survival of *L.*

monocytogenes at low temperatures (Bayles et al. 2000, Beumer et al. 1994, Ko et al. 1999, Mendum et al. 2002, Smith 2000). Although *L. monocytogenes* cannot synthesize these compatible solutes, glycine betaine and carnitine are readily available in the environment as they are present at high levels in foods from plant and animal origin (Rhodes et al. 1993). The uptake of these compatible solutes from the extracellular environment is achieved by three membrane transporter systems. Membrane transporter systems for glycine betaine uptake are BetL (betaine porter I), and GbuABC (betaine porter II) (Ko et al. 1999, Sleator et al. 1999, Sleator et al. 2000), and for carnitine uptake is OpuC (Fraser et al., 2000, Verhaul et al. 1995). BetL is a secondary uptake system by accumulating glycine betaine with Na^+-motive force; on the other hand, Gbu ABC and OpuC are ATP-dependent transportation system (Fraser et al. 2000, Ko et al. 1999).

1. 2. 1. 3. Protein production at low temperatures

Temperature is a key environmental signal for regulating the gene expression of the bacteria, which is essential for their survival in environment. Recently, the gene expression profile of *L. monocytogenes* serotype 1/2a strain 10403S was compared between the cultures grown at 4^{o}C and 37^{o}C by using whole genome microarray (Chan et al. 2007). The gene expression of cells grown at 4^{o}C until logarithmic or stationary growth phase was compared with the gene expression of cells grown at 37^{o}C until logarithmic or stationary phase. They reported increased expression in 245 genes when the cells were grown at 4^{o}C compared to cells grown at 37^{o}C (Table 2). Among these genes 30 were upregulated regardless of growth phase. Genes that showed increased expression levels at 4^{o}C included genes related to cold adaptation (such as *flaA, opuC,*

gbuC etc.). The cells grown at 4°C showed decreased expression of genes related to virulence (*hly, plcA* and*, plcB*) and heat shock response (*groES*) (Table 3). In addition, cells that were grown until stationary phase at 4°C showed decreased expression of alternative sigma factor, *sigL*, which was found to be important for cold growth in *Bacillus subtilis,* and additional virulence factors, *inlA, inlB,* and *inlC.* In a similar study, Liu et al. (2002) studied the expression of certain genes of *L. monocytogenes* serotype 1/2a strain 10403S involved in cold-adaptive responses, regulatory adaptive responses, general microbial stress responses, amino acid metabolism, cell surface alterations, and metabolism, and found increased expression of all these genes when the cells were grown at 10°C, when compared to cells grown at 37°C. In both studies identified genes as increased expression showed overlapping, with the exception of *groEL*, which is a heat shock protein. Liu et al. reported an increase in the expression of this gene at low temperatures. In another study, Wemekamp-Kamphius et al. (2002) reported that cold shock of *L. monocytogenes* serotype 1/2c strain LO28 at 10°C after growth at 37°C elevated the production of cold shock proteins (Csps) and resulted in increased protection against further stresses, including freezing, and high-pressure treatment.

Table 1. 2: *L. monocytogenes* 10403S genes upregulated at 4°C compared to 37°C. The genes that showed at least 7.00 fold increase in expression at 4°C compared to 37°C were chosen for this table (Chan et al. 2007).

Growth Phase	Gene	Function	Fold change
logarithmic	lmo0189	Highly similar to *B. subtilis*Veg protein	12.23
logarithmic	lmo0987	Similar to *Streptococcus agalactiae* CylB protein	7.55
logarithmic	lmo1364 (*cspL*)	Similar to cold shock protein	7.14
logarithmic	lmo2336 (*fruB*)	Fructose-1-phosphate kinase	10.63
logarithmic	lmo2335 (fruA)	Highly similar to phosphotransferase system (PTS) fructose-specific enzyme IIABC component	13.03
logarithmic	lmo2522	Similar to hypothetical cell wall binding protein from *B. subtilis*	10.57
stationary	lmo0823	Similar to oxidoreductases	16.99
stationary	lmo1364 (*cspL*)	Similar to cold shock protein	7.01
stationary	lmo1864	Similar to hemolysinIII proteins, putative integral membrane protein	8.26
stationary	lmo2211 (*hemH*)	Similar to ferrochelatase	7.58
stationary	lmo2668	Similar to transcriptional antiterminator (BglG family)	7.44
stationary	lmo2667	Similar to PTS system galactitol-specific enzyme IIA component	9.39
stationary	lmo2666	Similar to PTS system galactitol-specific enzyme IIB component	7.33
stationary	lmo2664	Similar to sorbitol dehydrogenase	9.87
stationary	lmo2663	Similar to polyol dehydrogenase	9.44

Table 1. 3: *L. monocytogenes* 10403S genes downregulated at 4°C compared to 37°C. The genes that showed at least 7.00 fold decrease in expression at 4°C compared to 37°C were chosen for this table (Chan et al. 2007).

Growth Phase	Gene	Function	Fold change
logarithmic	lmo0202 (*hly*)	Listeriolysin O precursor	-20.74
logarithmic	lmo0355	Similar to flavocytochrome C fumarate reductase chain A	-9.22
logarithmic	lmo2016 (cspB)	Similar to major cold-shock protein	-36.11
stationary	lmo0043	Similar to arginine deiminase	-8.61
stationary	lmo0202 (*hly*)	Listeriolysin O precursor	-26.09
stationary	lmo0434	Internalin B	-13.79
stationary	lmo0445	Similar to transcription regulator	-7.43
stationary	lmo0916	Similar to phosphotransferase system enzyme IIA	-7.50
stationary	lmo1068	unknown	-20.21
stationary	lmo1140	unknown	-14.51
stationary	lmo1348	Similar to aminomethyltransferase	-7.15
stationary	lmo1883	Similar to chitinases	-10.23
stationary	lmo2132	unknown	-7.92
stationary	lmo2175	Similar to dehydrogenase	-7.65
stationary	lmo2206 (*clpB*)	Similar to endopeptidase Clp ATP-binding chain B (ClpB)	-15.68
stationary	lmo2391	Conserved hypothetical protein similar to *B. subtilis* YhfK protein	-8.20
stationary	lmo2828	unknown	-17.93

1. 2. 2. Freeze-thaw stress

As a result of the environmental temperature drop or during the storage and preservation of foods, bacteria can be subjected to freezing stress. Freezing and thawing, which involves several stress conditions, cause injury to cells. During freezing the severity of freezing damage depends on several factors including the rate of freezing, in

which the slow freezing cause more severe damage than the rapid freezing as larger ice crystals can be formed (Gao et al. 2000). It was previously reported that gram-positive bacteria are more resistant against freezing than the gram-negative bacteria because of the high peptidoglycan composition in their cell wall (Chang et al., 2003).

It is now known that the oxidative stress occurring during thawing contributes to the freeze-thaw injury of the cells (Hermes-Lima et al. 1993, Stead et al. 2000). Stead et al. (2000) reported the importance of oxidative stress during freezing and thawing as superoxide dismutase deficient *Campylobacter coli* showed decreased freeze-thaw tolerance compared to wild type. Garénaux et al. (2008) also mentioned the involvement of oxidative stress during the freeze-thaw stress in *Campylobacter jejuni.* Vishnivetskaya et al. (2007) showed that cold acclimation of a psychrotrophic gram-positive bacterium, *Exiguobacterium,* resulted in increased tolerance against repeated freezing and thawing. They also reported that regardless of temperature, growth on solid media compared to liquid media resulted in increased tolerance of the bacteria against repeated freezing and thawing.

L. monocytogenes may be exposed to freezing as well as thawing in the course of its existence in natural environments (e.g. soils and water in temperate or cold regions) as well as during the storage and preservation of foods. However, cryotolerance in *Listeria* remains relatively understudied. El-Kest et al. (1991) showed that freezing caused the formation of intracellular and extracellular ice crystals that resulted in injury or death of bacteria. Wemekamp-Kamphuis et al. (2004) provided evidence for the role of *sigB* in cryotolerance of *L. monocytogenes* LO28. However, the impact of growth temperature

on cryotolerance of *L. monocytogenes* is poorly understood, and mechanisms underlying cryotolerance are unidentified.

1. 2. 3. Oxygen stress

L. monocytogenes is subjected to various stresses during its existence in the environment and during its intracellular lifecycle. One of these stresses is oxidative stress. During its intracellular lifecycle, *L. monocytogenes* is faced with oxygen stress during its escape from the phagocytic cells. Reactive oxygen species (ROS) are reactive agents that are the by-products of aerobic metabolism and produced during the oxidative burst in phagocytic cells (Latifi et al. 2009). ROS are generated as intermediates of O_2 reduction (Imlay, 2003). These reactive oxygen species (ROS) cause severe damage to DNA, protein, and lipids in the cell (Martindale and Holbrook, 2002). ROS includes powerful oxidizing agents such as singlet oxygen ($^1O_2$), superoxide anion (O_2^{-}.), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) (Latifi et al. 2009). The singlet oxygen ($^1O_2$) is resulted from the energy input to oxygen, and it is highly reactive. The singlet oxygen ($^1O_2$) has a short life in cells (Gorman & Rodgers, 1992), and it reacts with proteins, pigments, and lipids. The other three oxygen-reduction intermediates (O_2^{-}., H_2O_2 and $OH^{\cdot}$) shows different reactivities, toxicity levels, and targets as their intrinsic characteristics are different (Latifi et al. 2009). Both O_2^{-}., and $OH^{\cdot}$ are highly reactive with biomolecules as they have unpaired electron. The O_2^{-}. oxidizes the [4Fe–4S]$^{2+}$clusters to [3Fe–4S]$^{1+}$ by releasing iron (Fe^{2+}). It cannot diffuse through the membrane because of its negative charge. The reaction rate of $OH^{\cdot}$ is under the control of diffusion rate as it is highly reactive.

The H_2O_2 is less reactive than others however it can be reduced to hydroxyl radical by Fenton reaction ($Fe^{2+} + H_2O_2 \longrightarrow OH^- + FeO^{2+} + H^+ \longrightarrow Fe^{3+} + OH^- + OH^-$) and can be highly toxic. Both O_2^{-}. and H_2O_2 are mutagens as they lead to the release of the Fenton-active ferrous iron, leading to hydroxyl radical production that can cause extensive lesions on DNA (Imlay, 2003, & Latifi et al. 2009).

To survive under the damaging effect of ROS, living organisms developed various stress response mechanisms. These mechanisms can be grouped into two; enzymatic and nonenzymatic. Enzymatic defense mechanisms include the production of catalases, superoxide dismutases (SOD) and peroxidases. Nonenzymatic mechanisms include the production of antioxidant compounds such as glutathione, vitamin A, C, E, carotenoids, etc. When the cells cannot produce enough antioxidants to inactivate the oxidants, the organisms face with oxidative stress. The damaging effect of oxygen stress can range from cell death in bacteria to serious pathologies in higher organisms (Latifi et al. 2009).

L. monocytogenes initiates stress response mechanisms to cope with oxidative stress by producing enzymes such as superoxide dismutase, and catalase (Vazquez-Boland et al. 2001). Another oxygen stress response mechanism reported in *B. subtilis* is the production of an alkyl hydroperoxide reductase that has not yet been identified as a component of *Listeria*'s oxidative stress response (Hellmann et al. 2003). Previously, it was reported that catalase and superoxide dismutase (SOD) play role in the survival of *L. monocytogenes* during the intracellular life cycle. Both of these enzymes work together during the detoxification of ROS (Mostertz et al. 2004, and Vazquez et al. 2001).

Superoxide anions generated by the oxidative burst in phagocytic cells are converted to H_2O_2 by SOD, followed by conversion of toxic H_2O_2 into water (H_2O) and oxygen (O_2) by catalase (Mongkolsuk et al. 2002). Welch et al. (1979) reported that the two-catalase negative mutants of *L. monocytogenes* showed at least two-fold increased SOD activity indicating that catalase and SOD can take over the role of each other when one is inhibited. Leblond-Francilland et al. (1989) reported that the two catalase-negative mutants of *L. monocytogenes*, constructed by insertion of Tn*1545*, showed no difference from the wild type in the level of virulence in mice. Also, the mutants and the wild type were able to grow inside the host cells. Therefore, they stated that the catalase does not play a crucial role in the macrophage survival of *L. monocytogenes*. This finding was supported by some reported isolation of catalase-negative *L. monocytogenes* from listeriosis patients (Bubert et al. 1997, Elsner et al. 1996, Swartz et al. 1991). In addition, deletion of *sod* gene also resulted in a slight decrease in the survival capacity of *L. monocytogenes* in mouse bone marrow-derived macrophages and in the organs of infected mice (Brehm et al. 1992). On the contrary, van Dissel (1993) reported that murine macrophages stimulated by recombinant interferon gamma killed catalase-negative *L. monocytogenes* in the presence of relatively low serum concentrations (1-2.5%), whereas catalase-positive bacteria required much higher serum concentrations (10% for comparable killing). Such data suggest that under certain conditions (e.g. relatively low serum concentrations) catalase is required for bacterial resistance to killing by activated macrophages. As a result of these contradictory findings, the role of catalase in the intracellular survival and virulence of *L. monocytogenes* remains unclear.

1. 2. 4. Heavy metals

As a result of the human activities, environment is contaminated with heavy metals and the bacteria developed resistance mechanisms against them (Schützendübel et al. 2002). Most of the resistance systems of bacteria against heavy metal ions are present on the plasmid. On the other hand, frequently the resistance genes are found on the chromosome (Silver 1996). Previously, the resistance systems that are present on the bacterial plasmids against the toxic metal ions, including Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, TeO_3^{2-}, Tl^{+}, and Zn^{2+}, were characterized. The common resistance mechanism against the toxic metal ions is the use of energy-dependent efflux systems. Other resistance systems against toxic metal ions include enzymatic transformations (reduction, oxidation, methylation, and demethylation) and metal-binding proteins. The known efflux resistance systems are ATPases and chemiosmotic ion/proton exchangers (Silver, 1996).

L. monocytogenes is subjected to the stress caused by toxic metal ions during its survival in the environment. Cadmium is a heavy metal and considered as environmental pollutant because of the extensive use in industry. Cadmium particularly blocks the respiration and other metabolic mechanisms of bacteria. In *L. monocytogenes*, cadmium is pumped-out from the cell by using an energy-dependent efflux system. This cadmium efflux-system is associated with an operon consisting of two genes, *cadA* and *cadC* (Lebrun et al. 1994). CadA is a membrane protein that is P-type ATPase. It belongs to a family of related cation transport ATPase enzymes present in most living organisms. CadA is the major cadmium resistance determinant in *L. monocytogenes* (Wu et al.

2006). CadC is a soluble protein. These resistance mechanisms of *L. monocytogenes* against cadmium stress have been used for strain sub-typing (Harvey et al. 2001). The gene cassette location of the cadmium efflux system is diverse in *L. monocytogenes*. Lebrun et al. (1992: 1994a, b) reported that the cadmium efflux system (*cadAC*) is associated with a transposon (Tn*5422*). In addition, the sequencing of *L. monocytogenes* strains indicated that in some strains the cadmium efflux systems were present on plasmids; in *L. monocytogenes* H7858 and *L. monocytogenes* JO161 (Nelson et al., 2004) the efflux system was on a large (~80kb) plasmid, and in *L. monocytogenes* CLIP 11262 the efflux system was on pLI100 (Glaser et al. 2001). Furthermore, in *L. monocytogenes* EGD-e it was noticed that the efflux system was on the chromosome (Glaser et al. 2001).

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CHAPTER II: Role of Growth Temperature on Freeze-Thaw Tolerance of *Listeria*

Role of Growth Temperature on Freeze-Thaw Tolerance of *Listeria*

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Key words: *Listeria monocytogenes,* temperature, freeze-thaw stress, cold acclimation, cryotolerance

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2. 1. ABSTRACT

The foodborne pathogen *Listeria monocytogenes* can grow in a wide range of temperatures, and several key virulence determinants of the organism are expressed at 37^{o}C but are strongly repressed below 30^{o}C. However, the impact of growth temperature on the ability of the bacteria to tolerate environmental stresses remains poorly understood. In other microorganisms, cold acclimation resulted in enhanced tolerance against freezing and thawing (cryotolerance). In this study, we investigated the impact of growth temperature (4, 25 and 37^{o}C) on cryotolerance of 14 strains of *L. monocytogenes* from outbreaks and from food processing plant environments and four strains of non-pathogenic *Listeria* spp. (*L. welshimeri*, and *L. innocua*). Following growth at different temperatures, cells were frozen at -20^{o}C, and repeated freeze-thaw cycles were applied every 24 hours. Pronounced cryotolerance was exhibited by 37^{o}C-grown cells, with less than one log decrease following 18 cycles of freezing and thawing. In contrast, freeze-thaw tolerance was significantly reduced ($p<0.05$) when bacteria were grown at either 4 or 25^{o}C, with log decreases after 18 freeze-thaw cycles ranging from 2 to >4, depending on the strain. These findings suggest that growth at 37^{o}C, a temperature required for expression of virulence determinants of *L. monocytogenes*, is also required for protection against freeze-thaw stress. The negative impact of growth at low temperature against freeze-thaw stress was unexpected, and has not been reported before with this or other psychrotrophic microorganisms.

2. 2. INTRODUCTION

Listeria monocytogenes remains a leading cause of deaths due to foodborne illness in the United States and other industrialized nations. Neonates, pregnant women, and immunocompromised people are at high risk for infection (15, 28, 37). Outbreaks of listeriosis tend to involve a relatively small number of closely related strains ("epidemic clones"), primarily of serotype 4b. Several major outbreaks have been attributed to epidemic clone I (ECI) and epidemic clone II (ECII), both of serotype 4b (5, 21).

Unlike most other human foodborne pathogens, *L. monocytogenes* grows over a wide temperature range (1-45^{o}C) with optimal growth near around 37^{o}C (33). It has been known for some time that expression of several key virulence genes, including *hly*, encoding the hemolysin listeriolysin O (LLO) and *actA*, encoding a protein that mediates actin polymerization required for intracellular pathogenesis, is optimal at 37^{o}C but severely repressed at temperatures below 30^{o}C (22). In contrast, flagellin, motility, and chemotaxis genes are repressed at 37^{o}C, but optimally expressed at temperatures below 25^{o}C (9, 10).

For an organism like *L. monocytogenes*, which is commonly found in the environment but can also colonize and infect warm-blooded animals, temperature is likely to serve as a major signal differentiating environmental from vertebrate host-associated habitats (19). However, with the exception of the thermoregulated phenotypes described above (virulence factor production, motility and chemotaxis), the impact of growth at different temperatures on specific responses and adaptations of the pathogen remains poorly understood.

L. monocytogenes may be exposed to freezing as well as thawing in the course of its existence in natural environments (e.g. soils and water in temperate or cold regions) as well as during the storage and preservation of foods. The organism has been repeatedly isolated from frozen foods (e.g. ice cream) (7). Following freezing (-18^{o}C) in laboratory media or in foods and a single thawing cycle, survival depended on strain, freezing medium, and presence of glycerol as cryoprotectant (13, 14). Freezing and thawing of 30^{o}C-grown bacteria, in combination with essential oil, has been explored as one means to reduce the pathogen in foods (8). The possible role of the general stress sigma factor (Sigma B) in survival of bacteria grown at 30^{o}C and exposed to repeated freezing and thawing was also investigated (43). However, there is a surprising dearth of information on the possible impact of growth temperature on tolerance of *L. monocytogenes* to repeated freezing and thawing (cryotolerance).

Studies with another gram-positive psychrotrophic bacterium (*Exiguobacterium sibiricum* and other *Exiguobacterium* spp.*)* revealed that growth in the cold (4^{o}C), or growth on solid media regardless of temperature, resulted in increased tolerance of the bacteria against repeated freezing and thawing (40). It is not known whether low temperature or surface-associated growth may exert similar impacts on cryotolerance of *L. monocytogenes*. The objective of this study was to investigate the impact of growth temperature (4, 25 and 37^{o}C), and of planktonic vs. agar growth of *L. monocytogenes* serotype 4b on protection of the bacteria against repeated freezing and thawing.

2. 3. MATERIALS AND METHODS

Bacterial strains and growth conditions. *Listeria* strains used in this study were from the *Listeria* strain collection of our laboratory at N. Carolina State University. They included 14 strains of *L. monocytogenes* (10 of serotype 4b and two each of serotype 1/2a and serotype 1/2b) and two strains each of *L. welshimeri* and *L. innocua* (Table 1). Six of the serotype 4b strains were confirmed to be ECI, including five (F2365, F2381, G3982, G4011, G4030) implicated in outbreaks of listeriosis (37) and one isolated from a turkey processing plant environmental sample (12). Two serotype 4b strains were members of ECII, including strain H7550, implicated in the 1998-1999 hot dog outbreak of listeriosis (37) and strain L0226, isolated from a turkey processing plant environmental sample (12). Strain WS1 represented ECV, implicated in an outbreak of listeriosis in Winston-Salem, N.C. in 2000-2001 (5, 37), and strain 171A was isolated from a turkey processing plant and did not harbour markers specific to known epidemic clones (12). The serotype 1/2a strain J0161 represented ECIII, implicated in an outbreak of listeriosis traced to turkey deli meats in 2000 (37). Strains of *L. welshimeri* and *L. innocua* were isolated in 2004-2005 from a turkey processing plant, and were chosen so as to represent different strain types based on pulsed-field gel electrophoresis with AscI and ApaI.

Bacteria were grown on trypticase soy agar with 5% sheep blood (Remel, Lenexa, KS) at 37^{o}C for 36 h and liquid cultures were started by transferring a single colony into 5 ml of tryptic soy broth (TSB) (BBL, Cockeysville, MD) supplemented with 0.7% Yeast Extract (YE) (Becton, Dickinson & Co., Sparks, MD) (TSBYE) and incubating at 37^{o}C overnight. A sample (30 µl) of this culture was added to 30 ml of TSBYE and incubated

at the indicated temperature (4, 25, or 37°C). Growth phase was determined by monitoring optical density (OD) at 600 nm, using a spectrophotometer (BioRad SmartSpec 3000, Hercules, CA). Cultures at 37°C in mid-logarithmic and late logarithmic phase had OD_{600} approximately 0.6 and 1.5, respectively (approximately 11 h and 14 h, respectively). Stationary phase (OD_{600}~1.8) was reached after 24 or 36 h of growth for cultures grown at either 37°C or 25°C, respectively, whereas at 4°C cells reached stationary phase following 28 days of growth. Late stationary cultures (OD_{600}~1.7) were obtained after 48 h at 37°C. To evaluate the impact of growth in liquid cultures (planktonic cells) vs. solid media, strains F2365 and H7550 were grown at 4, 25, or 37°C on TSBYE containing 1.5% agar (Becton, Dickinson & Co) (TSAYE) until the diameter of single colonies reached 2-4 mm (approximately 48 h at 37°C and 25°C, and 30 days at 4°C). Colonies were then swabbed from the surface of the agar plate with a sterile cotton swab (Fisher Scientific, Houston, TX) and inoculated into 10 ml of TSBYE. OD_{600} of the cell suspension was measured as described above and adjusted so that it was comparable with the OD_{600} of cultures grown in broth. Cell enumerations were conducted by plating in duplicate on TSAYE after serial dilution in TSBYE, and incubation at 37°C for 36 h.

Freezing and thawing treatments. Bacteria grown at 4, 25 or 37°C (or the cell suspensions from agar-grown cultures, prepared as described above) were transferred (1.5 ml) into sterile cryovials (Nalgene, Rochester, NY) and frozen at -20°C. The freezing rate was 0.039 ml/min, and freezing temperature for TSBYE and the cultures was ca. 0°C. Freezing rates were determined by placing a thermocouple (0.254mm diameter Type T thermocouples, Omega Engineering Inc., Stamford, CT) equipped with a

Digisense 12-channel scanning thermometer (Cole-Parmer Instrument Co., Vernonhills, IL) inside the cryovial and measuring the temperature every 30 sec after placement of the vial at -20°C. Thawing was performed at room temperature for 10 min in a water bath. Freezing and thawing cycles were repeated every 24 h for 18 cycles. Every three cycles, cell enumerations were done in duplicate, as described above.

Assessment of injured cell prevalence following repeated freezing and thawing. Enumerations on Modified Oxford selective medium (Oxoid, Basingstoke, England) and on non-selective medium (TSAYE) were employed to assess prevalence of injured cells, as described (20). Following repeated freezing and thawing, *L. monocytogenes* F2365 (grown at 4°C and 37°C) was serially diluted in TSBYE as described above and plated both on TSAYE and on Modified Oxford selective medium. Colonies were enumerated following incubation at 37°C for 36 h.

Determination of possible impact of cryoprotectants in the culture supernatant on freezing and thawing tolerance. Stationary phase cultures grown at 4°C and 37°C were centrifuged at room temperature for 10 min at 16,110 × g (Eppendorf Centrifuge 5415-D, Westbury, NY), and the supernatant was filter-sterilized (0.2μm syringe filter; Fisher Scientific). Cells grown at 4°C were resuspended in filtered supernatants from 37°C-grown cells, and the cell suspensions were frozen at -20°C as described above. Cells resuspended in filtered supernatants from the cultures in which they were grown were used as controls. Repeated freezing and thawing cycles were applied and cell survival was determined as described above.

Statistical analysis. All treatment combinations were replicated at least twice. Log reduction was calculated following 18 freeze-thaw cycles. The experimental design accommodated all combinations of two temperatures and 18 strains in a complete, crossed two-factor layout, but with the additional temperature level of 25^{o}C for the two strains H7550 and F2365. Because of this incompleteness with regard to the third temperature level, we treated the 38 different temperatureXstrain combinations as levels of a single factor in a one-way analysis of variance with 80 observations. The lowest level of duplication of measurement was treated as subsampling and these two measurements were averaged to obtain 80 separate means for analysis. To investigate whether the impact of growth temperature on log reduction varied across strains, the difference (between cultures grown at 37^{o}C and 4^{o}C) in log reduction following 18 freeze-thaw cycles was computed for each strain, and these differences were compared pair-wise between strains. Significance was determined at unadjusted level of alpha = 0.05. All statistical analyses were performed using SAS v. 9.1 (Cary, NC).

2. 4. RESULTS

Cryotolerance was higher in stationary phase than logarithmic phase cultures. Impact of growth phase on the freeze-thaw tolerance of *L. monocytogenes* was investigated using *L. monocytogenes* F2365 (hereafter referred to as F2365) and *L. monocytogenes* H7550 (hereafter referred to as H7550) grown at 37^{o}C. Cells of F2365 in the mid-logarithmic growth phase were significantly more susceptible ($p<0.05$) to repeated (six or more cycles) freezing and thawing than cells from the late logarithmic, or

stationary phase (Fig. 1). Similar findings were obtained with H7550 (data not shown). For subsequent experiments to assess the impact of temperature on freeze-thaw tolerance, cultures in stationary phase of growth were used.

***L. monocytogenes* F2365 and H7550 grown at 37°C in liquid were more tolerant against freeze-thaw stress than liquid cultures grown at 4 or 25°C.** Repeated cycles of freezing and thawing appeared to have limited impact on survival of F2365 and H7550 grown at 37°C in liquid; following 18 cycles of freeze-thaw stress, log reduction values were 0.83±0.14 and 0.66±0.71, respectively (Fig. 2). However, tolerance of both strains to repeated freezing and thawing was severely impaired following growth of the bacteria at 4°C in liquid, with decline in survival becoming increasingly pronounced with increasing numbers of freeze-thaw cycles (Fig. 2). The rate of decline in CFU/ml of cultures grown at 4°C was significantly greater than observed with 37°C-grown cells ($p<0.0001$). Log decreases of 4°C-grown cultures after 18 freeze-thaw cycles were 4.39±0.85 and 3.09±0.46 for F2365 and H7550, respectively, suggesting significantly lower cryotolerance of F2365 ($p<0.05$) (Fig. 2). Plating of cell suspensions of F2365 (grown at 37°C and 4°C in liquid and subjected to repeated freezing and thawing) on TSAYE and on Modified Oxford selective medium did not provide evidence for injured cells in the cell suspensions. CFU/ml were similar on the two types of media (data not shown).

Rate of decline with increasing numbers of cycles was also significantly higher in cells grown at 25°C than in 37°C-grown cells ($p<0.05$), and did not differ significantly from that of 4°C-grown cells ($p>0.05$) (Fig. 2). Log decreases of 25°C-grown cells

following 18 cycles were 4.40±1.36 and 2.26±0.48, with F2365 again exhibiting lower cryotolerance than H7550 ($p<0.05$) (Fig. 2).

Impaired tolerance to repeated freezing and thawing was a general attribute of *L. monocytogenes* grown at low temperature, and was also observed among non-pathogenic *Listeria* spp. Strains F2365 and H7550 are members of two major epidemic clonal groups of *L. monocytogenes* serotype 4b, ECI and ECII, respectively. In order to investigate if the impact of growth temperature on tolerance to freezing and thawing could be seen with other strains of *L. monocytogenes* serotype 4b, a panel of eight additional strains were characterized. This panel included strains from different outbreaks and from the food processing plant environment (Table 1).

In all cases, growth of the bacteria at 37°C noticeably enhanced the tolerance of the cells to repeated freezing and thawing, compared to growth at 4°C (Fig. 3A) ($p<0.05$). When grown at 37°C cryotolerance levels of the different strains were not significantly different ($p>0.05$). However, we noted certain strain-specific differences in freeze-thaw tolerance of 4°C-grown cells. Specifically, following growth at 4°C strains G3982 and LO228 (both of ECI) and strain WS1 had significantly higher cryotolerance than F2365 (Fig. 3A). Significant differences among strains were also observed for the extent to which cryotolerance was reduced when cells were grown at 4°C in comparison to cells grown at 37°C. Relatively low differences (<100fold) were observed with strains WS1, LO228, G3982 and 171A (Fig. 3A). Within ECI, the difference in cryotolerance of cells grown at 37°C vs. 4°C was significantly higher for F2365 than for strains G3982 and LO228 ($p<0.05$) (Fig. 3A).

To determine whether growth at 37^{o}C also conferred protection against repeated freezing and thawing in *L. monocytogenes* of other serotypes, two strains each of serotype 1/2a and 1/2b were investigated. All four strains were significantly more tolerant against repeated freezing and thawing following growth at 37^{o}C than at 4^{o}C (Fig. 3B) ($p<0.05$). No significant differences in cryotolerance following growth at either 37^{o}C or 4^{o}C were noted among the tested strains; the cryotolerance-enhancing impact of growth at 37^{o}C (vs. growth at 4^{o}C) also did not vary significantly among these strains ($p>0.05$).

Examination of representatives of non-pathogenic *Listeria* spp. failed to reveal significant differences in the impact of growth temperature on cryotolerance between them and *L. monocytogenes*. For both tested strains of *L. welshimeri*, and both tested strains of *L. innocua*, freeze-thaw tolerance was significantly higher when bacteria were grown at 37^{o}C than at 4^{o}C (Fig. 3B) ($p<0.05$). Similarly to the serogroup 1/2 strains, no significant differences were noted among the tested strains of the non-pathogenic species ($p>0.05$).

Enhanced cryotolerance of agar-grown vs. planktonic *L. monocytogenes* grown at 25^{o}C. We determined whether agar-grown cultures differed in freeze-thaw tolerance from those grown planktonically. No significant differences were noted in cryotolerance of agar-grown vs. liquid cultures when bacteria were grown at 37^{o}C, or at 4^{o}C ($p>0.05$). F2365 and H7550 cells grown on agar media at 37^{o}C tolerated repeated freezing and thawing well, similarly to cultures grown at 37^{o}C in liquid. Cells grown on

agar at 4°C had markedly impaired survival, again similarly to what was observed with liquid cultures (Fig. 2A and 2B).

In contrast to the lack of impact of agar vs. liquid growth on cryotolerance of cells grown at 37°C or 4°C, both F2365 and H7550 grown at 25°C on solid media were significantly protected against repeated freezing and thawing when compared to cells grown at 25°C in liquid (Fig. 2) ($p<0.05$). Agar growth at 25°C affected cryotolerance differently for F2365 and H7550. Cryotolerance of H7550 grown on agar at 25°C was similar to that of cells grown at 37°C either in liquid or on agar (Fig. 2B) ($p>0.05$), whereas F2365 grown on agar at 25°C had impaired cryotolerance in comparison to cells grown at 37°C either in liquid or on agar (Fig. 2A) ($p<0.05$).

Lack of evidence for cryoprotectants in the supernatant of 37°C-grown cells. To address the possible involvement of cryoprotectants in enhanced freeze-thaw tolerance of 37°C-grown cells, cells (F2365; H7550) grown at 4°C in liquid were centrifuged and resuspended in the filtered supernatants from cultures of the same strain grown at 37°C in liquid. Freeze-thaw tolerance of 4°C-grown cells resuspended in the filtered supernatant of 37°C-grown cells remained impaired, similarly to the control (4°C-grown cells resuspended in their own filtered supernatant) (data not shown). However, cultures subjected to the centrifugation and resuspension steps had higher cryotolerance than untreated cultures of the same strain (data not shown), suggesting that centrifugation increased freeze-thaw tolerance.

2. 5. DISCUSSION

The observed impact of growth temperature on the cryotolerance of *Listeria* has not been reported previously, and was rather unexpected. In the case of psychrotrophic strains of *Exiguobacterium* spp. isolated from Siberian permafrost, planktonic growth at 4^{o}C conferred significant protection against repeated freezing and thawing than growth at higher temperatures (25^{o}C) (40). Following growth at 4^{o}C several other bacteria from permafrost were also found to have enhanced tolerance to long-term freezing (and subsequent thawing) (30).

Exiguobacterium spp. grown on solid media either at 4^{o}C or at 25^{o}C had enhanced cryotolerance, similar to that of cells grown planktonically at 4^{o}C (40). In contrast, we found that in the case of *L. monocytogenes* growth on solid media conferred enhanced cryotolerance in a temperature- and strain-specific fashion. H7550 and (to a lesser extent) F2365 grown on agar at 25^{o}C had higher cryotolerance than when grown in liquid at that temperature. Such findings suggest that the impact of growth temperature and planktonic vs. surface-associated growth varies among different psychrotrophic species, and even among different strains of the same species.

The mechanisms that underlie the observed markedly higher cryotolerance of planktonically grown *L. monocytogenes* following growth at 37^{o}C in comparison to growth at 4 or 25^{o}C remain unknown. Extracellular cryoprotectants such as trehalose and glycerol have been found to increase cryotolerance in *L. monocytogenes* and other bacteria, possibly by enhancing membrane stability (11, 14, 34). However, our experiments with resuspension of 4^{o}C-grown cells in filtered supernatants from 37^{o}C

grown cultures failed to provide evidence for extracellular cryoprotectants in the latter. Such experiments also suggested that cryotolerance was increased through the centrifugation and resuspension steps; impact on centrifugation and resuspension on stress responses of *L. monocytogenes* has been described by others as well (4).

A key finding was that cryotolerance of bacteria grown planktonically was severely impaired not only in cells grown at 4^{o}C but also in those grown at 25^{o}C. This suggests that impaired cryotolerance in cells grown in liquid at such temperatures was not an outcome of specific cold-induced membrane modifications or other cellular responses associated with cold stress or cold acclimation, such as those documented in numerous investigations (e.g. 2, 3, 23, 24, 42). The genes responsible for enhanced cryotolerance of 37^{o}C-grown cells may be components of a thermoregulated regulon, possibly also implicated in virulence in *L. monocytogenes*. The protective impact of 37^{o}C growth on cryotolerance was also observed with non-pathogenic *Listeria* spp. It is therefore tempting to speculate that the underlying thermoregulated mechanisms were present in the ancestral pathogenic *Listeria* lineage that is believed to have preceded the emergence of non-pathogenic species such as *L. innocua* and *L. welshimeri* (32).

Common mechanisms mediating responses of relevance both to pathogenesis and to cryotolerance may include those that allow cells to cope with reactive oxygen species produced during infection (17, 36), as well as during freezing and thawing (25). For instance, in *Campylobacter* superoxide dismutase (SOD) was found to play an important role in cryotolerance (18, 35) as well as in survival in macrophages (29) and in colonization of chickens (31). In *L. monocytogenes*, SOD is required for virulence in the

murine model and is controlled post-translationally by phosphorylation (1). Even though evidence for thermoregulated transcription of *sod* is lacking (3, 26), earlier studies suggested that SOD production was higher in cells grown at 37°C than 10°C (39). The possible role of SOD in *Listeria*'s cryotolerance remains to be characterized. Another determinant that may contribute both to cryotolerance and to virulence is the general stress sigma factor, sigma B. In *Bacillus subtilis* 168, sigma B was found to contribute to survival following freezing and subsequent thawing (41). In *L. monocytogenes,* sigma B is involved in oxidative stress responses as well as virulence (16) but its impact on freeze-thaw survival was only evaluated in logarithmic phase cells grown at 30°C; the observed impact was rather modest, and could be detected only in cells exposed to prior additional stresses such as cold and acid shock (43). The possible role of sigma B in cryotolerance of 37°C-grown cells vs. cells grown at lower temperature remains to be assessed.

Future comparative studies of the transcriptome and proteome of *L. monocytogenes* at different temperatures (e.g. 37°C, 4°C and 25°C) will assist the identification of candidate genes responsible for temperature and agar growth-dependent cryotolerance in this organism. Comparative studies employing different strains may also elucidate the mechanisms underlying the observed strain-specific differences. It was noteworthy that following growth at 4°C certain ECI strains (e.g. F2365) had markedly lower cryotolerance than others (e.g. G3982), suggesting differences in cryotolerance of cold-grown cells, even among strains of the same clonal group. Genetic and antigenic differences among ECI strains have been described before (6, 27). It is currently not clear

whether such differences, including those underlying the observed variable impact of growth temperature on cryotolerance, arose in the natural habitat of the strains, or subsequent to their isolation and in the course of their passage under laboratory conditions. It is also not clear whether the multiple frameshifts detected in the genome of F2365 (27) may contribute to the fact that this strain exhibited the greatest impairment in cryotolerance following 4^{o}C growth (vs. growth at 37^{o}C) among the tested strains.

Temperature-dependent cryotolerance may have evolved in response to specific selection pressures in *Listeria*'s natural habitats. For instance, bacteria amplified at body temperatures in mammalian blood or tissues as a result of listeric infection and subsequently released in the environment (e.g. in aborted fetuses or upon death of the hosts) might be better prepared to tolerate freezing and thawing that they might undergo in nature (e.g. in surface soil, water). Further studies are needed to determine whether growth at temperatures typically associated with animal infection also confers enhanced cryotolerance in other animal pathogens that have environmental lifestyles where freezing and thawing may be encountered, and, if this is found to be the case, to determine whether common molecular mechanisms may be involved.

ACKNOWLEDGMENTS

This project was partially funded by USDA grant 2006-35201-17377. S. Wilson was partially supported with a National Science Foundation minority research grant to St. Augustine's College. We thank J. Simunovic and B. Farkas for assistance with determinations of freezing rate. We are grateful to R. M. Siletzky for laboratory support

and to all other members of our laboratory for discussions, encouragement, and support in the course of this project.

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Table 2. 1: *Listeria* strains investigated in this study.

Strain	Source or reference
L. monocytogenes, serotype 4b	
171A	Turkey processing plant (12)
G3982	Food, 1983-1987 Switzerland outbreak, ECI (37)
F2365	Food, 1985 California outbreak, ECI (37)
F2381	Clinical, 1985 California outbreak, ECI (37)
G4011	Food, 1981 Canada outbreak, ECI (37)
G4030	Clinical, 1992 France outbreak, ECI (37)
H7550	Clinical, 1998-99 multistate outbreak, ECII (37)
L0226	Turkey processing plant, ECII (12)
L0228	Turkey processing plant, ECI (12)
WS1	Clinical, 2000 North Carolina outbreak, ECV (5, 37)
L. monocytogenes, serotype 1/2a	
10403S	Str^R isolate of 10403 (human skin lesion isolate, 1968)
SK2662	Turkey processing plant environment, 2004
JO161	Clinical, 2000 multistate outbreak, ECIII (37)
L. monocytogenes, serotype 1/2b	
2005-625	Clinical sporadic isolate, N.Carolina, 2005
G4008	Food
L. welshimeri	
SK1523	Turkey processing plant, environmental sample, 2004

Table 2. 1: Continued

SK1648	Turkey processing plant, product rinse, 2005
L. innocua	
SK1662	Turkey processing plant, environmental sample, 2005
SK1512	Turkey processing plant, environmental sample, 2004

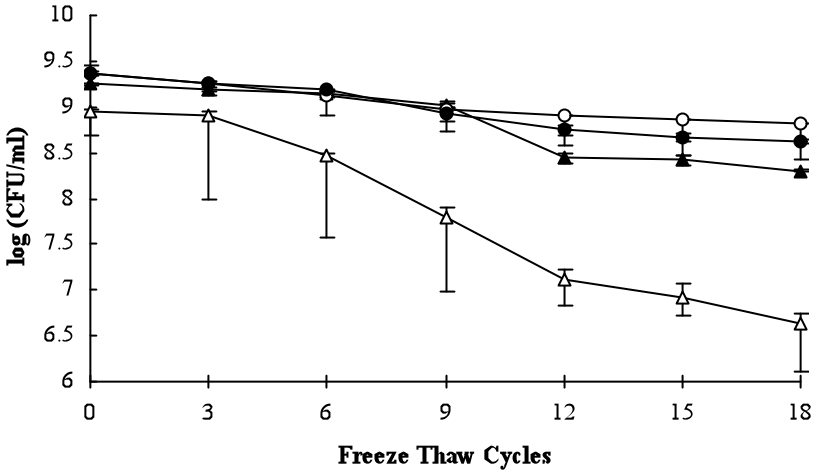
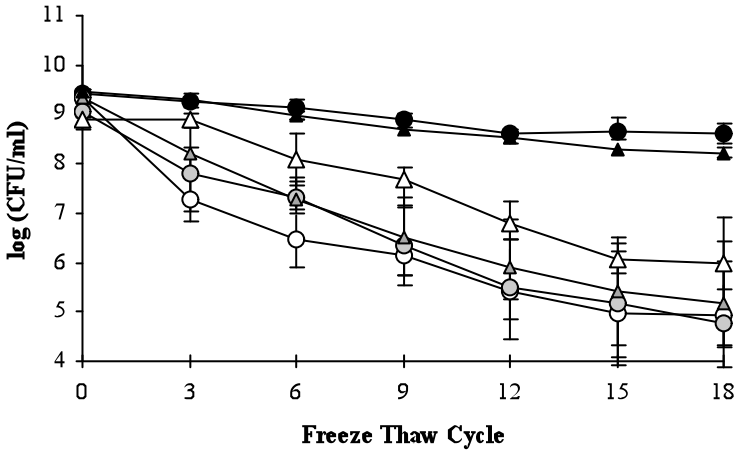


Fig. 2. 1. Impact of growth phase on the freeze-thaw tolerance of *L. monocytogenes* F2365. Data represent the means from two experiments, each done in duplicate. Symbols: Δ, mid-logarithmic phase; ▲, late logarithmic phase; ○, stationary phase; ●, late stationary phase.

(A)



(B)

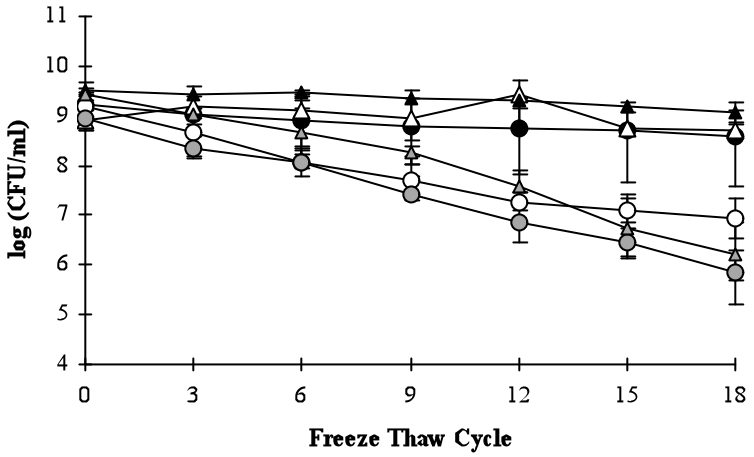
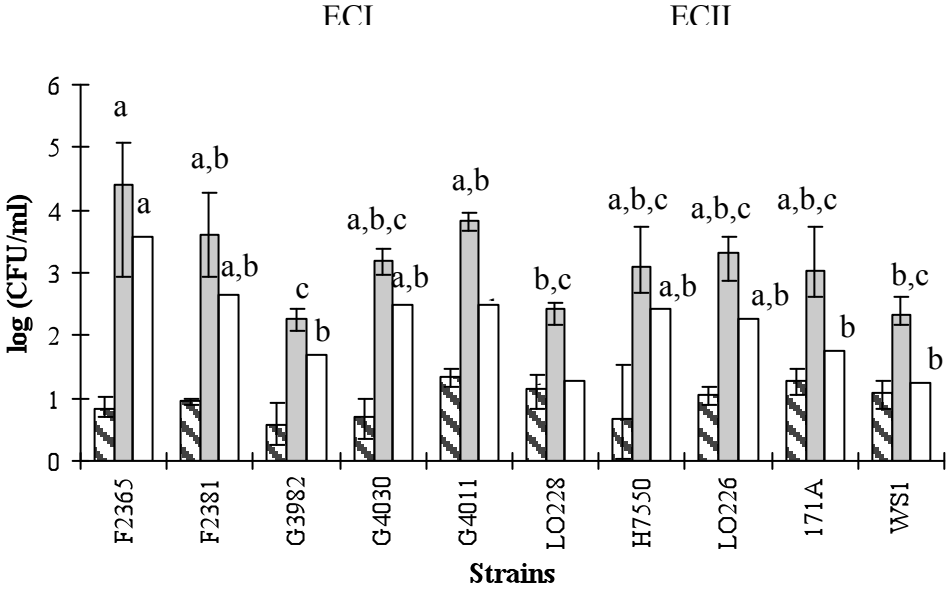


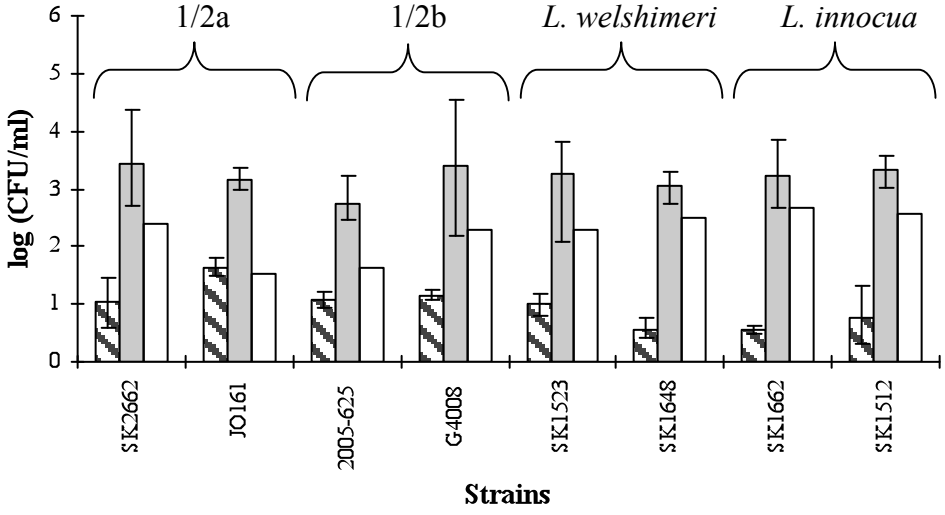
Fig. 2. 2. Impact of growth temperature and agar vs. liquid growth on the freeze-thaw tolerance of *L. monocytogenes* F2365 (A) and H7550 (B). Data represent the means from three experiments, each done in duplicate. Symbols: ●, liquid at 37 °C; ○, liquid at 25 °C; ●, liquid at 4°C; ▲, agar at 37 °C; Δ, agar at 25 °C; ▲, agar at 4 °C.

Fig. 2. 3. Log decrease for *Listeria* strains grown at 4°C and 37°C after 18 freeze-thaw cycles. A, *L. monocytogenes* serotype 4b; B, *L. monocytogenes* serotype 1/2a and 1/2b, and non-pathogenic *Listeria* spp. (*L. welshimeri*, *L. innocua*). Data represent the means from two experiments, each done in duplicate. Column patterns: Striated, cultures grown at 37°C; gray, cultures grown at 4°C; open, difference in CFU/ml (after 18 cycles) between cultures grown at 37°C and 4°C. Different letters on columns of the same pattern indicate statistically significant differences ($p<0.05$). No significant differences were noted among strains grown at 37°C (striated columns).

(A)


ECI
ECII
log (CFU/ml)
0
1
2
3
4
5
6
a
a
a,b
a,b
c
b
a,b,c
a,b
a,b
b,c
a,b,c
a,b
a,b,c
a,b
a,b,c
b
b,c
b
F2365
F2381
G3982
G4030
G4011
LO228
H7550
LO226
171A
WS1
Strains

(B)


1/2a
1/2b
L. welshimeri
L. innocua
log (CFU/ml)
0
1
2
3
4
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6
SK2662
JO161
2005-625
G4008
SK1523
SK1648
SK1662
SK1512
Strains

CHAPTER III: Role of Growth Temperature on Freeze-Thaw Tolerance of the Psychrotrophic Bacteria *Yersinia enterocolitica* and *Bacillus cereus*

3.1. ABSTRACT

In the previous chapter the impact of growth temperature on the cryotolerance of *Listeria monocytogenes* was characterized. *L. monocytogenes* cells grown at 37^{o}C showed increased cryotolerance compared to cells grown at 4^{o}C and 25^{o}C. This impact of growth temperature on cryotolerance was unexpected. Therefore, in this study, we characterized the impact of growth temperature on the cryotolerance of two other foodborne pathogens, *Yersinia enterocolitica and Bacillus cereus.* Impact of growth temperature on the cryotolerance of *Y. enterocolitica* was opposite to that observed with *L. monocytogenes*. When *Y. enterocolitica* was grown at 37^{o}C and subjected to 6 cycles of repeated freezing and thawing the cell concentration dropped to undetectable levels ($<10^{1}$ CFU/ml). On the other hand, when the bacteria were grown at 4^{o}C, their cryotolerance increased dramatically. We also tested the impact of growth temperature on the cryotolerance of *B. cereus*; however we were not able to grow the tested *B. cereus* strain at 4^{o}C. The cryotolerance of the 37^{o}C grown *B. cereus* was lower than *L. monocytogenes*, but higher than *Y. enterocolitica.* The findings from this study emphasize the impact of growth temperature of the bacteria on their cryotolerance, and demonstrate that this impact varies markedly among different psychrotolerant bacteria.

3. 2. INTRODUCTION

Foodborne bacteria are subjected to number of different stresses, including cold stress and freeze-thaw stress, both in the environment, and during food preservation and processing. To develop effective control measures for foodborne pathogens, it is important to understand the stress response mechanisms of these organisms. Cold tolerance of foodborne pathogens is one of the most important challenges to food safety. Cold-tolerant foodborne pathogens include *Bacillus cereus*, *Yersinia enterocolitica*, *L. monocytogenes,* and non-proteolytic *Clostridium botulinum* type E. These bacteria are able not only to survive but also to grow at temperatures typically found in cold-stored foods (4-10^{o}C). Non-proteolytic *Clostridium botulinum* type E and some *B. cereus* strains have the capability to produce enterotoxins (Annamalai et al. 2009, Bhaduri, 2006, Beuchat et al. 1997, Chan et al. 2009, Grecz et al. 1982, Tasara et al., 2006).

Foodborne pathogens are subjected to freezing and thawing during their survival in the environment and during food processing and preservation. Freezing and thawing stress involves several other stressors. Oxidative stress occurring during thawing contributes to the freeze-thaw injury of the cells (Hermes-Lima et al. 1993, Stead et al. 2000). The importance of oxidative stress during freezing and thawing was demonstrated by the finding that superoxide dismutase deficient *Campylobacter coli* showed decreased freeze-thaw tolerance compared to wild type bacteria.

Relatively few studies have addressed freeze-thaw tolerance of foodborne pathogens. El-Kest et al. (1991a & b) reported that the cryotolerance of *L. monocytogenes*

frozen in laboratory media or in foods was depended on strain, freezing medium, and the presence of cryoprotectants. Another study addressed the role of the general stress sigma factor (Sigma B) in survival of *L. monocytogenes* exposed to cold shock and to repeated freezing and thawing (Wemekamp-Kamphuis et al. 2004). Previously in this study, we reported the increase in the cryotolerance of *L. monocytogenes* when they were grown at 37^{o}C compared to lower temperatures (4^{o}C and 25^{o}C). Such findings were unexpected, and were opposite to what had been earlier described with another gram-positive psychrotrophic bacterium, *Exiguobacterium* spp. The freeze-thaw tolerance study in *Exiguobacterium* spp. showed that growth in the cold (4^{o}C), or growth on solid media regardless of temperature, resulted in increased tolerance of the bacteria against repeated freezing and thawing (Vishnivetskaya et al. 2007).

It is important to determine whether the impact of growth temperature found with *Listeria* on the cryotolerance was specific to this organism or common with other foodborne pathogens. Therefore, the objective of this study was to investigate the impact of growth temperature (4 and 37^{o}C), on the freeze-thaw tolerance of *Y. enterocolitica, B. cereus* and *L. monocytogenes*.

3. 3. MATERIALS AND METHODS

3. 3. 1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *Yersinia enterocolitica* strains used in this study were isolated from pork tongue and provided by Dr. Kumar Venkitanarayanan (Department of Animal Science, University of

Connecticut). *Bacillus cereus* strain was provided by N. C. Department of Health. Bacteria were grown on trypticase soy agar with 5% sheep blood (Remel, Lenexa, KS) at 37^{o}C for 36 h and liquid cultures were started by transferring a single colony into 5 ml of tryptic soy broth (TSB) (BBL, Cockeysville, MD) supplemented with 0.7% Yeast Extract (YE) (Becton, Dickinson & Co., Sparks, MD) (TSBYE) and incubating at 37^{o}C overnight. Thirty microliters of this culture was added to 30 ml of TSBYE and incubated at 4 or 37^{o}C for 3 weeks or 24 h, respectively (OD_{600}=1.049). Cell enumerations were conducted by plating in duplicate on TSAYE after serial dilution in TSBYE, and incubation at 37^{o}C for 36 h.

3. 3. 2. Freezing and thawing treatments

Bacteria grown at 4 or 37^{o}C were transferred (1.5 ml) into sterile cryovials (Nalgene, Rochester, NY) and frozen at -20^{o}C. Thawing was performed at room temperature for 10 min in a water bath. Freezing and thawing cycles were repeated every 24 h for 18 cycles. Every three cycles, cell enumerations were done in duplicate, as described above.

3. 4. RESULTS

3. 4. 1. Freeze-thaw tolerance of *Y. enterocolitica* grown at 4^{o}C and 37^{o}C

Following 6 cycles of freezing and thawing the cell concentration of all four tested strains of *Y. enterocolitica* grown at 37^{o}C decreased dramatically (<10^{1}cfu/ml). On the other hand, when the bacteria were grown at 4^{o}C, their cryotolerance was significantly increased, with less than one log reduction following 6 freeze-thaw cycles

(Fig. 1). A minimum of ca. 10^4 CFU/ml was detected following 18 freeze-thaw cycles of 4^oC-grown *Y. enterocolitica* (estimated average log reduction 2.12±1.53) (Fig. 1). However, noticeable differences were noted in freeze-thaw tolerance of different *Y. enterocolitica* strains grown at 4^oC ($p<0.05$).

3. 4. 2. Freeze-thaw tolerance of *B. cereus* grown at 37°C.

The *B. cereus* strain used in this study was unable to grow at 4^oC. When grown at 37^oC, *B. cereus* showed substantial tolerance to repeated freezing and thawing (Fig. 2). Interestingly, there was no further decrease in cryotolerance of *B. cereus* following the 6^{th} freeze-thaw cycle. *L. monocytogenes* grown at 37^oC showed noticeably higher freeze-thaw tolerance than either *B. cereus* or *Y. enterocolitica* at 37^oC ($p<0.05$).

3. 5. DISCUSSION

In this study, the impact of growth temperature on the freeze-thaw tolerance of *Y. enterocolitica and B. cereus* was characterized. In the previous chapter, we characterized the freeze-thaw tolerance of the cold tolerant gram-positive foodborne bacteria, *L. monocytogenes*. The cryotolerance of *L. monocytogenes* was significantly higher when they were grown in liquid media at 37^oC compared to 4^oC and 25^oC. On the other hand, in another gram-positive psychrotrophic bacteria, *Exiguobacterium* spp., isolated from Siberian permafrost, cells grown in liquid media at 4^oC showed increased tolerance against repeated freezing and thawing compared to cells grown at higher temperature (25^oC) (Vishnivetskaya et al. 2007). In another study, growth at 4^oC of several other bacteria from permafrost was also found to enhance tolerance to long-term freezing

(Ponder et al. 2005). Similarly, in this study we found that when *Y. enterocolitica* was grown at low temperatures (4^{o}C) they possessed significantly increased cryotolerance compared to cells grown at higher temperatures (37^{o}C). This varying impact of growth temperature on the cryotolerance of bacteria indicates the importance of other stresses that are involved during freezing and thawing. However the extent of the involvement of underlying stresses that are involved during freeze-thaw stress is still remains unknown. The differential stress response of each species to the underlying stresses of freezing and thawing might be responsible for the observed variation in cryotolerance.

Previously, it was reported that the cells were subjected to oxygen stress during thawing which results in the damaging effect of freezing and thawing (Garénaux et al. 2008 Gao et al., 2000). Also Stead et al. reported the role of SOD in the freeze-thaw tolerance of bacteria. Therefore, the observed opposite impact of growth temperature on the cryotolerance of *L. monocytogenes* compared to *Y. enterocolitica* or *Exiguobacterium* spp. might be because of differences in tolerance to oxidative stress following growth of these organisms at different temperatures.

The protective impact of cold shock to subsequent stresses, including freezing and thawing, was reported previously (Jones et al. 1994, Panoff et al. 1995). However, the impact of cold growth (cold acclimation) has not been fully understood, and in this study we observed varying impacts of cold growth on the cryotolerance of bacteria. Therefore, further studies are needed to understand how growth temperature impacts the cryotolerance of bacteria, including those foodborne pathogens with ability to grow at markedly different temperatures.

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Table 3. 1. Bacterial strains investigated in this study.

Strain	Source or reference
L. monocytogenes, serotype 4b	
F2365	1985 California outbreak, ECI (Swaminathan et al. 2007)
Y. enterocolitica	
Y. enterocolitica (DOH)	Department of Health, Raleigh, NC
Y. enterocolitica 9610	Pork tongue isolate (Annamalai et al. 2009)
Y. enterocolitica 14A	Pork tongue isolate (provided by Dr. Venkitanarayanan)
Y. enterocolitica 10A	Pork tongue isolate (provided by Dr. Venkitanarayanan)
Y. enterocolitica 7A	Pork tongue isolate (provided by Dr. Venkitanarayanan)
B. cereus	Department of Health, Raleigh, NC

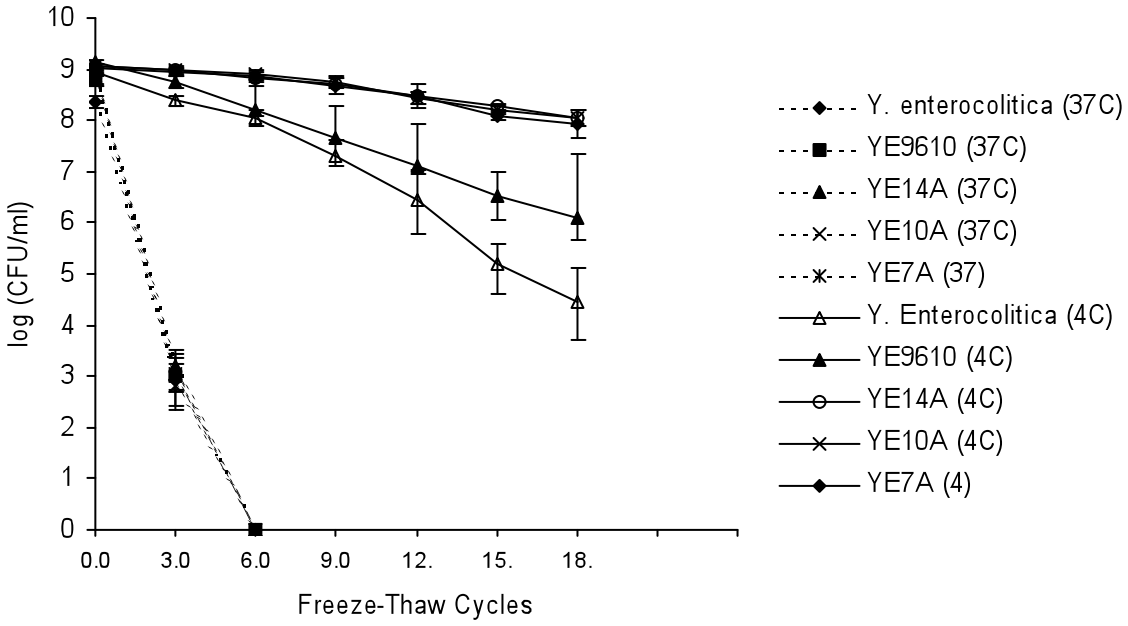

Fig. 3. 1: Freeze-thaw tolerance of 37^{o}C and 4^{o}C-grown *Y. enterocolitica* strains (*Y. enterocolitica* (DOH), YE 9610, YE14A, YE10A, and YE7A). Data are from two representative experiments, each done in duplicate.

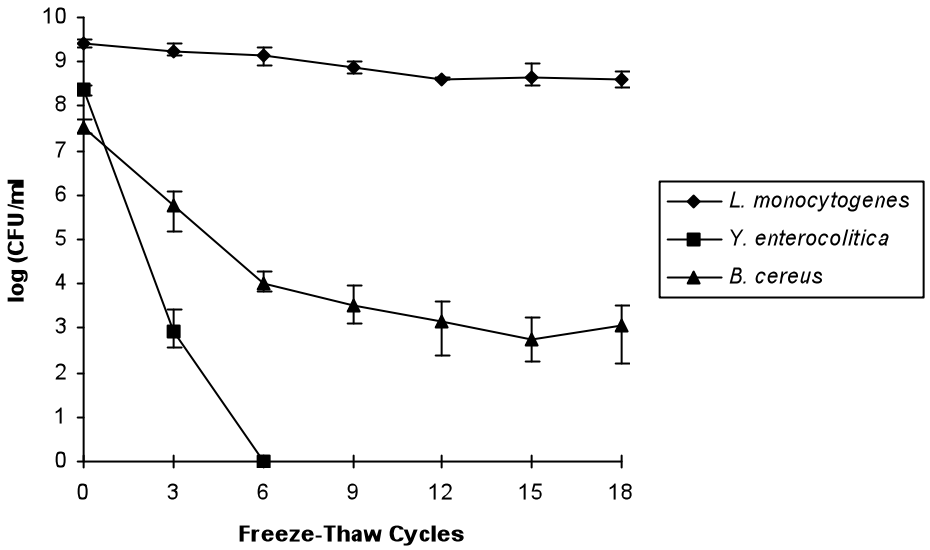

Fig. 3. 2: Freeze-thaw tolerance of 37^{o}C grown *Y. enterocolitica* strains, *B. cereus* and *L. monocytogenes* Data are from two representative experiments done in duplicate.

CHAPTER IV: Construction of the Mutant Library of *Listeria monocytogenes*

4. 1. ABSTRACT

Previously, we described the impact of growth temperature on the cryotolerance of *Listeria monocytogenes*, in which the cells grown at 37^{o}C were more cryotolerant that the cells grown at 4^{o}C or 25^{o}C. In this study, we used a genetic approach to determine the responsible genes for this unexpected phenotype. We constructed mutant libraries of *L. monocytogenes* serotype 4b strain F2365 and serotype 1/2a strain 10403S by using a *mariner*-based transposon system. High efficiency of transposon insertion was achieved. Integration frequency in *L. monocytogenes* strains 10403S and F2365 was 100% (960 of 960) and 99% (3329 of 3360), respectively. Because of challenges in screening the mutant libraries directly for impairment of freeze-thaw tolerance, we screened for loss or reduction of tolerance to stresses that may take place during the freeze-thaw stress. These stresses include oxidative, cold, and osmotic stress. We also screened for loss of hemolysin activity.

4. 2. INTRODUCTION

Construction and screening of mutant libraries is an effective approach to identify genes responsible for particular stress response mechanisms in bacteria. Transposons are valuable tools for mutant library construction. In order to determine the genes responsible for the freeze-thawing tolerance of *L. monocytogenes*, mutant libraries of *L. monocytogenes* strain F2365 (serotype 4b) and 10403S (serotype 1/2a) was constructed by using a *mariner*-based transposition system, *Tc1/mariners*, described by Cao et al. (2007). This transposition system has a self-encoded transposase and was earlier shown not to require any other factors for transposition (Lampe et al. 1996). In addition, this transposition system requires dinucleotide TA for insertion, which provides a highly random transposition in low-GC content organisms, such as *L. monocytogenes*. Cao et al. (2007) compared this transposition system with a Tn*917*-based system and showed that the *mariner*-based transposition system had low rates of plasmid retention and higher efficiency for random transposition (Cao et al., 2007).

In this study, we used *mariner*-based transposon mutant libraries of *L. monocytogenes* to identify genes responsible for resistance to selected environmental stresses including cold and sensitivity against sub-lethal concentrations of paraquat, cadmium, and NaCl. In addition, mutants negative in hemolytic activity and in catalase were identified.

4. 3. MATERIALS AND METHODS

4. 3. 1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Mutant libraries were constructed in the serotype 4b strain F2365 (cheese isolate of the California outbreak) and in the serotype 1/2a strain 10403S (streptomycin resistant derivative of a human skin lesion isolate). Bacteria were grown in brain heart infusion broth (BHI, Difco, Sparks, MD), at 37^{o}C for 36 hours, and stored at –80^{o}C in the presence of 20% glycerol. Following the construction of mutant libraries (see below) mutants that grew on brain heart infusion agar with 5 μg/ml erythromycin (BHI-Em) but not on BHI with 10 μg/ml (BHI-Km) were inoculated individually with sterile toothpicks in fresh BHI and grown at 37^{o}C for 36 hours. Mutant libraries were stored in 96-well microtiter plates at –80^{o}C.

4. 3. 2. Construction of mutant libraries

In the construction of mutant library of F2365 and 10403S, plasmid pMC38 carrying a mariner-based transposon system (*TC1/mariner*) (Cao et al. 2007) was used. The pMC38 plasmid DNA was kindly provided by Dr. Marquis (Cornell University). Plasmid (pMC38), was first transformed into *Escherichia coli* DH5α by electroporation and a transformant was picked on LB plates supplemented with erytromycin (5 μg/ml) to start a culture for plasmid purification. Tha transformant was incubated at 37^{o}C for at most 18 hours, as the plasmid appears to be somewhat unstable in *E. coli*. Following incubation the plasmid was purified by using plasmid extraction kit (Qiagen, Valencia, CA). The selective marker for the transposon is erythromycin resistance, whereas the vector harbors resistance to kanamycin (Cao et al, 2007; Fig. 1). The plasmid was

introduced into *L. monocytogenes* F2365 and 10403S by electroporation. For electroporation, the cultures were grown in BHI at 37°C overnight. The cultures (1 ml) were diluted (1:100) into 99 ml BHI containing 0.5 M sucrose and incubated at 37°C with shaking (100 rpm) until the OD_{600} reached around 0.2 (4 hours). Penicillin G (10 μg/ml; Sigma-Aldrich, St. Louis, MO) was added and cultures were further incubated for 2 h at 37°C. The cells were pelletted by centrifugation (8,000 rpm; 10 min; 4°C), followed by washing 3 times (4 ml, 3 ml and 3 ml, respectively) with electroporation buffer (1 mM Hepes, pH 7.0 and 0.5 M sucrose). Final pellets were suspended in electroporation buffer (250 μl) and aliquots (150 μl) of the suspension were kept at -80°C (fresh cell suspensions were typically used). The cell suspension (150 μl) was mixed with plasmid DNA (10 μl), and incubated on ice for 1 h. The mixture was placed in pre-chilled 1mm gap cuvettes (Eppendorf, Madison, WI) and electroporated (Eppendorf) at 1.0 kV (10kV/cm). The electroporated suspension was immediately mixed with 1 ml BHI with 0.5 M sucrose supplemented with 0.1 μg/ml erythromycin (Em), and incubated at 30°C for 1 h for the expression of the transposon-encoded *ermC* gene. Following incubation the culture was plated on BHI supplemented with 5 μg/ml Em and incubated at 30°C for 3-4 days. Plasmid retention was confirmed by screening for Em and kanamycin (Km) resistance. Growth of colonies on both media confirmed plasmid retention.

A colony resistant to both antibiotics was picked and inoculated in 3 ml BHI-Em and incubated overnight at 30°C. The overnight culture (10 μl) was inoculated in 2 ml BHI-Em and incubated at 30°C with shaking for 1 h; then the temperature was shifted to 40°C until OD_{600}=0.3-0.5 (about 5 hours), to construct the mutant library. Temperature

shift-up was performed for the integration of the transposon into the chromosome, as pMC38 is a temperature-sensitive plasmid (Cao et al., 2007). Integration of the transposon into the chromosome and loss of the plasmid is expected to result in mutants that are Em resistant but Km sensitive. Plasmid retention rate was calculated by dividing the Km-resistant colonies by the total number of Em-resistant colonies.

The culture was plated on BHI-Em and incubated at 30^{o}C for 2 days. Screening for the transposon insertion was done by streaking individual colonies in duplicate onto BHI-Em (5μg/ml), and BHI-Km (10 μg/ml) plates (Fig 2). Transposon mutants were inoculated in 250 μl BHI in 96-well microtiter plates (Costar, Corning, NY) and grown at 37^{o}C overnight. The mutants were transferred to 96-well microtiter plates with fresh BHI (250 μl/well) using an 8-channel pipette (Eppendorf, Westbury, NY) and incubated at 37^{o}C overnight. Initial 96-well plates were stored at -80^{o}C without glycerol. The mutants were screened following overnight growth at 37^{o}C as described below.

4. 3. 3. Mutant library screening procedures

Mutant libraries were screened as described below for hemolytic activity, cold sensitivity, catalase activity, freeze-thawing survival and sensitivity against sub-lethal concentrations of paraquat, cadmium, and NaCl.

4. 3. 3. 1. Screening for non-hemolytic mutants

For the loss of hemolysin activity, 3,360 mutants of F2365 and 960 mutants of 10403S were screened. The mutants grown in BHI on 96-well microtiter plates were spotted on blood agar (Remel Inc., TSA supplemented with 5% sheep blood) by using a sterile 48-pin replicator. The plates were air-dried and incubated at 37^{o}C for 2 days.

Following incubation plates were observed for absence of zones of hemolysis around the colonies.

4. 3. 3. 2. Screening for cold sensitivity

For growth at 4^{o}C, 3,360 mutants of F2365 and 960 mutants of 10403S were screened. The mutants grown in BHI on 96-well microtiter plates were spotted on BHI agar by using the sterile 48-pin replicator. The plates were air-dried, incubated at 4^{o}C for 30 days, and screened for growth.

4. 3. 3. 3. Screening for catalase activity

For the loss of catalase activity, 3,360 mutants of F2365 and 960 mutants of 10403S were screened. Initial BHI-Em plates used for screening of transposon integration were used for the screening for catalase activity. Hydrogen peroxide (H_2O_2) (2µl of a 30% v/v solution) (Superoxol®, J. T. Baker Chemical Co., Phillipsburg, NJ) was dropped at the edge of the streaked cultures. Absence of bubbling indicated the loss of catalase activity.

4. 3. 3. 4. Screening for repeated freezing and thawing survival

For repeated freezing and thawing survival, 960 mutants of F2365 were screened. The mutants of F2365 were grown in BHI on 96-well microtiter plates at 37^{o}C for 36 hours. Following growth at 37^{o}C mutants were frozen at -20^{o}C. After 24 hours the mutants on 96-well microtiter plates were thawed at 25^{o}C for 1 hour and re-frozen immediately at -20^{o}C. Following 18 cycles of repeated freezing and thawing, the mutants were spotted on BHI-agar plates by using the sterile 48-pin replicator. The plates were incubated at 37^{o}C for 48 hours, and screened for growth.

4. 3. 3. 5. Screening for paraquat sensitivity

For paraquat sensitivity, 2,400 mutants of F2365 and 960 mutants of 10403S were screened. Mutants grown in BHI in 96-well microtiter plates were spotted on BHI-agar plates supplemented with 0.05 mM paraquat (methyl viologen dichloride hydrate, 98%) (Sigma-Aldrich, St. Louis, MO) with the sterile 48-pin replicator. This concentration of paraquat was chosen as a sub-lethal concentration following preliminary tests with increasing concentrations of paraquat (0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM and 1 mM). The plates were incubated at 37^{o}C for 48 hours and screened for absence or reduction of growth on BHI-agar plates supplemented with paraquat.

4. 3. 3. 6. Screening for cadmium sensitivity

For cadmium sensitivity, 2,400 mutants of F2365 and 960 mutants of 10403S were screened. The mutants grown in BHI in 96-well microtiter plates were spotted on both isosensitest agar (Oxoid Ltd., Hampshire, England) and isosensitest agar supplemented with 10 μg/ml cadmium-chloride (Fisher Scientific, Fair Lawn, NJ) with the sterile 48-pin replicator. This concentration of cadmium was chosen as a sub-lethal concentration following preliminary tests with increasing concentration of cadmium (5 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml). The plates were incubated at 37^{o}C for 48 hours and screened for absence or reduction of growth on isosensitest agar supplemented with 10 μg/ml cadmium-chloride.

4. 3. 3. 7. Screening for NaCl sensitivity

For NaCl (Fisher Scientific, Fair Lawn, NJ) sensitivity, 2,400 mutants of F2365 and 960 mutants of 10403S were screened. The mutants grown in BHI in 96-well

microtiter plates were spotted on BHI-agar plates supplemented with 7.5% NaCl (w/v) with the sterile 48-pin replicator. This concentration of NaCl was chosen as a sub-lethal concentration following preliminary tests with increasing concentration of NaCl (5%, 7.5%, 10% and 15%). The plates were incubated at 37^{o}C for 48 hours and screened for the loss of growth on BHI-agar plates supplemented with NaCl.

4. 3. 4. Southern blots

The number of transposon insertion for selected mutants were determined by Southern blots. The PCR products from primers Maq205 (5'-GGT ATA GCA TAT GAA TCG CAT CCG ATT GCA G-3') and Maq206 (5'-TGT CAG ACA TAT GGG CAC ACG AAA AAC AAG T-3') were used to obtain a *mariner*-based transposon probe. The primers for the DNA probe were designed to amplify a 400-bp fragment within the erythromycin resistance gene (e*rmC*) of pMC38. The PCR products were labeled with digoxigenin (Genius kit; Roche, Indianapolis, IN). Labeling was done by adding sterile water to 10 ng-1 μg DNA to have a final volume of 15 μl. The DNA was denatured by placing in boiling water for 10 minutes, and placing immediately on ice. Next, 2 μl of 10×Hexanucleotide Mix, 2 μl 10× DIG DNA labeling mix and 1 μl Klenow (5 U/μl) were mixed with the denatured DNA and incubated at 37ºC overnight. The reaction was stopped by the addition of 2 μl of 0.2 M EDTA (pH 8.0). The labeled DNA was stored at –20^{o}C. The genomic DNA of selected mutants were isolated by using DNeasy kit (Qiagen, Valencia, CA). The genomic DNAs were digested by restriction enzyme, *Eco*RI (New England Biolabs, Waverly, MA). The digested genomic DNAs were electoporated in TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, and pH 8.0) with 0.8% agar at

85 V for 2.5 hours. The DNA fragments were transferred onto nylon membranes (Osmonics Inc. Westborough, MA) in 10 × SSC buffer (diluted from 20 × SSC, 20 × SSC: 175. 3 g NaCl, 88. 2 g Na-Citrate, H7.0) by capillary action, overnight. CSPD (Chemiluminescent substrate, Roche) (diluted 1:100 ml in detection buffer 0.1 M Tris, 0.1 M NaCl, pH 9.5) was used as the substrate for anti-DIG-alkaline phosphatase. X-ray film (Fuji) was exposed to the chemiluminescent light resulting from hybridization of DNA fragments labeled with the probe.

4. 3. 5. Determination of transposon insertion sites

Transposon insertion site was determined by sequencing DNA fragments amplified by arbitrary PCR. In arbitrary PCR, DNA was amplified from one end of the transposon by applying two successive PCRs as described by Cao et al. (2007). In each round of PCR one transposon-specific and one arbitrary primer was used. The arbitrary primers, Marq207 (5'–GGC CAC GCG TCG ACT AGT ACNNNNNNNNNGTAAT–3') and Marq208 (5'–GGC CAC GCG TCG ACT AGTAC–3'), used in this study were previously reported by Cao et al. (2007). In the primary PCR, primer pairs Marq207 & Marq255 (5'–CAG TAC AAT CTGCTC TGA TGC CGC ATA GTT–3') and Marq 207 & Marq269 (5'–GCT CTG ATA AAT ATG AAC ATG ATG AGT GAT–3') were used to amplify the DNA fragments from the left and right end of the transposon, respectively. In the secondary PCR, 5 µl of the 4% dilution from the primary PCR was used in a 20-µl reaction. Primer pairs Marq208 Marq256 (5'–TAG TTA AGC CAG CCC CGA CAC CCG CCA ACA–3') and Marq 208 & Marq270 (5'–TGT GAA ATA CCG CAC AGA TGC GAA GGG CGA–3') were used to amplify the DNA fragments from the left and

right ends of the transposon, respectively. After purification of the PCR products by QIAquick® PCR Purification Kit (Qiagen, MA), they were sequenced using Marq257 and Marq271 for the left and right end of the transposon, respectively. The sequencing of the PCR products were performed at Genomic Science Laboratory at North Carolina State University (Raleigh, NC) and at Genewiz, Inc. (South Plainfield, NJ).

4. 4. RESULTS

4. 4. 1. Construction of Mutant Libraries

The plasmid pMC38 (8.3kb) was introduced into *L. monocytogenes* by electroporation, resulting in erythromycin and kanamycin resistant cells. Shifting the temperature to 40^{o}C selected for transposon integrants into the chromosome that were erythromycin-resistant but kanamycin-sensitive. Integration of the transposon was determined by streaking single colonies on BHI plates supplemented by erythromycin and kanamycin (Fig 2). Integration frequency in *L. monocytogenes* strains 10403S and F2365 were 100% (960/960) and 99% (3329/3360), respectively. The number of transposon copies in selected mutants was determined by Southern blot analysis, and single insertions were detected in all tested mutants (Fig. 3).

4. 4. 2. Screening for non-hemolytic mutants

During the screening of mutants two non-hemolytic mutants were found. One of these mutants (ROA2) has the transposon integrated at the *hly* (listeriolysin O) (Fig. 4), whereas the other (ROA9) has the insertion at LMOf2365_2800 (pseudogene). In addition, seven mutants showed decreased hemolytic activity (e.g. ROA7, Fig. 5) and

harbored the transposon insertion in LMOf2365_2760 (bifunctional glutamate—cysteine ligase/glutathione synthetase). Among these mutants the transposon was integrated in two different locations in LMOf2365_2760 (Table 1). In addition to the mutants that were non-hemolytic or showed decreased hemolytic activity, we found one mutant that could not grow on blood agar (Fig. 6). In this mutant transposon was integrated in LMOf2365_0065 (*purA*, adenylosuccinate synthetase). Repeated trials showed that this mutant could not grow on the commercial blood agar plates (Remel Inc.), but grew normally on TSA plates without blood. The mutant had decreased growth on TSA plates supplemented with 5%(v/v) Sheep blood, prepared in our laboratory.

4. 4. 3. Screening for cold sensitivity

Screening of the mutant library of *L. monocytogenes* strains F2365 and 10403S for cold sensitivity was performed by spotting them on BHI-agar plates without antibiotics and incubating at 4^{o}C for 30 days. One mutant with the transposon inserted on LMOf2365_1746 (helicase domain protein) lost its ability to grow at 4^{o}C (Fig. 7). In addition, we found 7 other mutants that showed impaired growth at 4^{o}C. Among these 7 mutants 5 were derived from F2365. These had transposon insertions in *kat* (catalase), LMOf2365_2443 (leucine rich repeat domain protein), LMOf2365_0246 (PIN/TRAM domain protein), LMOf2365_2517 (thymidine kinase) and LMOf2365_0999 (branched-chain amino acid aminotransferase). The other two were mutants of 10403S having transposon insertions in lmo0136 (oligopeptide ABC transporter, permease protein), and lmo0084 (protein similar to oxidoreductases) (Table 1).

4. 4. 4. Screening for catalase activity

One F2365 mutant showed loss of catalase activity and harbored a transposon insertion in *kat* (catalase) gene. Interestingly, this mutant also showed impaired growth on agar plates.

4. 4. 5. Screening for freeze-thaw survival

Freeze-thaw survival screening was performed with about 1000 mutants of F2365. At the end of 18 freeze-thaw cycles no predictable difference were observed on any mutants and the positive and negative controls. F2365 grown at 37^{o}C and 4^{o}C were used as positive and negative controls, respectively. Because of the inefficiency of this protocol to differentiate between positive and negative controls, this testing was not further performed (Fig. 8).

4. 4. 6. Screening for paraquat sensitivity

Paraquat sensitivity of F2365 and 10403S mutants was screened on plates containing 0.5mM paraquat. One mutant of 10403S (ROA6) and one of F2365 (ROA3) showed impaired growth on paraquat plates. In the paraquat-sensitive mutant ROA6 of 10403S the transposon was integrated in lmo0136 (oligopeptide ABC transporter, permease protein) and in the F2365 mutant ROA3 the transposon was integrated in *kat* (Fig. 9).

4. 4. 7. Screening for cadmium sensitivity

Cadmium sensitivity screening was conducted on Isosensitest agar plates with 10 μg/ml cadmium. Among the mutants screened; nine mutants of F2365 and one of 10403S showed reduced tolerance to cadmium. The sequencing results indicated in the

cadmium sensitive F2365 mutants the transposon was integrated in 3 different genes. In seven of these nine mutants the transposon insertion was in LMOf2365_2760 (bifunctional glutamate—cysteine ligase/glutathione synthetase), at two different locations (five mutants had insertion on the same location and two in another location of the gene) (Fig. 10a&b); in one mutant the insertion was in LMOf2365_1716 (conserved hypothetical protein) (Fig. 10a&b); and one mutant harbored an insertion in LMOf2365_2800 (pseudogene) (Fig. 10b). In the cadmium-sensitive 10403S mutant the transposon was integrated in oligopeptide ABC transporter, permease protein (Fig. 11).

4. 4. 8. Screening for NaCl sensitivity

In the screening for NaCl sensitivity TSA plates with 7.5% NaCl were used. Among the strains tested two F2365 mutants having the same transposon insertion site showed impaired growth (Fig. 12). In these mutants the transposon was integrated in LMOf2365_1875 (ABC transporter, manganese-binding protein).

4. 5. DISCUSSION

In this part of the study our objective was to identify genes responsible for freeze-thaw tolerance of *L. monocytogenes*. To achieve this goal we constructed mutant libraries of two *L. monocytogenes* strains, F2365 (serotype 4b), and 10403S (serotype 1/2a), with the *mariner*-based transposon plasmid pMC38. In the process of mutant library construction we achieved a high frequency of integrants by using pMC38 with both strains used (100% in 10403S, and 99% in F2365). Previously, it was also reported that the efficiency of this transposition system was 84%, higher than others used for construction of mutant libraries, such as Tn*917*-based systems (Cao et al. 2007). This

high frequency of integrants showed that this system is a valuable tool for the future mutagenesis studies.

Initially, we screened the mutants of F2365 for the loss of their freeze-thaw tolerance. As the survival differences between positive and negative controls could not be consistently differentiated, and we concluded that mutants would not be reliably identified with this method. Therefore, we tested for mutants in tolerance to selected stresses expected to be involved during freezing and thawing. Previously, it was reported that freezing and thawing stress involves a number of different stresses, including osmotic, cold, and oxidative stress (Gao et al. 2000). In light of this, we screened our mutants for loss of tolerance to salt, cold, cadmium, and paraquat. We hypothesized that if a mutant lost its tolerance to one of these stresses, it would also be susceptible against freeze-thaw stress. In the screening of the mutant libraries we identified two mutants sensitive against salt stress, eight mutants that either could not grow or showed inhibited growth at low temperatures, four mutants that were susceptible to cadmium and two that were susceptible to paraquat. However, with the exception of one mutant susceptible to paraquat, further freeze-thaw tolerance of the mutants was not significantly impaired.

Previous gene expression comparisons of 4^{o}C-and 37^{o}C-grown *L. monocytogenes* by microarray analysis indicated that the expression of *kat* decreased at low temperature both in logarithmic and stationary phase. However, the change in expression level of this gene was relatively low, 3.50, and 2.45 during logarithmic and stationary phases, respectively (Chan et al. 2007). In the same study LMOf2365_1746 (helicase domain protein) showed increased expression at low temperature both in logarithmic and

stationary phases. Again, the changes in the expression levels were relatively low, 2.40, and 3.86 for logarithmic and stationary phases, respectively (Chan et al. 2007). This increased expression of LMOf2365_1746 (helicase domain protein) indicated that this gene may be involved in growth at low temperatures, which supports the phenotype that we observed in this study. The transposon insertion in oligopeptide ABC transporter, permease protein in the 10403S mutant (ROA6) led to sensitivity to cadmium and paraquat, as well as to cold. The microarray comparison study indicated that this gene showed decreased expression at logarithmic phase during growth at 4^{o}C (5.48-fold decrease). On the other hand, no significant change in expression was observed at stationary phase during growth at low temperature (Chan et al. 2007). Another gene that we found in this study that showed differential expression was *purA*. The expression of *purA* increased at low temperatures, although the changes in expression levels were relatively low, 2.18 and 3.05 for logarithmic and stationary phases, respectively (Chan et al. 2007). On the other hand, the gene expression comparison between 4^{o}C and 37^{o}C by microarray, showed no difference in expression levels of the other genes found in this study between these two temperatures (Chan et al. 2007).

Although, we were not able to screen directly for mutants impaired in growth temperature-dependent tolerance to repeated freezing and thawing, we were able to identify novel genes putatively related to tolerance of *L. monocytogenes* against different stresses. In the following chapter, the mutants that had transposon insertions in *kat* (catalase), LMOf2365_1746 (helicase domain protein), and LMOf2365_2760

(bifunctional glutamate—cysteine ligase/glutathione synthetase) will be further characterized.

4. 6. REFERENCES

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Table 4. 1: *L. monocytogenes* mutants analyzed in this study.

Strains	Insertion nucleotide	Genotype and Features	Homolog in EGD-e	Homolog in *Listeria innocua* Clip11262	Screening Phenotype
ROA2	211210	Transposon mutant of F2365 *hly* (LMOf2365_0213)::transposon (pMC38), Em^R Km^S	*hly* lmo0202	-	Blood agar Non-hemolytic
ROA3	2833280	Transposon mutant of F2365 *kat* (LMOf2365_2776)::transposon (pMC38), Em^R Km^S	*kat* lmo2785	*kat* lin2920	H_2O_2 Catalase negative, impaired growth at low temperatures, and on regular media, growth was restored on blood agar.
ROA4	1765482	Transposon mutant of F2365 LMOf2365_1746 (helicase domain protein)::transposon (pMC38), Em^R Km^S	lmo1722	lin1833	4^oC growth Cold sensitive, no growth at 4^oC.
ROA6	151145	Transposon mutant of 10403S (LMOf2365_0154) (oligopeptide ABC transporter, permease protein)::transposon (pMC38), Em^R Km^S	lmo0136	lin0183	Paraquat plates (0.05mM) Cad10 sensitive, impaired growth on 0.05mM paraquat, impaired growth at 4^oC.
ROA7 ROA11 ROA15 ROA20 ROA21	2812912	Transposon mutant of F2365 LMOf2365_2760 (putative glutamate--cysteine ligase/amino acid ligase)::transposon (pMC38), Em^R Km^S	lmo2770	lin2913	Cadmium plates (10µg/ml) Cad10 sensitive, decreased hemolytic activity, impaired growth at 4^oC.
ROA12 ROA17	2812908	Transposon mutant of F2365 LMOf2365_2760 (putative glutamate--cysteine ligase/amino acid ligase)::transposon (pMC38), Em^R Km^S	lmo2770	lin2913	Cadmium plates (10µg/ml) Cad10 sensitive, decreased hemolytic activity.
ROA8 ROA18 ROA19	1737774	Transposon mutant of F2365 LMOf2365_1716 (conserved hypothetical protein); upstream of LMOf2365_1715 (dUTPase family protein), Em^R Km^S	lmo1692	lin1800	Cadmium plates (10µg/ml) Cad10 sensitive.

Table 4. 1: Continued.

ROA9	2852598	Transposon mutant of F2365 LMOf2365_2800 (pseudogene)::transposon (pMC38), Em^R Km^S			Cadmium plates (10μg/ml) Impaired growth on Cad10,non-hemolytic
ROA13 ROA16	1903255	Transposon mutant of F2365 LMOf2365_1875 (ABC transporter, manganese-binding protein)::transposon (pMC38), Em^R Km^S	lmo1847	lin1961	NaCl plates (7.5% w/v) Impaired growth on 7.5% NaCl.
ROA14	65882	Transposon mutant of F2365 LMOf2365_0065 (adenylosuccinate synthetase) *purA*::transposon (pMC38), Em^R Km^S	*purA* lmo0055	lin0048	Blood agar No growth on blood agar. Normal growth on TSAYE.
ROA22	97265	Transposon mutant of 10403S LMOf2365_0101 (F2365) (oxidoreductase, aldo/keto reductase family) ::transposon (pMC38), Em^R Km^S	lmo0084	-	4^oC growth Impaired growth at 4^oC.
ROA23	2499285	Transposon mutant of F2365 LMOf2365_2443 (leucine rich repeat domain protein)::transposon (pMC38), Em^R Km^S	lmo2470	-	4^oC growth Impaired growth at 4^oC.
ROA24	260164	Transposon mutant of F2365 LMOf2365_0246 (PIN/TRAM domain protein)::transposon (pMC38), Em^R Km^S	lmo0234	lin0266	4^oC growth Impaired growth at 4^oC.
ROA25	2575470	Transposon mutant of F2365 LMOf2365_2517 (thymidine kinase)::transposon (pMC38) upstream of *prfA* (vicinity of *rpmE2-rho)*, Em^R Km^S	lmo2544	lin2688	4^oC growth Impaired growth at 4^oC.
ROA26	1010710	Transposon mutant of F2365 LMOf2365_0999 (branched-chain amino acid aminotransferase)::transposon (pMC38), Em^R Km^S	lmo0978	Lin977	4^oC growth Impaired growth at 4^oC.

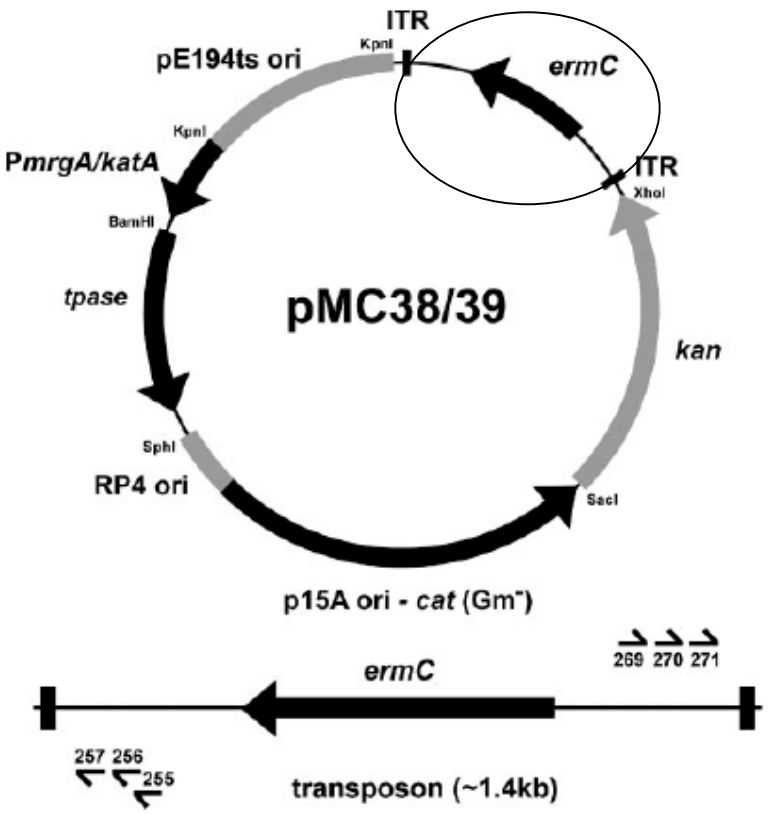


Fig. 4. 1: Map of the *mariner*-based transposon delivery vector pMC38/39 (modified from Cao et al. 2007).

2a **2b**

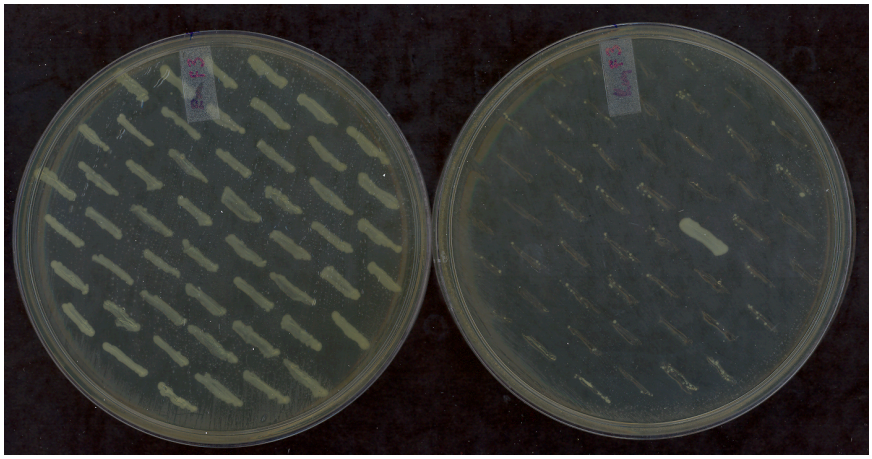


Fig.4. 2: Screening of putative mutants for plasmid retention. Screening was done by streaking a colony in duplicate onto BHI-Em (5μg/ml) (2.a), and BHI-Km (10 μg/ml) (2.b) plates. Growth on both plates indicates plasmid retention.

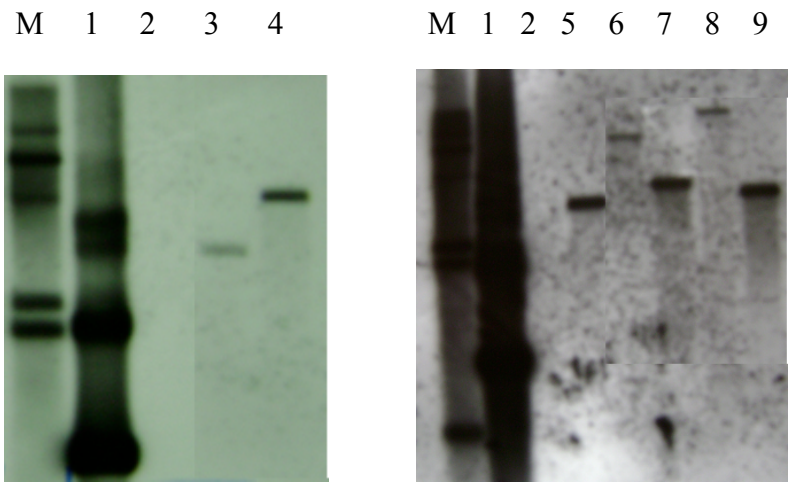


Fig. 4. 3: Southern blot for determination of transposon copy number; M, the molecular size marker, Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 are; *ermC,* F2365, ROA2, ROA3, ROA4, ROA6, ROA7, ROA8, and ROA9, respectively. The mutant DNAs were all digested by *HindIII* and hybridized with digoxigenin-labeled probes derived from the erythromycin resistance gene (*ermC*).

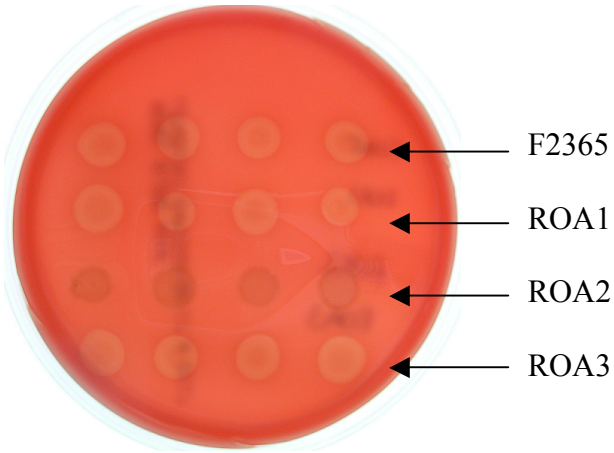


Fig. 4. 4: Loss of hemolytic activity in the F2365 mutant ROA2. Transposon integration in ROA2 was in *hly*.

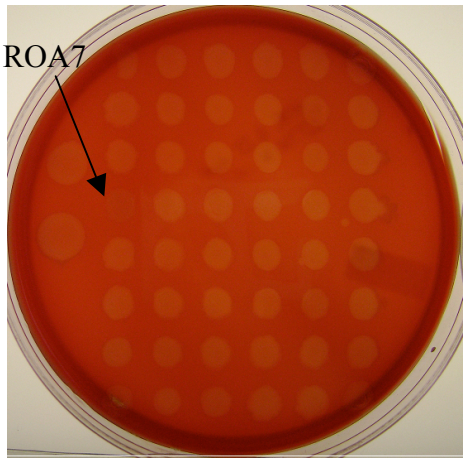
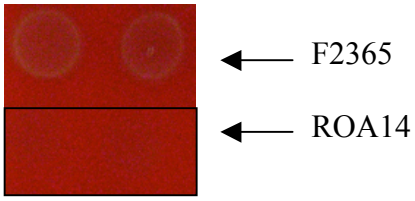


Fig. 4. 5: Reduced hemolytic activity in the F2365 mutantROA7. Transposon insertion in ROA7 was in LMOf2365_2760 (bifunctional glutamate-cysteine ligase/glutathione synthetase).

A



B

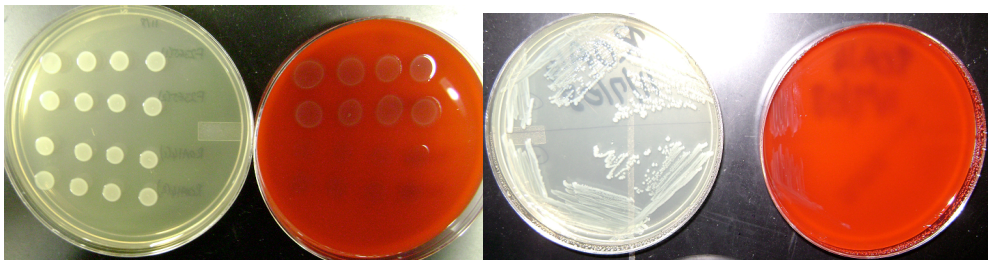


Fig. 4. 6: Inhibition of growth on blood agar of F2365 mutant ROA14. Transposon insertion in ROA14 was in LMOf2365_0065 (*purA*, adenylosuccinate synthetase).

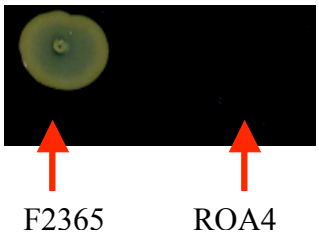


Fig. 4. 7: Loss of ability to grow at 4°C in the F2365 mutant ROA4. Transposon insertion in ROA4 was in LMOf2365_1746 (helicase domain protein).

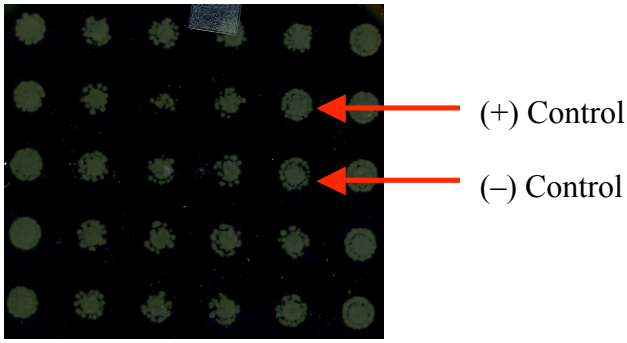


Fig. 4. 8: Impact of 18 cycles of repeated freezing and thawing on the mutant library.

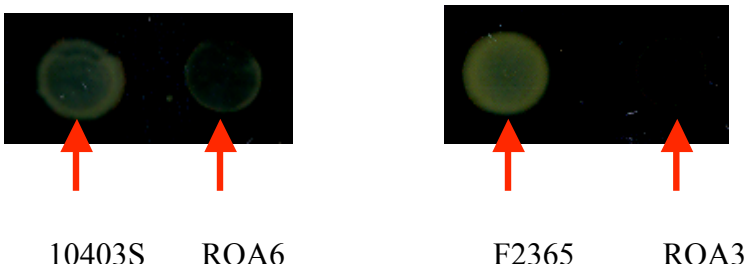


Fig. 4. 9: Decrease in paraquat tolerance in 10403S mutant ROA6 and F2365 mutant ROA3 (also catalase-negative). Transposon insertion in ROA6 was in oligopeptide ABC transporter, permease protein (ROA6), whereas transposon insertion in ROA3 was in *kat*.

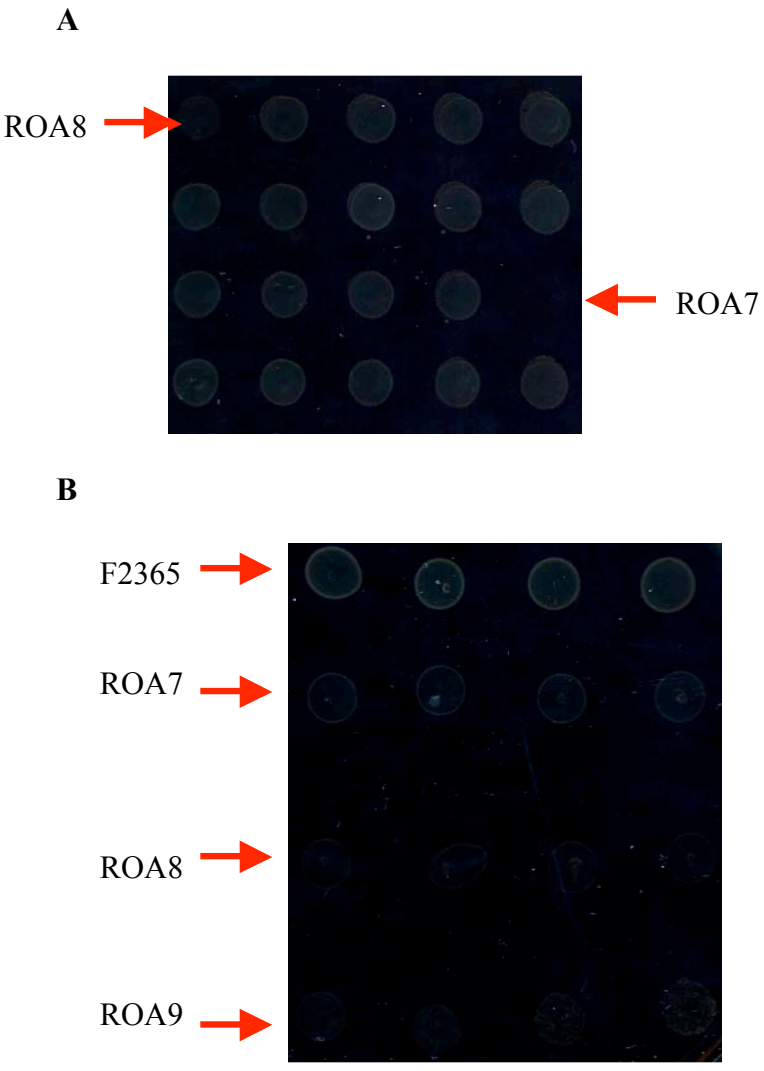


Fig. 4. 10: Impaired tolerance to cadmium in F2365 mutants ROA7, ROA8, and ROA9. Transposon insertions in these mutants were in LMOf2365_2760 (bifunctional glutamate—cysteine ligase/glutathione synthetase), LMOf2365_1716 (conserved hypothetical protein), and LMOf2365_2800 (pseudogene), respectively.

Fig. 4. 11: Impaired tolerance to cadmium in 10403S mutant ROA6. Transposon insertion in ROA6 was in oligopeptide ABC transporter, permease protein.

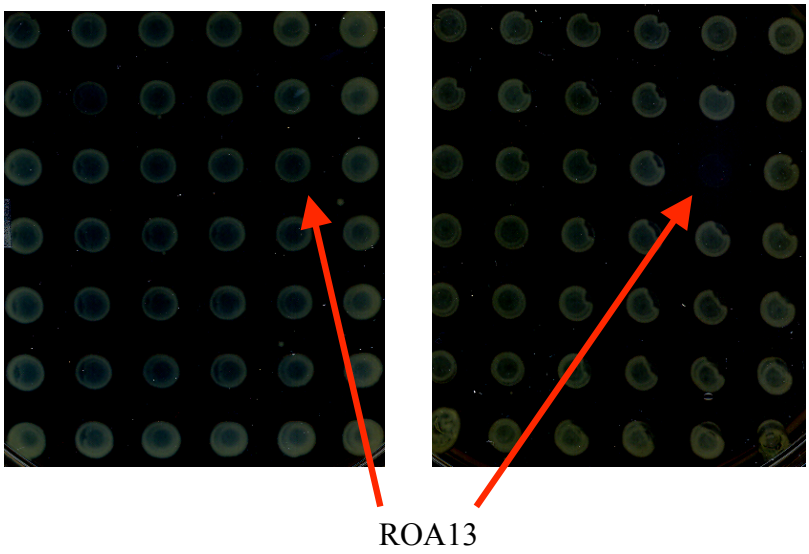


Fig. 4. 12: Impaired tolerance to NaCl in F2365 mutant ROA13**.** Transposon insertion in ROA13 was in LMOf2365_1875 (ABC transporter, manganese-binding protein).

CHAPTER V: Characterization of the Catalase Gene in *L. monocytogenes* strain F2365

5. 1. ABSTRACT

Listeria monocytogenes is a gram-positive, psychrotrophic, facultative intracellular foodborne pathogen responsible for severe illness (listeriosis). *L. monocytogenes* can grow in a wide range of temperatures and several key virulence determinants of the organism are expressed at 37^{o}C but are strongly repressed below 30^{o}C. Recently, we discovered that the freeze-thaw tolerance of *Listeria* was dependent on growth temperature, being much stronger when bacteria were grown at 37^{o}C than when grown at 25^{o}C or 4^{o}C. In order to understand the mechanisms underlying this temperature dependent freeze-thaw tolerance, we constructed a mutant library of *L. monocytogenes* strain F2365, and screened for mutants with impaired resistance to a number of different stresses. One interesting mutant found during the screening of the mutant library had a transposon insertion in the catalase gene (*kat*). In this study, we characterize the growth attributes and freeze-thaw tolerance of this mutant. Freeze-thaw tolerance of 37^{o}C and 25^{o}C cells was similar between the catalase mutant and the wild type parental strain. However, the growth of the catalase-negative mutant was impaired compared to the wild type. Impaired growth was more noticeable when the cells were grown on the surface of agar plates compared to growth in liquid, especially at lower temperatures, suggesting that they were subjected to increased oxygen stress under such conditions. By complementing *kat* into the catalase-negative mutant, we were able to confirm that inactivation of this gene was responsible for these phenotypes. Further studies are needed to fully understand the role of catalase in the ecology of *L. monocytogenes*.

5. 2. INTRODUCTION

Listeria monocytogenes is a gram-positive foodborne pathogen that has the ability to cause severe illness (listeriosis) in both humans and animals. Common symptoms of listeriosis are meningitis, septicemia, and abortion. Neonates, pregnant women, elderly and immunocompromized people are at high risk for listeriosis (Farber et al., 1991, Schlech, 2000).

L. monocytogenes is ubiquitously distributed in the environment and has the ability to survive over a wide range of temperatures (between 1 to 45^{o}C) (Farber et al., 1991). During its survival in the environment *L. monocytogenes* is subjected to number of stress conditions including repeated freezing and thawing. Therefore, characterization of freeze-thaw stress tolerance (cryotolerance) of *L. monocytogenes* will be valuable for understanding the organism's ecology. In the previous chapter, construction and screening of mutant libraries of two *L. monocytogenes* strains (F2365 and 10403S) was described. The screening of the mutant libraries for loss of cryotolerance did not provide significant differentiation between the cryotolerant and the cryosensitive bacteria. Therefore, the mutant library was screened for underlying stress mechanisms that take place during freezing and thawing. Previously, it was reported that during thawing oxidative burst occurred (Hermes-Lima et al. 1993, Park et al. 1998) and 60% of the *Saccharomyces cerevisiae* mutants that were cryosensitive were also oxidative stress-sensitive (Garénaux et al. 2008, Park et al. 1998). In addition to oxidative stress, it is also

known that freeze-thaw stress involves other stresses such as osmotic and cold stress (Gao et al.. 2000).

L. monocytogenes is subjected to oxidative stress during both extracellular and intracellular growth. It is subjected to oxidative burst in phagocytic cells during its intracellular life cycle. Also, *Listeria* is a facultative microaerophile, and when the bacteria are growing aerobically they are faced with oxygen stress. During oxygen stress, reactive oxygen species (ROS) cause damage to DNA, protein, and lipids in the cell (Martindale et al. 2002). In order to overcome these stress conditions, *L. monocytogenes* initiates the production of superoxide dismutase and catalase (Vazquez-Boland et al. 2001). Another oxygen stress response mechanism reported in *B. subtilis* is the production of an alkyl hydroperoxide reductase that has not yet been identified as a component of *Listeria*'s oxidative stress response (Hellmann et al. 2003). Previously, it was reported that catalase and superoxide dismutase (SOD) worked together during the detoxification of ROS and play major role in the survival of *L. monocytogenes* during its intracellular life cycle (Mostertz et al. 2004, and Vazquez et al. 2001). Briefly, superoxide anions generated by the oxidative burst in phagocytic cells were converted to H_2O_2 by SOD, which was followed by conversion of toxic H_2O_2 into water (H_2O) and oxygen (O_2) by catalase (Mongkolsuk et al. 2002). Welch et al. (1979) reported that catalase-negative mutants of *L. monocytogenes* showed at least two-fold increased SOD activity, suggesting that catalase and SOD can take over the role of each other when one is inhibited.

During its intracellular lifecycle *L. monocytogenes* has to overcome the listericidal effect of macrophages, thought to involve reactive nitrogen intermediates (RNI) generated during the oxidative burst of reactive oxygen intermediates (ROI) and via the interaction of the superoxide anion with nitric oxide (NO) (Vazquez et al. 2001). In vivo studies conducted with immunodeficient mice (Beckerman et al. 1993), mouse peritoneal and human monocyte-derived macrophages (Bermudez, 1993) supported the significant contribution of ROI and RNI in the macrophage-mediated killing of *L. monocytogenes* (Vazquez et al. 2001). As SOD and catalase are involved in the intracellular survival and colonization of *L. monocytogenes,* they are considered to be accessory virulence factors (Haas et al. 1991, Vazquez et al. 2001). Leblond-Francilland et al. (1989) reported that catalase-negative mutants of *L. monocytogenes*, constructed by insertion of Tn*1545*, showed no difference from the wild type in virulence in intravenous infections of mice. Also, the mutants and the wild type were able to grow inside the host cells. Therefore, they stated that the catalase did not play a crucial role in the macrophage survival of *L. monocytogenes*. This finding was supported by reported isolation of catalase-negative *L. monocytogenes* from listeriosis patients (Bubert et al. 1997, Elsner et al. 1996, Swartz et al. 1991). On the contrary, van Dissel (1993) reported that murine macrophages stimulated by recombinant interferon gamma killed catalase-negative *L. monocytogenes* in the presence of relatively low serum concentrations (1-2.5%), whereas catalase-positive bacteria required much higher serum concentrations (10% for comparable killing. Such data suggest that under certain conditions (e.g. relatively low serum concentrations) catalase is required for bacterial resistance to killing by activated

macrophages. As a result of these contradictory findings, the role of catalase in the intracellular survival and virulence of *L. monocytogenes* remains unclear. Furthermore, the deletion of *sod* resulted in a slight decrease in the survival capacity of *L. monocytogenes* in mouse bone marrow-derived macrophages and in the organs of infected mice (Brehm et al. 1992). On the other hand, Archambaud et al. (2006) reported that deletion of *sod* resulted in impaired survival in macrophages and markedly attenuated virulence in mice, indicating the importance of tolerance against ROS in *L. monocytogenes* virulence.

In this study, a catalase negative mutant found during the screening of the mutant library was complemented with *kat*. The mutant strains and the wild type F2365 were further characterized in terms of their freeze-thaw tolerance and other phenotypes.

5. 3. MATERIALS AND METHODS

5. 3. 1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. Mutant library of cheese isolate of the California outbreak, F2365, was constructed as described in the previous chapter. Selected mutants and *L. monocytogenes* strain F2365 were grown in brain heart infusion broth (BHI, BBL, Sparks, MD) or tryptic soy broth (TSB) (BBL, Sparks, MD) supplemented with 0.7% Yeast Extract (YE) (BBL, Sparks, MD) (TSBYE), at 37^{o}C for 36 hours, and stored at –80^{o}C in the presence of 20% glycerol. Before each experiment bacteria were grown on trypticase soy agar with 5% sheep blood (Remel, Lenexa, KS) at 37^{o}C for 36 h and liquid cultures were started by transferring a single colony into 5 ml of TSBYE and incubating at 37^{o}C overnight. For motility assays soft

agar medium, TSBYE with 0.4% agar, was used. Briefly, 5µl of the culture was spotted on the soft agar medium, air dried and incubated at 25^{o}C for up to 96 hours. Antibiotics used for selection of *Listeria* mutants were chloramphenicol (6 µg/ml), and nalidixic acid (20 µg/ml). For complementation, *Escherichia coli* strains (DH5α and SM10) were grown in Luria-Bertani (LB) (BBL, Sparks, MD) broth or agar medium supplemented with chloramphenicol (25 µg/ml).

5. 3. 2. Genetic complementation

To confirm the role of *kat* in the stress response of *L. monocytogenes*, the mutant (ROA3) was complemented with the wild type gene using the site-specific temperature-sensitive integration vector, pPL2 (Lauer et al. 2002) (Fig. 1). The promoter and the transcription termination regions of the *kat* were identified previously in *L. seeligeri* (Haas et al. 1991). The sequence comparison of *kat* between EGD-e, F2365 and *L. seeligeri* indicated that the promoter and the transcription termination sites were conserved among these three strains (Fig. 2). Therefore, these regions were also included in the fragment cloned for genetic complementation of ROA3.

Forward and reverse primers were designed based on the DNA sequence of *kat*. The fragment within two primers included the target gene (*kat*), putative promoter region, and the putative transcription termination site. The *kat* was amplified with primers ROA3F (5'- CTAA CCCGGG CGA TGA ATT AGG TCG TCT GT-3', XmaI site underlined) and ROA3R2 (5'- GTAA GAGCTC TTA CTC CAA TCT TCT AGC C-3', SacI site underlined). At the 5' end of each primer, restriction enzyme recognition sequences were incorporated for cloning purposes. Following the amplification, the PCR

product was digested by restriction enzymes and purified using PCR purification kit (Qiagen Valencia, CA). The purified PCR product was ligated into the *L. monocytogenes* site-specific integration vector pPL2 (Lauer et al. 2002), which was digested with the same restriction enzymes. The recombinant plasmid was transformed into *E. coli* DH5α or SM10 by electroporation. In electroporation 50 µl of competent cells were mixed with 1 µl of plasmid ligation mixture. The mixture was placed in pre-chilled 1mm gap cuvettes (Eppendorf, Madison, WI) and electroporated (Eppendorf) at 1.25 kV (12.5 kV/cm). The selection of the transformants was done on LB plates supplemented with chloramphenicol (25 µg/ml). Positive colonies were confirmed by PCR with primers specific to the target genes.

5. 3. 3. Bacterial conjugation

E. coli SM10 transformants were grown in LB supplemented with chloramphenicol (25 µg/ml) to mid-log phase (OD_{600}= ~0.55) with shaking (100 rpm) at 30^{o}C. Recipient *L. monocytogenes* strains were grown overnight at 37^{o}C, and were treated at 45^{o}C for 10 min in water bath. Donor culture (3 ml) and heat-treated recipient culture (1.5 ml) were mixed and filtered through 0.45-µm-pore-size HA-type filters (22 mm; Millipore; 10ml syringe, BD). The filter was washed with BHI (10ml). Following washing, the filter was placed onto a BHI plate without antibiotic and incubated overnight at 30^{o}C. The bacteria on the filter were resuspended in 2.5-4.0 ml of BHI and 25 µl or 50 µl of the suspension were plated on BHI plates supplemented with chloramphenicol (6 µg/ml) and nalidixic acid (20 µg/ml). Nalidixic acid was added to BHI for selection of *L. monocytogenes*. Plates were incubated at 30^{o}C for 2 to 3 days. In

addition, 50 µl of the suspended bacterial cells was transferred into 30 to 50 ml BHI broth supplemented with chloramphenicol (6 µg/ml) and nalidixic acid (20 µg/ml) and grown at 30^{o}C overnight. Overnight culture (50µl) was plated on BHI plates supplemented with chloramphenicol (6 µg/ml) and nalidixic acid (20 µg/ml) and the plates were incubated at 30^{o}C for 2 to 3 days

5. 3. 4. Phenotypic characterization of mutants

5. 3. 4. 1. Hemolytic activity

Hemolytic activity of the mutants, complemented strains and the wild type were determined as described in previous chapter.

5. 3. 4. 2. Catalase activity

Catalase activity of the mutants, complemented strains and the wild type were determined as described in previous chapter.

5. 3. 4. 3. Motility

The ROA3, complemented strains and the wild type strain of *L. monocytogenes* were grown in TSBYE at 37^{o}C for 36 h. Five microliters of the cultures were spotted in duplicate on a soft agar plate, TSBYE with 0.4% agar, and incubated at 4^{o}C, 10^{o}C, 25^{o}C, and 37^{o}C for 30 days, 7 days, 48h and 36 h, respectively.

5. 3. 4. 4. Growth on agar plates

The mutant ROA3, the complemented strain and F2365, the wild type strain of *L. monocytogenes* were grown in TSBYE at 37^{o}C for 36 h. After serial dilution they were plated on TSAYE and incubated at 37^{o}C for 36 h and 25^{o}C for 48 h. Also, ROA3 and wild type were grown under microaerobic conditions generated by the CampyPak

Microaerophilic System (BBL, Sparks, MD) at 37°C for 36 h and 25°C for 48 h. The gas mix generated by these packs consist of 80% nitrogen, 7.5% hydrogen, 7.5% carbon dioxide, and 5% oxygen (Bolton et al. 1997). The size of the colonies on the plates was compared. In addition, 5 µl of mutant and wild type were spotted on TSAYE and incubated at 37°C for 36 h and 25°C for 48 h.

5. 3. 4. 5. Cold Growth

Cold growth of the mutant ROA3, the genetically complemented mutant and the wild type of *L. monocytogenes* were determined by inoculating 1 ml of 37°C grown culture into 99ml of TSBYE and growing them at 10°C and 4°C. Growth phase was determined by monitoring optical density (OD) at 600 nm, using a spectrophotometer (BioRad SmartSpec 3000, Hercules, CA). The OD_{600} readings were taken every 24 h, and every 3 days at 10°C and 4°C, respectively. Also, 5 µl of the mutant and the wild type were spotted on TSAYE and incubated at 10°C and 4°C.

5. 3. 4. 6. Freeze-thaw tolerance of mutants

Freeze-thaw tolerance of ROA3 and the wild type strain F2365 were determined by following the protocol described in previous chapters. Briefly, ROA3 and the wild type were grown at 25°C or 37°C for 48 or 36 h respectively, followed by 18 freeze-thaw cycles. The cultures were enumerated every 3 cycles by plating the appropriate dilutions on TSAYE.

5. 3. 4. 7. Growth and survival of ROA3 in food

To determine the role of *kat* in the growth and survival of *L. monocytogenes*, we have conducted growth and survival tests for ROA3 and the wild type strain F2365 in

raw or pasteurized milk and on surface of tomatoes. The growth of ROA3 and F2365 in milk was determined by inoculating 1ml of overnight culture in 99 ml of raw or pasteurized milk followed by incubation at 25^{o}C or 4^{o}C. The pasteurized and raw milk were provided from NCSU Food Science Dairy & Process Applications Laboratory. The growth of the cells was determined by plating appropriate dilutions on Modified Oxford selective medium (Oxoid, Basingstoke, England).

The survival of ROA3 and F2365 on the surface of tomatoes was determined by spotting overnight cultures on the surface (bottom half) or the stalk scar of organic cherry tomatoes purchased from a local grocery. First the stalk and bottom parts of the tomato were cut, then 5 µl of ROA3 and F2365 were spotted and air-dried. Following overnight incubation on the tomato surface at room temperature (25^{o}C) the survival of the cells was determined by suspending the cultures by vortexing in phosphate buffered saline (PBS) (Sigma, St. Louis, MO) and plating the appropriate dilutions on Modified Oxford selective medium (Oxoid).

5. 3. 4. 8. Sequence comparison of inactivated genes between F2365 and EGD-e

The genomic region including *kat* was compared by using an online sequence comparison tool, WebACT. WebACT uses the Artemis Comparison Tool (ACT) developed by the Sanger Institute (Carver et al. 2005).

5. 4. RESULTS

5. 4. 1. Complementation of ROA3 with *kat*

Integration of pPL2 with or without *kat* was confirmed by PCR with primer NC16, which binds upstream of $tRNA^{Arg}$ in the host genome, and primer PL95, which

binds to PSA *int* in pPL2. The size of the PCR product is 499 bp (Fig.3A). Confirmation of the complementation of *kat* was performed by PCR by using primers specific for *kat* (ROA3F and ROA3R2) (Fig. 3B).

5. 4. 2. Hemolytic activity

No difference in the hemolytic activity between the wild type, ROA3, ROA3E, and ROA3C were detected.

5. 4. 3. Catalase activity

Catalase activity of ROA3 was restored following the complementation with the *kat* gene, using the integration vector pPL2. On the other hand, no catalase activity was observed on ROA3, or ROA3 harboring the integrated pPL2 (ROA3E).

5. 4. 4. Motility

The motility test for the catalase negative mutant, ROA3, was done at different temperatures (4^{o}C, 10^{o}C, 25^{o}C, and 37^{o}C) by using soft agar plates (TSBYE with 0.4% agar). It was observed that the ROA3 mutant, and ROA3E was slightly less motile at 25^{o}C. Following 96 hrs incubation at 25^{o}C the radius of the spots for ROA3 and ROA3E were around 0.95, and for F2365 and ROA3C were around 1.15cm (Fig. 4). On the other hand, at other temperatures no difference was seen on the radius of the spots, although less growth was observed with the catalase-negative mutants.

5. 4. 5. Growth on agar plates

When the catalase negative mutant, ROA3, were spotted or plated on TSAYE at 4^{o}C, 10^{o}C, 25^{o}C, and 37^{o}C, it was observed that they formed smaller colonies, compared to wild type. Complementation of the *kat* gene in ROA3 restored the formation of normal

size colonies, whereas integrating the empty vector pPL2 in ROA3 did not restore normal growth (Fig. 5). On the other hand when the mutant, complemented strains, and the wild type were spotted or plated on blood agar no observable difference in colony size were noticed. Furthermore, no differences was noted in colony size were noted among the mutant and the wild type when the bacteria were spotted or plated on TSAYE and incubated under microaerobic conditions.

5. 4. 6. Cold growth

In comparison to F2365, the surface-grown ROA3 and the ROA3 harboring the integrated empty vector pPL2 (ROA3E) showed decreased growth on agar regardless of the temperature in which the impairment in growth was more extensive at lower temperatures. Complementation of ROA3 with kat (ROA3C) fully restored growth, whereas no restoration was noted with ROA3 harboring the integrated empty vector (ROA3E) (Fig. 5). Restoration of growth was also observed when the bacteria were grown in liquid at $4^{o}C$ (Fig. 6).

5. 4. 7. Freeze-thaw tolerance

The freeze-thaw tolerance of ROA3 and F2365 grown at $25^{o}C$ and $37^{o}C$ was assessed over 18 freeze-thaw cycles. No pronounced differences were noted in survival of F2365 vs. ROA3 (Fig. 7).

5. 4. 8. Growth and survival of ROA3 in foods

The growth and survival comparison of ROA3 and F2365 in raw or pasteurized milk (Fig. 8) or on tomato surface (Fig. 9) did not reveal any difference between ROA3 and F2365. However, we noticed the impact of pasteurization of milk in the survival of

cultures at 25^{o}C (Fig. 8). Following 21 days of incubation the number of both ROA3 and F2365 cultures started to increase when they were inoculated in raw milk. However, no such growth was observed when the cultures were inoculated in pasteurized milk.

No difference in the survival of the cultures on the tomato surface was seen between ROA3 and F2365 (Fig. 9). However, a difference in the survival of the mutants on the bottom or the stalk of tomato was observed. When the cultures were spotted at the bottom of the tomato they showed less survival than when they were spotted on the stalk (Fig. 9).

5. 4. 9. Sequence comparison of *kat* between F2365, EGD-e, *L. welshimeri*, and *L. innocua*

An online sequence comparison tool, WebACT was employed to compare the *kat* genomic region among different *Listeria* spp. strains. Interesting differences was noticed upstream of *kat* (Fig. 10). In EGD-e the *bvr* locus composed of three genes was present immediately upstream of *kat*, whereas the *bvr* locus was absent from F2365 and other serotype 4b strains, as well as from *L. welshimeri* serovar SLCC5334, and *L. innocua* Clip11262 (Fig. 10). On the other hand, *L. innocua* Clip11262 lacked five genes downstream of *kat* compared to F2365, EGD-e, and *L. welshimeri* SLCC5334. These five genes (LMOf2365_2775, LMOf2365_2774, LMOf2365_2773, *bglX_2*, and LMOf2365_2771) were putatively involved in β-glucoside metabolism.

5. 5. DISCUSSION

In this study, the role of catalase in growth and freeze-thaw tolerance of *L. monocytogenes* was characterized by using a catalase-negative transposon mutant of *L.*

monocytogenes strain F2365, and a genetically complemented derivative. The catalase-negative mutant showed impaired growth on solid media at all temperatures tested, except when the bacteria were grown microaerobically, or when they were grown on blood agar. Thus, catalase protects the cells against damaging effects of reactive oxygen species produced during aerobic growth. This was also supported by the finding that the mutants grew normally on blood agar in which the heme may quench some of the oxygen, and under microaerobic conditions.

To confirm that the catalase was responsible for this growth impairment the catalase-negative mutant was genetically complemented with the wild type *kat* cloned in the site-specific temperature-sensitive integration vector, pPL2. In addition the empty vector (pPL2) was also integrated in the catalase-negative mutant as a negative control. Complementation of *kat* confirmed the role of catalase in this phenotype. Even though previous studies investigated the role of catalase in virulence of *L. monocytogenes* (Leblond-Francillard et al. 1989, Welch et al. 1979), the formation of smaller colonies has not been reported. The study of Leblond-Francillard et al. (1989) involved strain EGD, of serotype 1/2a, and the serotype of the strains employed by Welch et al. (1979) was not indicated. The sequence comparison of different *Listeria* strains showed that the genomic region around *kat* varied between *L. monocytogenes* serotypes. Therefore, this difference in the genomic region might lead to difference in catalase expression and function between the serotypes of *L. monocytogenes,* which might explain the formation of normal colonies in other serotypes. Catalase-negative mutants of *L. monocytogenes* strain F2365 have not been described before, and the observed phenotype of the catalase-

negative mutant described here might be specific to serotype 4b, or to this strain. Nightindale et al. (2007) previously reported that F2365 carries 20 authentic mutations that lead to premature stop codons, and these frameshifts might inhibit the production of proteins that take over the role of catalase in other strains of *L. monocytogenes.* Rea et al. (2005) observed the formation of small colonies in *L. monocytogenes* in which *perR* was deleted. In addition, this mutant showed decreased growth in liquid media at 37^{o}C. However, this study did not address the impact of growth temperature on the growth impairment of *perR* deleted mutant. PerR is responsible for the regulation of genes involved in defense against peroxide and ROS stress. Interestingly, the *perR* deletion mutants had increased sensitivity to peroxide stress, but transcriptional analysis showed that catalase was upregulated in these mutants. It was speculated that the increased expression of catalase was toxic to the cells and resulted in decreased colony size and decreased peroxide tolerance (Rea et al. 2005). However, the increased transcription of catalase does not necessarily mean increased production of catalase; the deletion of *perR* might be inhibiting the translation of catalase, which might lead to increased sensitivity to peroxide and formation of smaller colonies. In this study, we observed a similar phenotype although *kat* was inactivated. The growth of the catalase negative mutant in liquid media showed decrease only at 4^{o}C compared to wild type and the catalase-complemented mutant. This suggests that at relatively high temperatures the cells were subjected to less oxygen stress in liquid media compared to the surface of agar. On the contrary at low temperatures, the impact of oxygen stress may be more severe resulting in slower rate of growth, and decreased final concentration of cells. Thus, catalase may be

especially important for surface associated growth aerobically, and in the presence of additional stresses, such as low temperature. During screening of the mutant library we observed that the catalase-negative mutant showed impaired growth on plates containing sublethal levels of salt, cadmium, and paraquat. However, we still do not know if this impaired growth is because of the oxygen stress they are faced on the surface of the plates or because of the stress agents. Therefore, further characterization of *kat* will be valuable to fully understand its role in the stress response mechanism of *L. monocytogenes*. During screening of the mutant library we observed that the catalase-negative mutant showed impaired growth on plates containing sublethal levels of salt, cadmium, and paraquat. However, we still do not know if this impaired growth is because of the oxygen stress they are faced on the surface of the plates or because of the stress agents. Therefore, further characterization of *kat* will be valuable to fully understand its role in the stress response mechanism of *L. monocytogenes*.

The motility test showed that the catalase-negative mutants were less motile than the wild type strain at 25^{o}C. In addition, it was observed that the cell density of the catalase-negative mutants on the soft agar was less than the wild type and the complemented mutant. This suggests that the observed decrease in the motility of the catalase-negative mutants might be due to the impaired growth.

Analysis of the catalase genomic region revealed that serogroup 1/2a bacteria harbored three genes (*bvrA*, *bvrB*, and *bvrC*) immediately upstream of *kat*, whereas F2365 lacked these genes. Previously, it was reported that the *bvr* locus encodes a β-glucoside-specific sensor, which is responsible for the repression of the virulence genes

in the presence of cellobiose and salicin (Brehm et al. 1999). Low-stringency Southern blots suggested that this locus is present in some *L. monocytogenes* strains, however, absent in other *Listeria* spp. Low-stringency Southern blots suggested that this locus is present in some *L. monocytogenes* strains, however, absent in other *Listeria* spp. (Brehm et al. 1999). β-glucosides are sugars of plant origin. Park and Kroll (1993) showed that when the β-glucoside cellobiose was the only fermentable carbohydrate in the growth medium, the expression levels of the virulence genes, *hly* and *plcA*, were significantly repressed. These findings indicate that this region plays a role in environmental regulation of the virulence genes. The presence of this region in *L. monocytogenes* serotype 1/2a strain EGD-e and absence in other *Listeria* serotypes can be because of the horizontal transfer of this locus from a different species into EGD-e during its survival in environment. However the average GC content of this region was around 38%, typical of the genome average for *L. monocytogenes*, suggesting that such acquisition may have been an ancient event. Alternatively, this locus may have been acquired from a bacterium with GC content similar to *Listeria*'s.

When the sequence comparison was made between F2365, EGD-e, *L. welshimeri* and *L. innocua* it was observed that immediately downstream of *kat* five genes involved in β-glucoside metabolism were present in all, except *L. innocua*. As mentioned previously, β-glucoside metabolism plays a role in the transcriptional control of virulence gene, and the absence of this metabolism might be related to the non-pathogenic nature of *L. innocua*. On the other hand, the genes were present downstream of kat in another non-pathogenic *Listeria* species, *L. welshimeri.*

It was previously reported that cells were subjected to oxygen stress during thawing (Garénaux et al. 2008, Gao et al. 2000). In addition, the role of SOD in the freeze-thaw tolerance of bacteria was also reported (Stead et al. 2000). No pronounced loss in the freeze-thaw tolerance of the catalase-negative mutant was noted in our study, suggesting that other oxygen stress enzymes, especially SOD, might be enough for protecting the bacteria against the damaging effects of the reactive oxygen species. In order to address this, further studies are needed such as construction of the double-deletion of both SOD and *kat*, along with single deletion of each of these genes. By this way we would be able to better understand the significance of oxygen stress during the freeze-thaw tolerance of *L. monocytogenes.*

5. 6. REFERENCES

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Table 5. 1. *Listeria monocytogenes* strains used in this study.

Strains	Genotype and features	Source and reference
F2365	Cheese isolate from California outbreak	(Swaminathan and Gerner-Smidt 2007)
ROA3	Transposon mutant of F2365 *kat*::transposon (pMC38), Em^R Km^S, catalase negative, impaired growth at low temperatures, and on regular media, growth was restored on blood agar.	This study
ROA3C	Genetically complemented ROA3 with integrated pPL2::*kat*	This study
ROA3E	Genetically complemented ROA3 with pPL2, negative control	This study

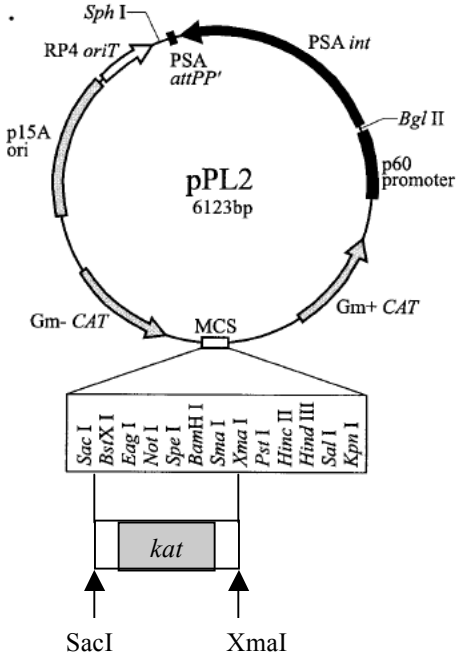


Fig. 5. 1: Plasmid map of pPL2 and construction of the recombinant plasmid, pPL2::*kat*.

(pPL2 map modified from Lauer et al. 2002).

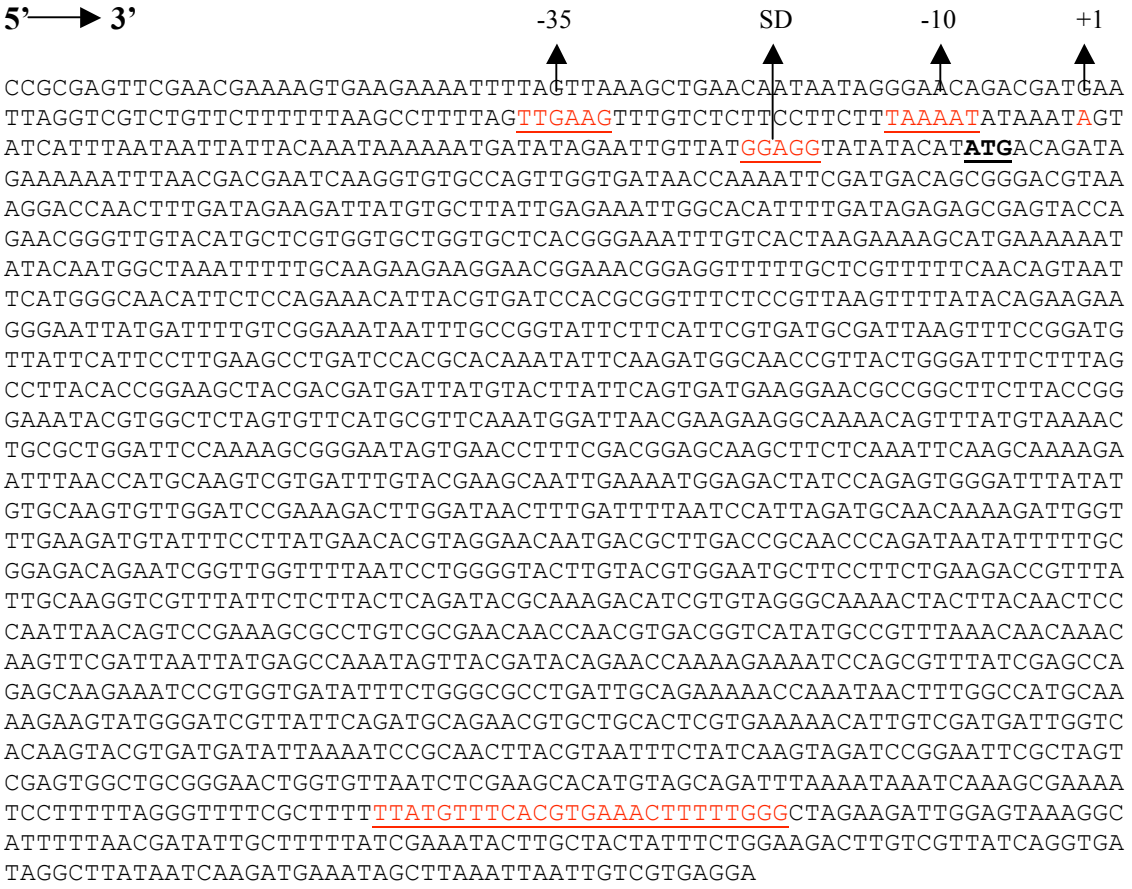


Fig. 5. 2: Sequence of *kat* including the putative promoter and the putative termination region. The upstream and downstream sequences of *kat* (GI:85700163) was included and the putative transcription initiation and termination sites were determined by comparing the genomic region with that of *L. seeligeri* (Haas et al. 1991). Promoter region is underlined and shown in red. Start codon of *kat* is bold and underlined. Putative transcription termination site is also underlined and shown in red.

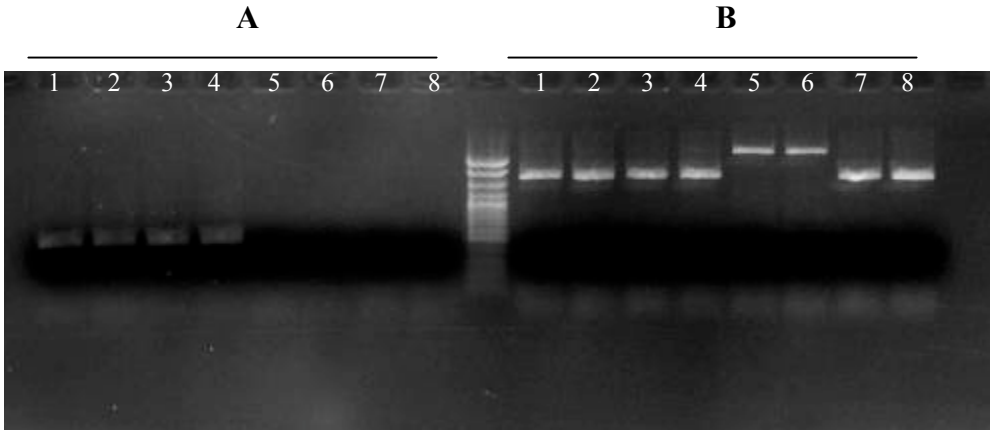
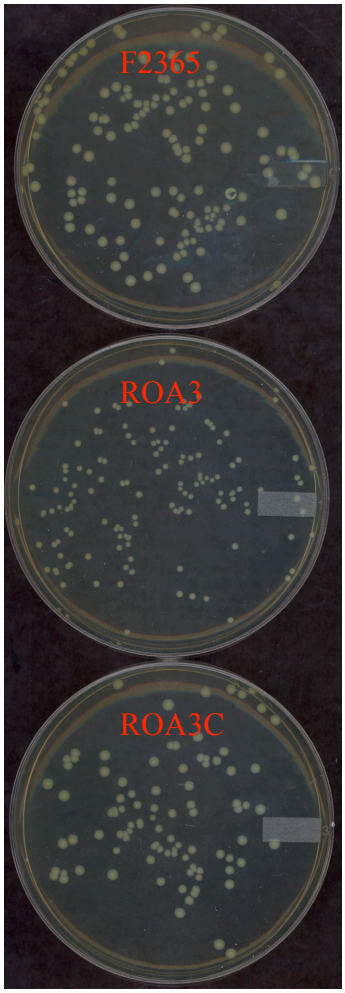


Fig. 5. 3: PCR confirmation for the integration of the pPL2::*kat* into chromosomal DNA of complementation constructs in ROA3. (A) Confirmation of integration by primers NC16 and PL95; (B) Confirmation of the complementation of *kat* by primers ROA3F and ROA3R2. Lanes 1-4 ROA3 with integrated pPL2::*kat*, lanes 5 and 6 ROA3, and lanes 7 and 8 F2365. Larger size bands in lane B5 and B6 indicates the transposon integration on *kat.*

Fig. 5. 4: Growth of wild type F2365, catalase-negative ROA3, *kat* complemented ROA3C, and empty vector integrated ROA3E on soft agar (TSAYE with 0.4% agar) at 25^{o}C incubated for 96 h.

Fig. 5. 5: Growth of F2365, ROA3 and ROA3C on TSAYE following (A) 48 h incubation at 37^{o}C (B) 1 week incubation at 10^{o}C; ROA3E was also included for this test.

(A)



(B)

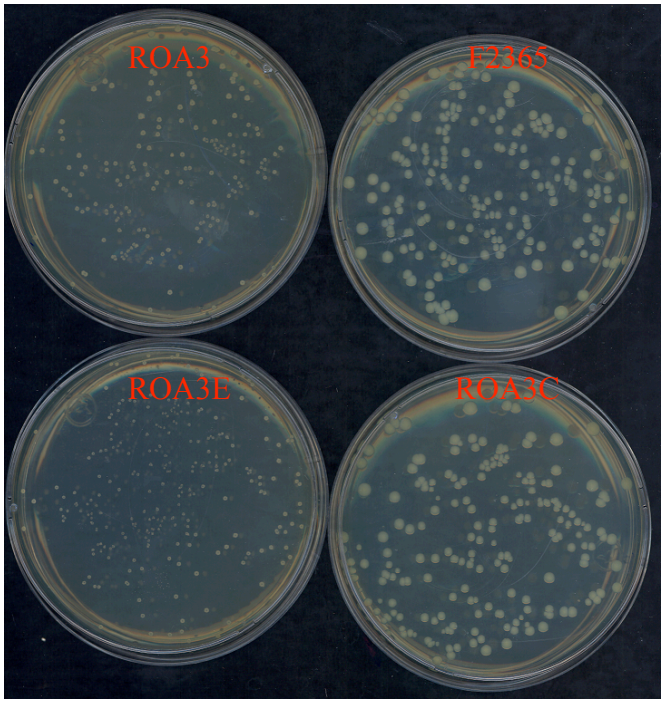
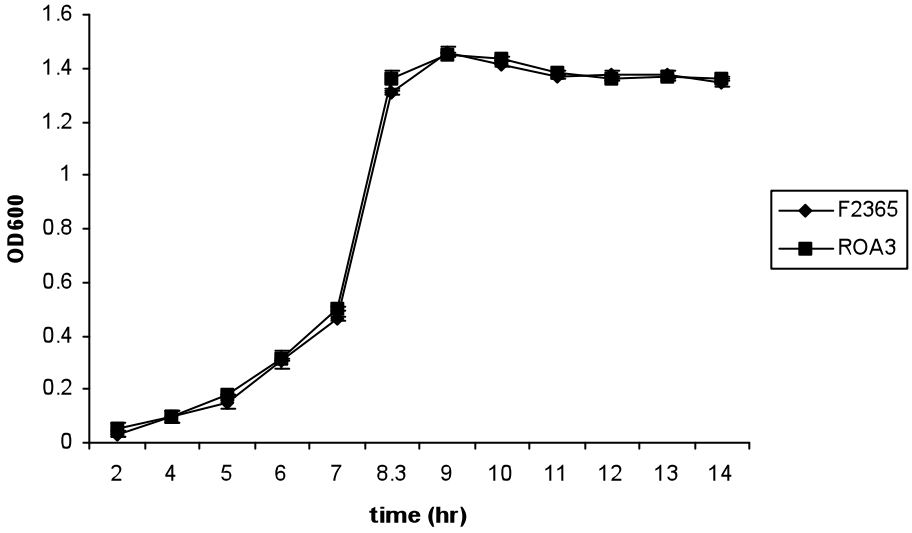
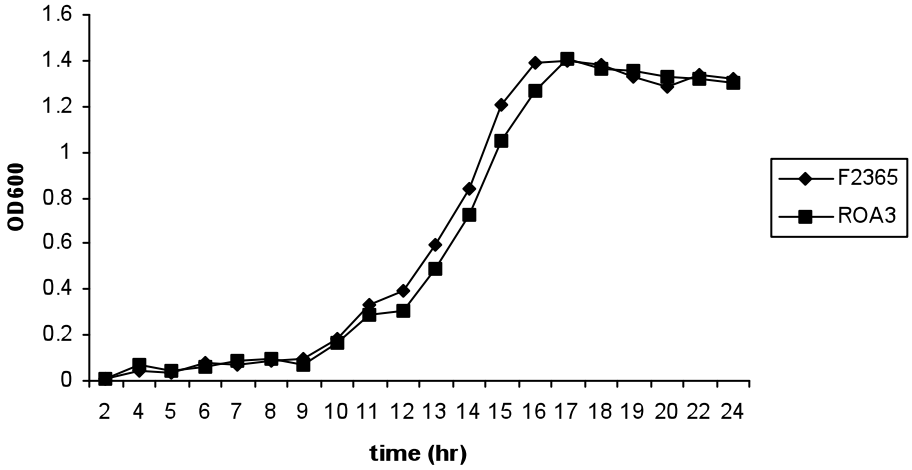


Fig. 5. 6: Growth curves of F2365, ROA3, at (A) 37^{o}C, and (B) 25^{o}C, and F2365, ROA3, ROA3C, and ROA3E at (C) 10^{o}C, and (D) 4^{o}C. Data are from one representative experiment done in duplicate.

(A)


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
2 4 5 6 7 8.3 9 10 11 12 13 14
time (hr)
F2365
ROA3

(B)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1.4
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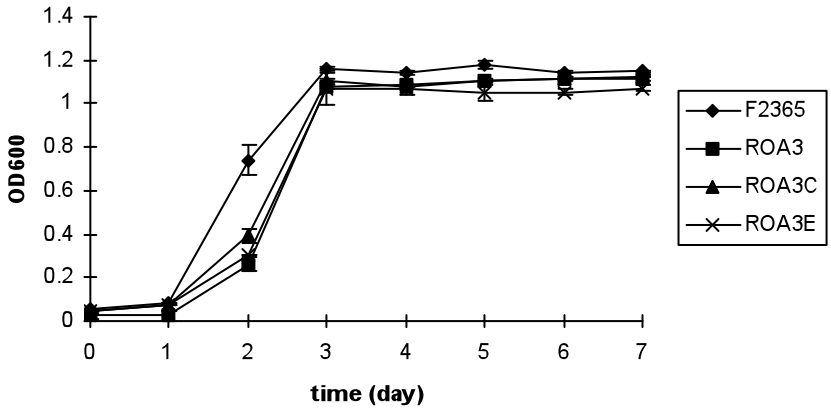
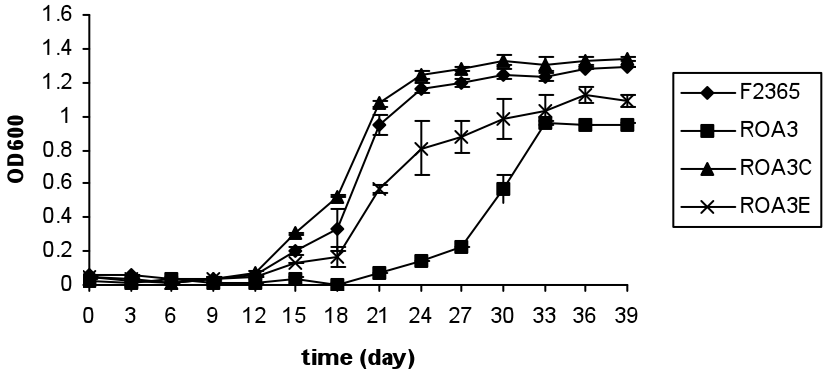
1
0.8
0.6
0.4
0.2
0
OD600
2 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 22 24
time (hr)
F2365
ROA3

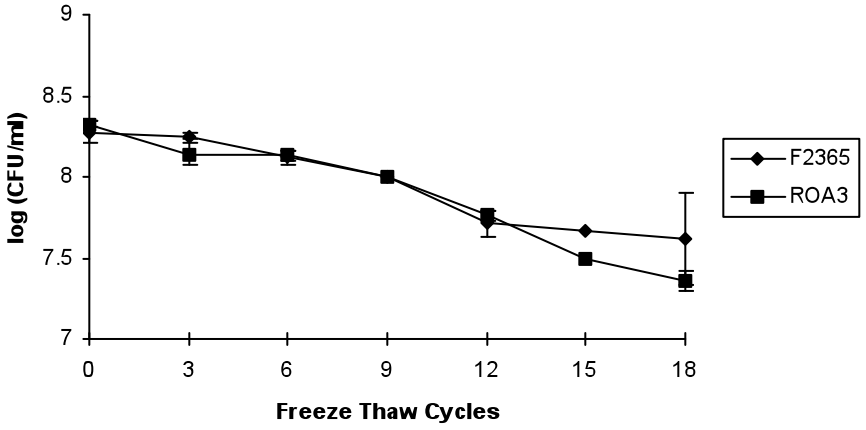
(C)


1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
1
2
3
4
5
6
7
time (day)
F2365
ROA3
ROA3C
ROA3E

(D)


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
3
6
9
12
15
18
21
24
27
30
33
36
39
time (day)
F2365
ROA3
ROA3C
ROA3E

(A)



(B)

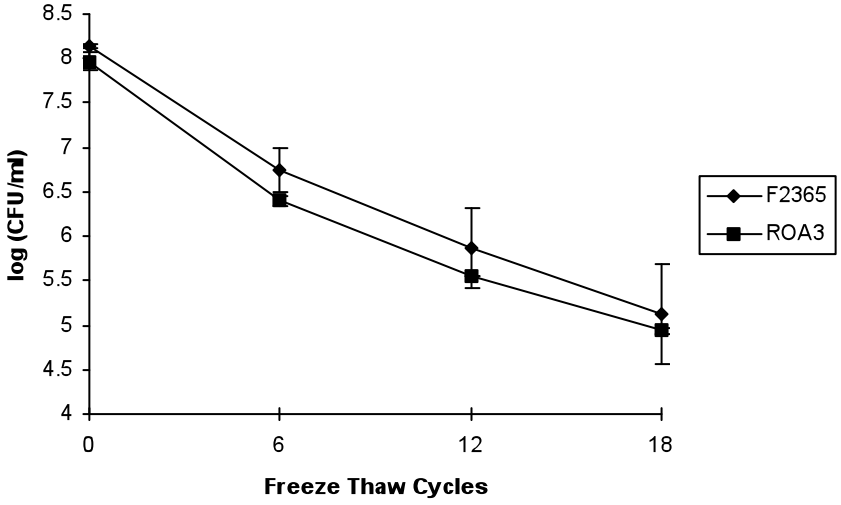
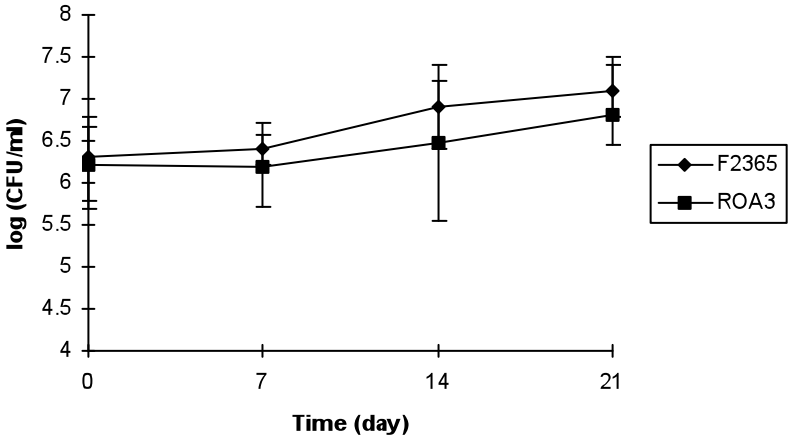


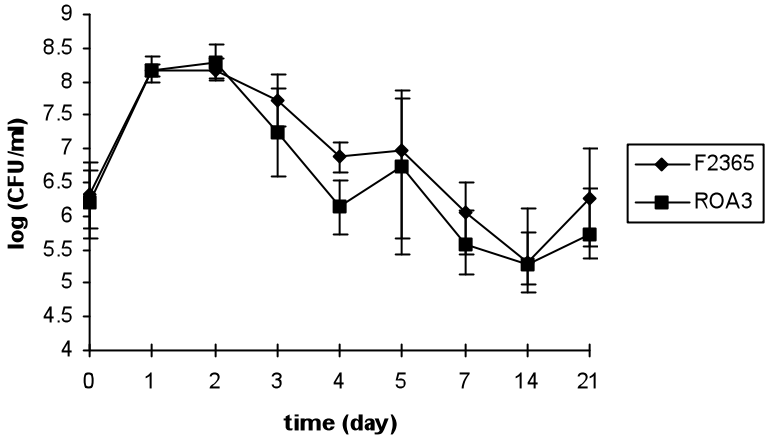
Fig. 5. 7: Freeze-thaw tolerance of ROA3 and F2365 after growth at (A) 25^{o}C and (B) 37^{o}C. Freeze-thaw test for 37^{o}C grown cells was conducted over one represented experiment done in duplicate.

Fig. 5. 8: Growth of ROA3 and F2365 in (A) raw or (B) pasteurized milk at (i) 4^{o}C and (ii) 25^{o}C. Data are from two representative experiments done in duplicate.

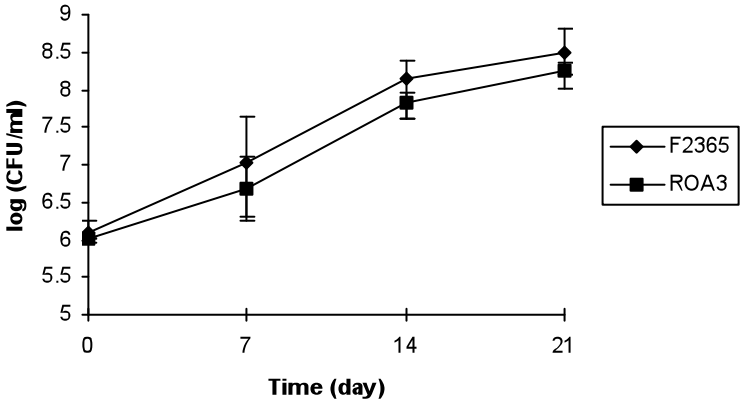
(A) (i)


8
7.5
7
6.5
6
5.5
5
4.5
4
log (CFU/ml)
0
7
14
21
Time (day)
F2365
ROA3

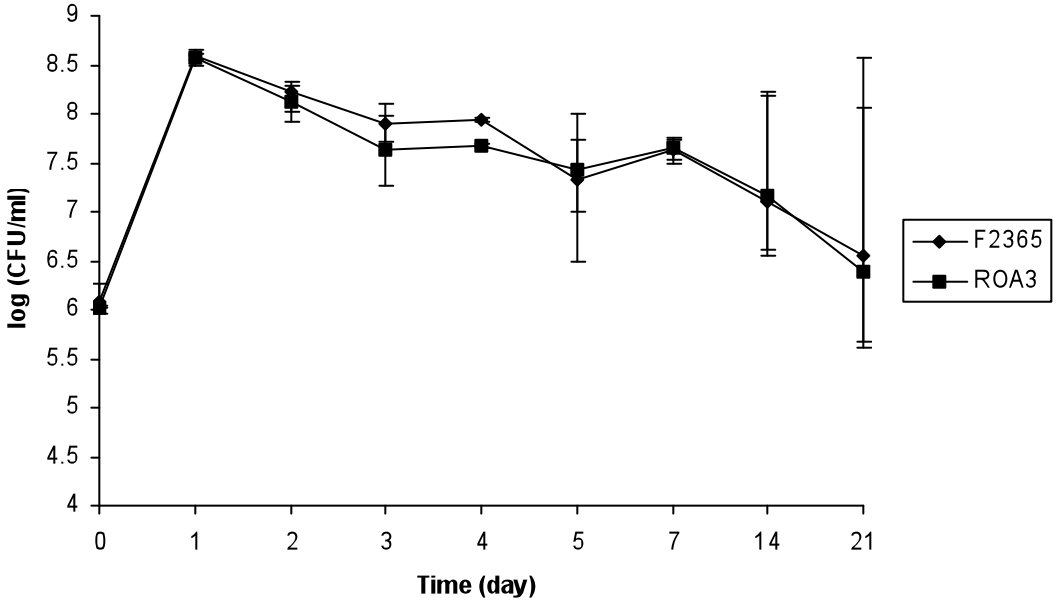
(ii)


9
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
log (CFU/ml)
0
1
2
3
4
5
7
14
21
time (day)
F2365
ROA3

(B) (i)


log (CFU/ml)
9
8.5
8
7.5
7
6.5
6
5.5
5
0
7
14
21
Time (day)
F2365
ROA3

(ii)


log (CFU/ml)
9
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
0
1
2
3
4
5
7
14
21
Time (day)
F2365
ROA3

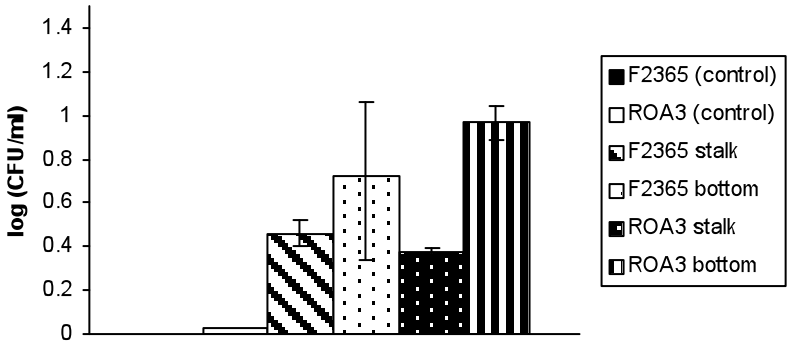
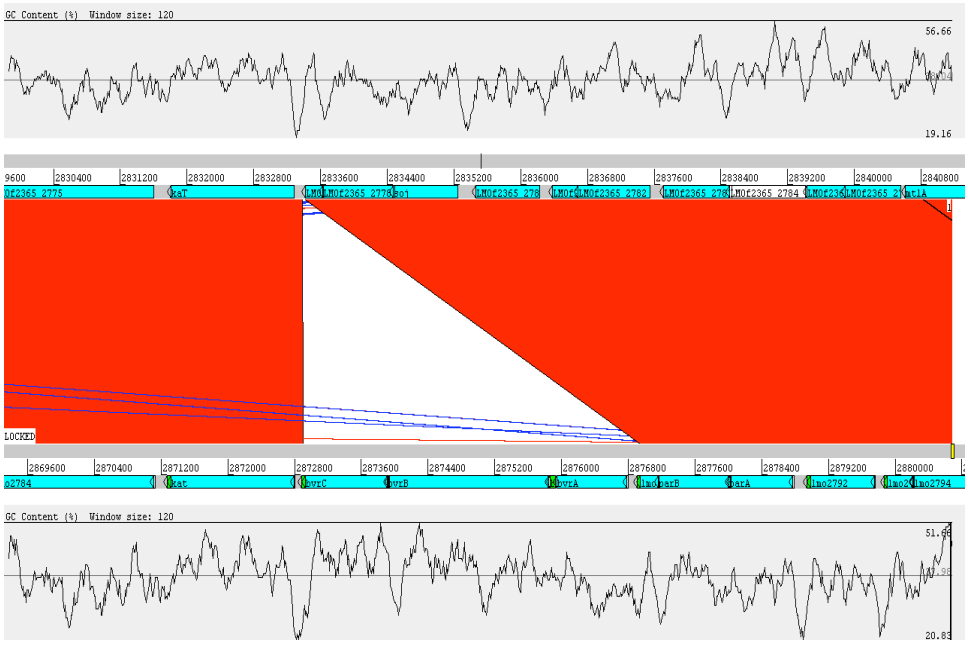


Fig. 5. 9: Survival of ROA3 and F2365 on tomato surface at 25^{o}C. Data are from one representative experiments done in duplicate.

Fig. 5. 10: Comparison of *kat* genomic region among different *Listeria* strain. (A) F2365 and EGD-e with GC content indicated above and below each strain, respectively; (B) F2365, EGD-e, *L. innocua* Clip11262, and *L. welshimeri* serovar 6b SLCC5334.

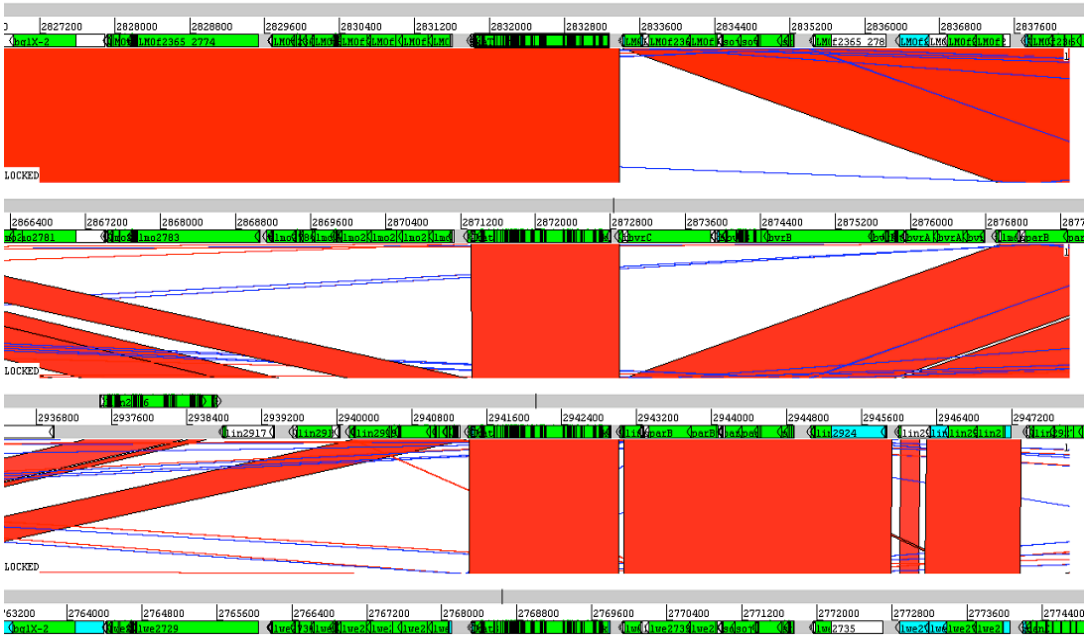
A



F2365

EGD-e

B



F2365

EGD-e

L. innocua Clip11262

L. welshimeri serovar 6b SLCC5334

CHAPTER VI: Analysis of Freeze-Thaw Tolerance of Mutants in Genes with Putative Involvement in the Oxidative Stress Response of *L. monocytogenes*

6. 1. ABSTRACT

Listeria monocytogenes is a gram-positive and facultative intracellular bacterium and it is ubiquitous in the environment. We have found that the ability of *L. monocytogenes* and other *Listeria* spp. to tolerate freeze-thaw stress depends on the growth temperature of the bacteria, with bacteria grown at 37^{o}C being significantly more tolerant to freeze-thaw stress than bacteria grown at 25^{o}C or 4^{o}C. Freeze-thaw stress involves different types of stresses, including oxidative stress. To elucidate the genetic mechanisms underlying freeze-thaw tolerance in *L. monocytogenes,* we characterized transposon mutants with impaired tolerance to cadmium or paraquat, agents that are known to induce formation of reactive oxygen species. One paraquat-susceptible mutant of *L. monocytogenes* 10403S had a single transposon insertion in LMOf2365_0154, oligopeptide ABC transporter, permease protein. Three cadmium susceptible mutants of *L. monocytogenes* harbored single insertion in LMOf2365_2760 encoding a putative glutamate--cysteine ligase/amino acid ligase), LMOf2365_1716 (conserved hypothetical protein), and LMOf2365_2800 (pseudogene). The paraquat susceptible mutant ROA6 had significant decrease in freeze-thaw tolerance compared to the wild type strain, 10403S ($p<0.05$), but freeze-thaw tolerance of the mutants with insertions in LMOFf2365_2760 was not noticeably different from that of the wild type strain F2365. Genetic complementation of one of the mutants harboring a transposon insertion in LMOf2365_2760 with an intact copy of LMOf2365_2760 in the integration vector pPL2 partially restored the ability of the bacteria to grow in the presence of cadmium. Further

studies are needed to fully understand the involvement of these genes in the oxidative stress response of *L. monocytogenes.*

6. 2. INTRODUCTION

Listeria monocytogenes has the ability to cause severe foodborne illness with symptoms such as meningitis, septicemia, and abortion (listeriosis) in both humans and animals. It is a gram-positive, non-sporeforming, facultative anaerobic, facultative intracellular rod. It has the ability to grow at temperatures from 1 to 45^{o}C. Individuals at high risk for listeriosis include; neonates, pregnant women, elderly and immunocompromised people (Farber et al., 1991, Schlech, 2000).

L. monocytogenes may be exposed to freezing as well as thawing in environment as well as during the storage and preservation of foods. Bacterial cells are subjected to oxidative burst during thawing (Hermes-Lima et al. 1993, Mazur, 1970), and genes such as *sod*, encoding superoxide dismutase, mediating inactivation of reactive oxygen species have been shown to be required for freeze-thaw tolerance in *Campylobacter* (Park et al. 1998, Stead et al. 2000). However, our knowledge about the freeze-thaw tolerance of *L. monocytogenes* is limited. It was previously reported that the survival of *L. monocytogenes* following freezing (-18^{o}C) in laboratory media or in foods (followed by a single thawing cycle), was depended on strain, freezing medium, and presence of glycerol as cryoprotectant (El-Kest et al. 1991). In addition, earlier in this study we reported the impact of growth temperature on the cryotolerance of *L. monocytogenes*, bacteria grown at 37^{o}C showed increased tolerance against repeated freezing and thawing

compared to cells grown at 25°C or 4°C. Therefore, to identify the genes essential for freeze-thaw tolerance of *L. monocytogenes*, we screened the mutant library for the loss of oxidative stress tolerance. Paraquat and cadmium are compounds that induce oxidative stress and have been used to screen for mutants with reduced resistance to oxygen stress (Ballal et al. 2009, Garénaux et al. 2008, Pacheco et al. 2008).

Paraquat is used as a herbicide and mainly generates superoxide inside the cell by NADPH-mediated reduction of oxygen (Hassan et al. 1977, Blanchard et al. 2007). Cadmium is a heavy metal that blocks respiration and other metabolic mechanisms of bacteria (Pacheco et al. 2008). In *L. monocytogenes*, cadmium is pumped-out from the cell with an energy-dependent efflux system associated with two genes, *cadA* and *cadC* (Lebrun et al. 1994).

In this study, we addressed the freeze-thaw tolerance of mutants that had enhanced susceptibility to paraquat and cadmium.

6. 3. MATERIALS AND METHODS

6. 3. 1. Bacterial strains and culture conditions

The mutant libraries of F2365 and 10403S were constructed as described in the previous chapter. The bacterial strains used in this study are listed in Table 1. Selected mutants and *L. monocytogenes* strains F2365 and 10403S were grown in brain heart infusion broth (BHI, BBL, Sparks, MD) or tryptic soy broth (TSB) (BBL, Sparks, MD) supplemented with 0.7% Yeast Extract (YE) (BBL, Sparks, MD) (TSBYE), at 37°C for 36 hours, and stored at –80°C in the presence of 20% glycerol. Before each experiment

bacteria were grown on trypticase soy agar with 5% sheep blood (Remel, Lenexa, KS) at 37^{o}C for 36 h and liquid cultures were started by transferring a single colony into 5 ml of TSBYE and incubating at 37^{o}C overnight. For complementation, *Escherichia coli* strains (DH5α and SM10) were grown in Luria-Bertani (LB) (BBL, Sparks, MD) broth or agar medium supplemented with chloramphenicol (25 μg/ml).

Paraquat sensitivity was determined by using BHI-agar plates supplemented with 0.05 μg/ml paraquat (methyl viologen dichloride hydrate, 98%) (Sigma-Aldrich, St. Louis, MO). For cadmium sensitivity screening, mutants were spotted on both isosensitest agar (Oxoid Ltd., Hampshire, England) and isosensitest agar supplemented with 10μg/ml cadmium-chloride (Cad10) (Fisher Scientific, Fair Lawn, NJ).

6. 3. 2. Genetic complementation of LMOf2365_2760

Primers ROA7F and ROA7R were used to amplify LMOf2365_2760 from *L. monocytogenes* strain F2365. Forward and reverse primers were designed based on the DNA sequence of LMOf2365_2760 (putative glutamate--cysteine ligase/amino acid ligase). The LMOf2365_2760 was amplified with primers ROA7F (5'- CTAA CCCGGG ACA TAT TGC GGT AAA ATA TC-3', XmaI site underlined) and ROA7R (5'- GTAA GAGCTC GAT GTG ATT AGT TCA AAT GG-3', SacI site underlined). At the 5' end on each primer, restriction enzyme recognition sequences were incorporated for cloning purposes. The PCR product was digested by the indicated restriction enzymes and purified using a PCR purification kit (Qiagen Valencia, CA). The purified PCR product was ligated into the *L. monocytogenes* site-specific integration vector pPL2 (Lauer et al. 2002), which was digested with the same restriction enzymes. The recombinant plasmid

was transformed into *E. coli* DH5α or SM10 by electroporation as described in previous chapters.

6. 3. 3. Bacterial conjugation

The conjugation of the plasmid carrying the gene of interest was performed by using the procedure described in previous chapters. After the conjugation of the plasmid into ROA7, the transconjugants were selected on BHI plates supplemented with chloramphenicol (6 μg/ml) and nalidixic acid (20 μg/ml) following incubation at 30^{o}C for 2-3 days.

6. 3. 4. Freezing and thawing treatments

Bacteria grown at 37^{o}C were transferred in 1.5 ml aliquots into cryovials (Nalgene, Rochester, NY) and frozen at –20^{o}C. Freezing and thawing cycles were repeated every 24 h for 18 cycles. Every three or six cycles, cell enumerations were done in duplicate, as described above.

6. 3. 5. Statistical analysis

The log reduction of ROA6 and the wild type 10403S following 18 cycles of repeated freezing and thawing was analysed statistically by using unpaired *t*-test (dof=6) (http://www.physics.csbsju.edu/cgi-bin/stats/t-test).

6. 4. RESULTS

6. 4. 1. Impact of LMOf2365_0154 on the stress response of *L. monocytogenes* strain 10403S

During the screening of the mutant library of *L. monocytogenes* strain 10403S, the mutant that had the transposon insertion in LMOf2365_0154 (oligopeptide ABC

transporter, permease protein) showed impaired growth on plates containing sublethal levels of paraquat (0.05 μg/ml) and cadmium (10μg/ml) as well as some loss on cold tolerance. In this study, we addressed the impact of LMOf2365_0154 on the tolerance of *L. monocytogenes* against repeated freezing and thawing. Interestingly we observed that the mutant ROA6 showed impaired freeze-thaw tolerance compared to wild type 10403S following 18 cycles of repeated freezing and thawing ($p<0.05$) (Fig. 1).

6. 4. 2. Impact of LMOf2365_2760, LMOf2365_1716, and LMOf2365_2800 on the stress response of *L. monocytogenes* strain F2365

The Cad10 sensitive mutants of F2365 did not showed any impairment on plates supplemented with paraquat. Also these genes did not impact the cold tolerance of the mutants. In addition the freeze-thaw tolerance test showed that the tolerance of ROA7, ROA8, and ROA9 against repeated freezing and thawing did not change compared to the wild type (Fig. 2). On the other hand, we observed a decrease in the hemolytic activity of ROA7, and loss of hemolytic activity on ROA9.

6. 4. 3. Genetic complementation of ROA7 with LMOf2365_2760 (putative glutamate--cysteine ligase/amino acid ligase)

To address the role of LMOf2365_2760 (putative glutamate--cysteine ligase/amino acid ligase) in cadmium tolerance and other stress response mechanisms of *L. monocytogenes*, this gene was integrated into ROA7 by using the site-specific temperature-sensitive integration vector, pPL2. Integration of the pPL2 with LMOf2365_2760 was confirmed by PCR with primer NC16, which binds upstream of $tRNA^{Arg}$ in the host genome, and primer PL95, which binds to PSA *int* in pPL2. The size

of the PCR product is 499 bp (Fig.3A). Complementation of LMOf2365_2760 was confirmed by PCR by using primers ROA7R and ROA7F (Fig. 3B). The complementation of LMOf2365_2760 into ROA7 did not show full restoration of tolerance against Cad10, however it was observed that the complemented ROA7 mutants showed formation of single colonies when spotted on Cad10 plates (Fig. 4).

6. 5. DISCUSSION

The paraquat sensitive mutant of *L. monocytogenes* strain 10403S showed a moderate decrease in freeze-thaw tolerance when compared to wild type 10403S. In this mutant the transposon insertion was in the gene encoding a putative oligopeptide ABC transporter, permease protein. As the genome for 10403S has been sequenced the homolog in F2365 was identified (LMOf2365_0154). The peptide transporter systems play important role for the nutrition of the cell. In addition they are involved in the various signaling processes, such as the regulation of gene expression in proteolytic systems, DNA transfer by conjugation, chemotaxis, and virulence. Furthermore, these peptide transporters are involved in the defense against antimicrobial peptides. More importantly, their regulatory roles through their involvement in signaling processes are important for the adaptation to the environmental conditions (Detmers et al. 2001, Dunny et al. 1997). In agreement with such diverse impacts, the mutant harboring a transposon insertion in oligopeptide ABC transporter lost its tolerance against several different stresses, including stress induced by cadmium and paraquat. Also this mutant became cold sensitive, and lost some of its tolerance against repeated freezing and thawing.

Further studies are needed to confirm the role of this gene in the response mechanisms of environmental stress conditions. Complementation of this gene in the mutant will provide evidence if this gene is really responsible for the observed phenotypes. However, because of the location of the gene in the genome complementation studies may be challenging. There is possibility for polar effects, in which the transcription of the downstream genes is also inhibited in this mutant.

The mutant that had the transposon insertion in LMOf2365_2760 (bifunctional glutamate—cysteine ligase/glutathione synthetase) was sensitive to cadmium. The possible role of this gene in cadmium stress in *L. monocytogenes* has not been reported previously. Masip et al. (2006) noted in their review that glutathione play a key role in maintaining the proper oxidation state of protein thiols as well as in protection of the cells exposed to low pH, chlorine compounds, and oxidative and osmotic stresses. They also reported the posttranslational regulator role of gluthathiones during the oxidative stress response by modifying the proteins. Glutathione production in prokaryotes is not common with the exception of cyanobacteria, proteobacteria and certain strains of gram-positive bacteria (Fahey et al. 1991). Prokaryotes that lack glutathione produce low molecular thiols that function like glutathione. On the other hand, glutathione is present in almost all eukaryotes except for those that lack mitochondria or chloroplasts (Fahey & Sundquist 1991, Newton et al. 1990). In *E. coli*, glutathione was found to play a critical role against environmental stresses, such as osmotic shock, acidity, protection against toxins such as methylglyoxal, chlorine compounds such as hypochlorous acid and oxidative stress induced by peroxides (Masip et al., 2006, Pacheco et al. 2008). As the

freeze-thaw stress involves oxidative stress, it is viable to speculate that this gene might be playing a role in freeze-thaw tolerance of *L. monocytoges*. However, in this study we did not detect any impact of this gene on freeze-thaw tolerance of *L. monocytogenes.* One possible explanation is that; other compounds might take over the possible role of glutathione in freeze-thaw tolerance and therefore the mutant was able to survive under these damaging condition. Further studies are needed to address the role of this gene in the cadmium tolerance mechanism in *L. monocytogenes*. In addition, the other two cadmium-sensitive mutants did not show loss of cryotolerance in this study. Cadmium sensitivity of the mutant harboring a transposon insertion in LMOf2365_1716 (conserved hypothetical protein) may be due to polar effects of the insertion. This gene is upstream of Lmof2365_1715 (dUTPase family protein), which is involved in the chemical reactions and pathways of dUTP metabolic processes. The other cadmium-sensitive mutant harbored a transposon insertion in LMOf2365_2800 (pseudogene). Pseudogenes are not functional and the ORF downstream of this pseudogene appears to be transcribed in the opposite direction, which eliminates the possibility of a polar effect. Therefore, the observed phenotype of this mutant is difficult to explain. Further complementation and deletion studies are needed to identify the gene responsible for the observed phenotypes of these mutants

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Table 6. 1. *Listeria monocytogenes* strains used in this study.

Strains	Genotype and features	Source and reference
F2365	Cheese isolate from 1985 California outbreak	(Swaminathan and Gerner-Smidt 2007)
10403S	Str^R isolate of 10403 (human skin lesion isolate, 1968)	(Edman, et al. 1968)
ROA6	Transposon mutant of 10403S in LMOf2365_0154 (oligopeptide ABC transporter, permease protein), Em^R Km^S, sensitive to cadmium (10μg/ml cadmium chloride anhydrous), impaired growth on 0.05mM paraquat.	This study
ROA7	Transposon mutant of F2365 in LMOf2365_2760 (putative glutamate--cysteine ligase/amino acid ligase), Em^R Km^S, sensitive to cadmium (10μg/ml cadmium chloride anhydrous), decreased hemolytic activity.	This study
ROA8	Transposon mutant of F2365 in LMOf2365_1716 (conserved hypothetical protein), Em^R Km^S, sensitive to cadmium (10μg/ml cadmium chloride anhydrous).	This study
ROA9	Transposon mutant of F2365 in LMOf2365_2800 (pseudogene), Em^R Km^S, decreased growth on cadmium (10μg/ml cadmium chloride anhydrous), non-hemolytic.	This study
ROA7C	Genetically complemented ROA7 with integrated pPL2::LMOf2365_0154	This study

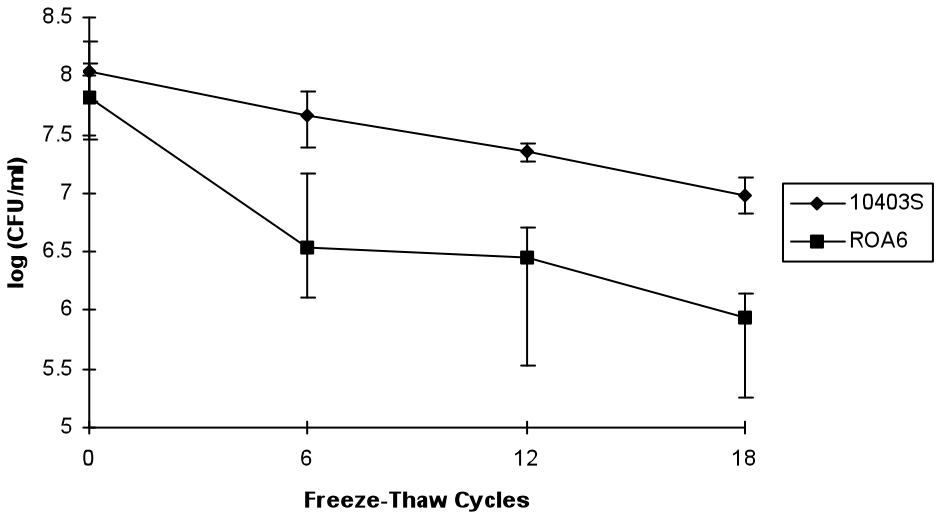


Fig. 6. 1. Freeze-thaw tolerance of ROA6 and 10403S after growth at 37^{o}C. Data represent two independent experiments, each done in duplicate.

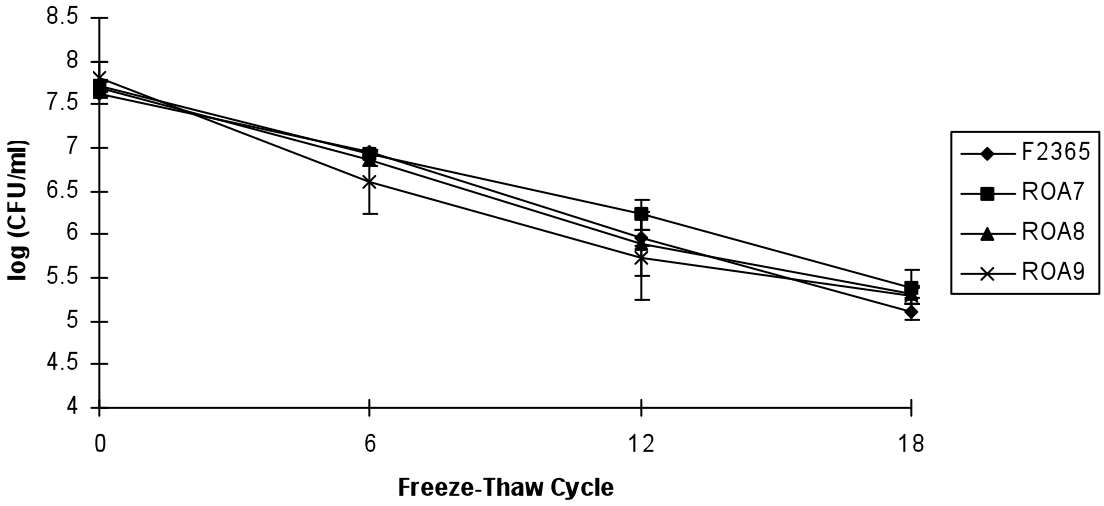
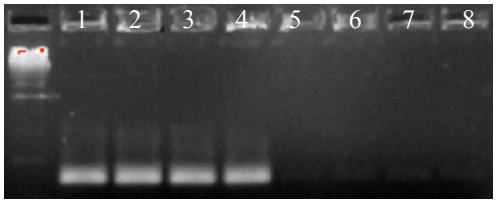


Fig. 6. 2: Freeze-thaw tolerance of ROA7, ROA8, ROA9 and F2365 after growth at 37^{o}C. Data are from one representative experiment done in duplicate.

(A)



(B)

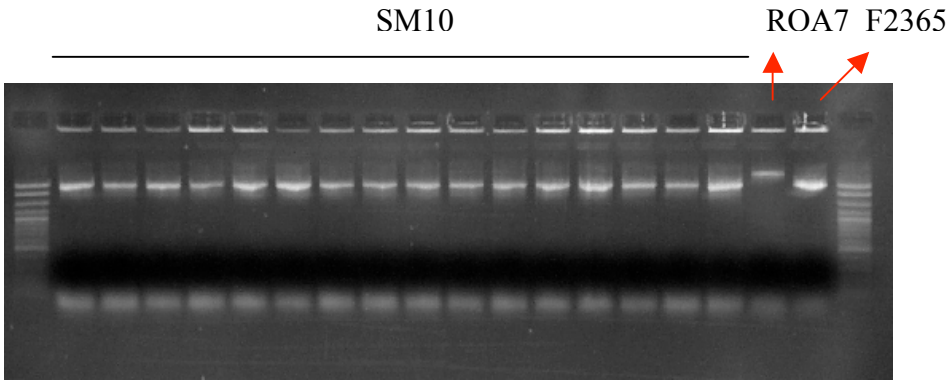


Fig. 6. 3: PCR confirmation for the integration of the pPL2::LMOf2365_2760 (putative glutamate--cysteine ligase/amino acid ligase) into chromosomal DNA of complementation constructs in ROA7. (A) Confirmation of integration by primers NC16 and PL95. Lanes 1-4 ROA7 with integrated pPL2::LMOf2365_2760, lanes 5 and 6 ROA7, and lanes 7 and 8 F2365. (B) Confirmation of the complementation of LMOf2365_2760 by primers ROA7F and ROA7R.

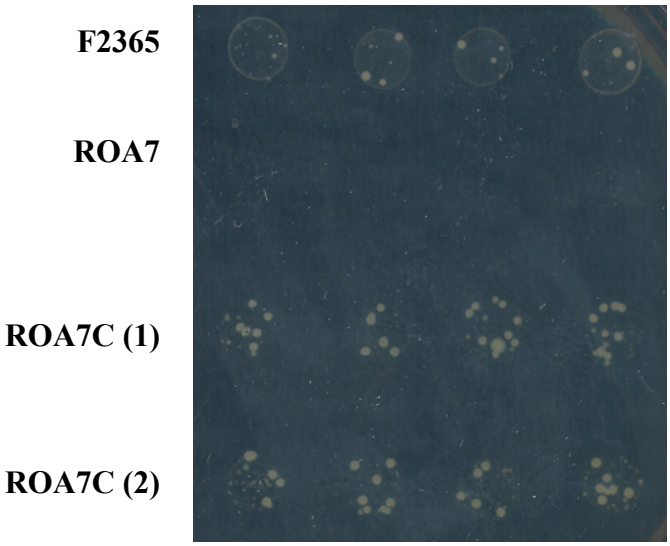


Fig. 6. 4: Growth of selected mutants and wild type parental strain F2365 on plates with cadmium following 96 hrs of incubation at 25^{o}C. Cadmium (cadmium chloride anhydrous) was used at 10µg/ml. ROA7C (1) and ROA7C (2) represent cultures derived from two independent colonies of complemented ROA7.

CHAPTER VII: Characterization of the Cold Sensitive Mutants of *L. monocytogenes* strain F2365

7. 1. ABSTRACT

Listeria monocytogenes is ubiquitous in the environment and can experience different stress conditions, including freeze-thaw stress. Understanding the freeze-thaw tolerance mechanisms is challenging as freezing and thawing involves several stresses such as oxidative, osmotic, and cold stress. Previously, during the screening of a mutant library of *L. monocytogenes* strain F2365, two mutants harboring transposon insertions in genes that might play a role in the freeze-thaw tolerance were identified. One of these mutants had a single transposon insertion in LMOf2365_1746 (encoding a putative helicase domain protein) and lost its ability to grow at lower temperatures (4^{o}C and 10^{o}C). In addition, it showed impaired growth at 25^{o}C. The other mutant had a single transposon insertion in LMOf2365_1875 (encoding a putative ABC transporter, manganese-binding protein) and lost its tolerance against 7.5% NaCl. In this study we characterized freeze-thaw tolerance of these two mutants. Following 18 cycles of repeated freezing and thawing survival of the mutants was similar to the wild type parental strain F2365. The cold sensitive mutant was further studied by genetic complementation with LMOf2365_1746. However, ability to grow at low temperature was not restored, possibly due to polar effects of the transposon insertion. Both of these mutants need to be further studied to fully understand the role of the inactivated genes in stress response mechanisms of *L. monocytogenes.*

7. 2. INTRODUCTION

Listeria monocytogenes is a gram-positive, non-sporeforming, facultative anaerobic, facultative intracellular rod. It is ubiquitous in the environment and has the ability to survive over a wide range of temperatures (between 1 to 45^{o}C) (Farber et al., 1991). *L. monocytogenes* has the ability to cause severe illness (listeriosis) in both humans and animals. Common symptoms of listeriosis are meningitis, septicemia, and abortion. Neonates, pregnant women, elderly and immunocompromised people are at high risk for listeriosis (Farber et al., 1991, Schlech, 2000).

L. monocytogenes is subjected to number of different stress conditions during its existence in the environment and has long been known for its ability to tolerate low temperatures. In the previous chapters, the construction and screening of mutant libraries for the characterization of the stress response mechanisms of two *L. monocytogenes* strains (F2365 and 10403S) were described. As the screening of the mutant libraries for the loss of cryotolerance was not successful, the mutant libraries were also screened for other stresses expected to be involved during freezing and thawing, including screening for the loss of ability to grow at low temperatures and under high salt concentrations (7.5% NaCl).

Ability to grow at low temperatures is important for the survival of *L. monocytogenes* in the environment. Some response mechanisms of *L. monocytogenes* against cold stress have been characterized. These mechanisms include changes in the cell membrane fatty acid composition (Annous et al. 1997), uptake of compatible solutes (Smith, 1996, Verhaul et al., 1997, Ko et al. 1999, Smiddy et al., 2004), and production

of cold stress proteins (Csps) and cold acclimation proteins (Caps) (Wemekamp-Kamphius et al. 2002, Liu et al. 2002, Chan et al. 2007, Tasara et al., 2006).

The gene expression profile of *L. monocytogenes* serotype 1/2a strain 10403S grown at 4^{o}C and 37^{o}C has been characterized and compared using whole genome microarrays (Chan et al. 2007). Genes that showed increased expression levels at 4^{o}C included genes related to cold adaptation (such as *flaA, opuC, gbuC* etc.). On the other hand, the cells grown at 4^{o}C showed decreased expression of genes related to virulence (*hly, plcA* and*, plcB*) and heat shock response (*groES*). In addition, cells at stationary phase at 4^{o}C showed decreased expression of the gene encoding an alternative sigma factor (*sigL*) and of the additional virulence factors, *inlA, inlB,* and *inlC* (Chan et al. 2007).

Osmotic stress is another stress that the cells are subjected to during freeze-thaw stress (Gao et al. 2000). Microorganisms are subjected to osmotic stress in foods that are high in salt, sugar or in dried foods. Therefore, the constructed mutant library was also screened for reduced growth in the presence of 7.5% NaCl. One mutant that had a transposon insertion in LMOf2365_1875 (encoding a putative ABC transporter, manganese-binding protein) lost its ability to survive under such salt stress.

In this study, we further characterize the cold and NaCl sensitive *L. monocytogenes* mutants and their possible involvement in the freeze-thaw tolerance of *L. monocytogenes*.

7. 3. MATERIALS AND METHODS

7. 3. 1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. Mutant library of cheese isolate of the California outbreak, F2365, was constructed as described in the previous chapter. Selected mutants and *L. monocytogenes* strain F2365 were grown in brain heart infusion broth (BHI, BBL, Sparks, MD) or tryptic soy broth (TSB) (BBL, Sparks, MD) supplemented with 0.7% Yeast Extract (YE) (BBL, Sparks, MD) (TSBYE), at 37^{o}C for 36 hours, and stored at –80^{o}C in the presence of 20% glycerol. Before each experiment bacteria were grown on trypticase soy agar with 5% sheep blood (Remel, Lenexa, KS) at 37^{o}C for 36 h and liquid cultures were started by transferring a single colony into 5 ml of TSBYE and incubating at 37^{o}C overnight. Antibiotics used for selection of *Listeria* mutants were chloramphenicol (6 μg/ml), and nalidixic acid (20 μg/ml). For complementation, *Escherichia coli* strains (DH5α and SM10) were grown in Luria-Bertani (LB) (BBL, Sparks, MD) broth or agar medium supplemented with chloramphenicol (25 μg/ml).

7. 3. 2. Genetic complementation

Forward and reverse primers were designed based on the DNA sequence of LMOf2365_1746 (helicase domain protein). For the cold sensitive mutant, ROA4, only the target gene (LMOf2365_1746 (helicase domain protein) was included in the PCR product. The LMOf2365_1746 was amplified with primers ROA4F (5'- CTAA CCCGGG ATG ACA GAA TCA AAT ATC CCA AGC -3', XmaI site underlined) and ROA4R (5'- GTAA GAGCTC CGC ATC TTA AGA TCT TTT GAA ATT AT -3', SacI

site underlined). Complementation was performed as described in previous chapter. The purified PCR product was ligated into the *L. monocytogenes* site-specific integration vector pPL2AP, which was digested with the same restriction enzymes.

7. 3. 3. Bacterial conjugation

Bacterial conjugation was performed as describe in previous chapter.

7. 3. 4. Phenotypic characterization of mutants

7. 3. 4. 1. Cold growth

Cold growth of the ROA4, complemented strains and the wild type of *L. monocytogenes* were determined by spotting 5 µl of the mutants and the wild type on TSAYE and incubating at 10^{o}C and 4^{o}C.

7. 3. 4. 2. Freeze-thaw tolerance of mutants

Freeze-thaw tolerance of ROA4, ROA13 and the wild type were determined by following the protocol described in previous chapters. Briefly, ROA4, ROA13 and the wild type were grown at 37^{o}C for 36 h, followed by 18 freeze-thaw cycles. The cultures were enumerated every 3 or 6 cycles by plating the appropriate dilutions on TSAYE.

7. 3. 4. 3. Sequence comparison of inactivated genes between F2365 and EGD-e

The genomic region including LMOf2365_1746 (helicase domain protein) gene was compared by using an online sequence comparison tool, WebACT. WebACT uses the Artemis Comparison Tool (ACT) developed by Sanger Institute (Carver et al. 2005).

7. 4. RESULTS

7. 4. 1. Complementation of LMOf2365_1746 in ROA4

The role of LMOf2365_1746 in the stress response of *L. monocytogenes* was characterized by the complementation of the gene into transposon-integrated mutant (ROA4) by using site-specific temperature-sensitive integration vector pPL2AP. LMOf2365_1746 gene expression after integration needed to be assured by a promoter along with the gene as this gene does not have its own promoter in the upstream region. Previously, in our laboratory, the *gtcA* promoter region (303 nucleotide) was cloned in pPL2 and designated pPL2AP. The PCR product for LMOf2365_1746 gene was digested with restriction enzymes XmaI and SacI and cloned in pPL2AP, which was also digested with the same enzymes.

Integration of the pPL2AP with or without LMOf2365_1746 (helicase domain protein) were confirmed by PCR with primers NC16, which binds upstream of $tRNA^{Arg}$ in the host genome, and primer PL95, which binds to PSA *int* in pPL2 or pPL2AP. The size of the PCR product is 499 bp (Fig. 1A). The confirmation of the LMOf2365_1746 (helicase domain protein) was performed by PCR by using primers NC16 and ROA4R (Fig. 1B).

7. 4. 2. Cold growth

Survival of ROA4, ROA4C and ROA4E at low temperatures was determined both on agar surface and in liquid media. The complementation of the LMOf2365_1746 (helicase domain protein) in ROA4 did not complement the phenotype of the cold

susceptible ROA4 (Fig. 2). The ROA4 cells grown at 4^{o}C or 10^{o}C showed no growth and the cells grown at 25^{o}C showed impaired growth on TSAYE plates.

The ROA13 did not show any loss in its ability to grow at low temperatures.

7. 4. 3. Freeze-thaw tolerance of mutants

The tolerance of the wild type and the ROA4 and ROA13 did not show any difference after 18 cycles of repeated freezing and thawing following growth at 25^{o}C (for ROA4) or 37^{o}C (Fig. 3).

7. 4. 4. Sequence comparison of LMOf2365_1746 (helicase domain protein) between F2365, EGD-e

The sequence comparison between F2365 and EGD-e around LMOf2365_1746 (helicase domain protein) showed no difference in the gene organization (Fig. 4).

7. 5. DISCUSSION

The mutant that had the transposon insertion in the LMOf2365_1746 (helicase domain protein) gene lost its ability to grow at low temperatures (4^{o}C and 10^{o}C). Furthermore, it showed impaired growth at 25^{o}C. The deduced product of LMOf2365_1746 is a member of DEAD-like helicase superfamily. This diverse helicase protein family is involved in ATP-dependent RNA or DNA unwinding and plays a key role in optimal cell growth at low temperature (Tanner et al. 2001). Hunger et al. (2006) reported that cold induced putative DEAD box RNA helicases, CshA and CshB, were playing an essential role in the cold tolerance of *Bacillus subtilis.* They suggested that the

cold-shock proteins and these helicases work together to unfold mRNA at low temperatures.

Previous whole genome microarray comparison of the gene expression profile of *L. monocytogenes* serotype 1/2a strain 10403S grown at 4^{o}C and 37^{o}C revealed that the expression of lmo1722 (homolog of LMOf2365_1746 in *L. monocytogenes* strain 10403S) was increased when the cells were grown at low temperature (~4 log increase) (Chan et al 2007). This gene was one of only few genes up-regulated at low temperatures at both log and stationary phases. This increased expression of LMOf2365_1746 along with our finding suggests that the LMOf2365_1746 plays key role in the ability of *L. monocytogenes* to grow in the cold. As cold stress is one of the underlying stresses during freezing and thawing, we compared the freeze-thaw tolerance of ROA4 and F2365 grown at 25^{o}C and 37^{o}C. No difference between the mutant and the wild type was observed in the freeze-thaw tolerance. However, as this gene is essential for growth at low temperatures, it might impact the freeze-thaw tolerance of cells grown at much lower temperatures. Conducting this test with lower temperatures was not feasible as the mutants were not able to grow at these temperatures.

To confirm the impact of LMOf2365_1746 in cold growth, it was introduced into ROA4 by using the site-specific temperature-sensitive integration vector, pPL2AP. However, the complemented ROA4 was still unable to grow at low temperatures. The lack of complementation of the cold-sensitive phenotype of the ROA4 mutant suggests that either LMOf2365_1746 had a polar effect by affecting the transcription of the downstream genes or that the promoter on the vector used, pPL2AP, was not adequate for

transcription of LMOf2365_1746. Genes downstream of LMOf2365_1746 include two components of the phosphotransferase system (PTS) permeases. When sufficient amounts of glucose, fructose or sucrose are present in the growth medium, the synthesis of the enzymes necessary for transport and metabolism of other carbon sources are repressed (Stoll et al. 2008). This is known as carbon catabolite repression (CCR). The bacterial PTS, which catalyzes the uptake and phosphorylation of numerous carbohydrates, plays a major role in CCR (Deutscher et al. 2006). The reverse-trascriptase PCR analysis of the helicase gene region will be valuable to determine possible co-transcription of the downstream PTS genes.

In conclusion, no evidence of loss of freeze-thaw tolerance of *L. monocytogenes* was observed with the inactivation of LMOf2365_1746 (helicase domain protein) or LMOf2365_1875 (ABC transporter, manganese-binding protein). However, further characterization of these genes would be helpful in understanding the ecology of *L. monocytogenes* as these genes have not been characterized previously.

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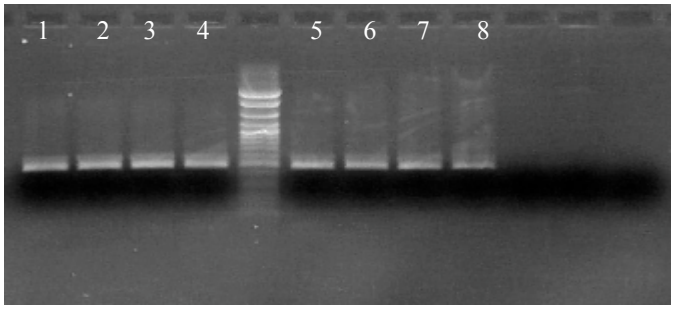
Table 7. 1: *Listeria monocytogenes* strains used in this study.

Strains	Genotype and features	Source and reference
F2365	Cheese isolate from California outbreak	(Swaminathan and Gerner-Smidt 2007)
ROA4	Transposon mutant of F2365 LMOf2365_1746 (helicase domain protein)::transposon (pMC38), Em^R Km^S, cold sensitive, no growth at 4^oC.	This study
ROA4C	Genetically complemented ROA4 with integrated pPL2AP::LMOf2365_1746	This study
ROA4E	Genetically complemented ROA4 with pPL2AP, negative control	This study
ROA13	Transposon mutant of F2365 LMOf2365_1875 (ABC transporter, manganese-binding protein)::transposon (pMC38), Em^R Km^S, 7.5% NaCl sensitive.	This study

(A)



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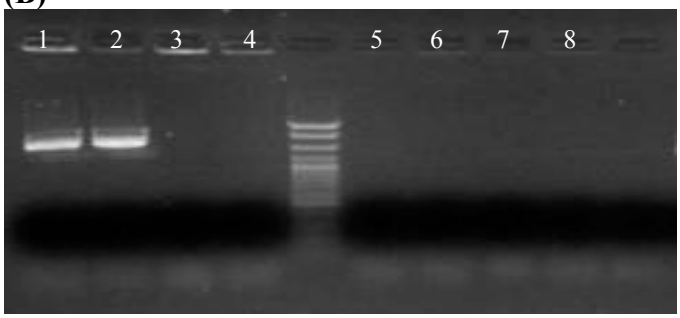
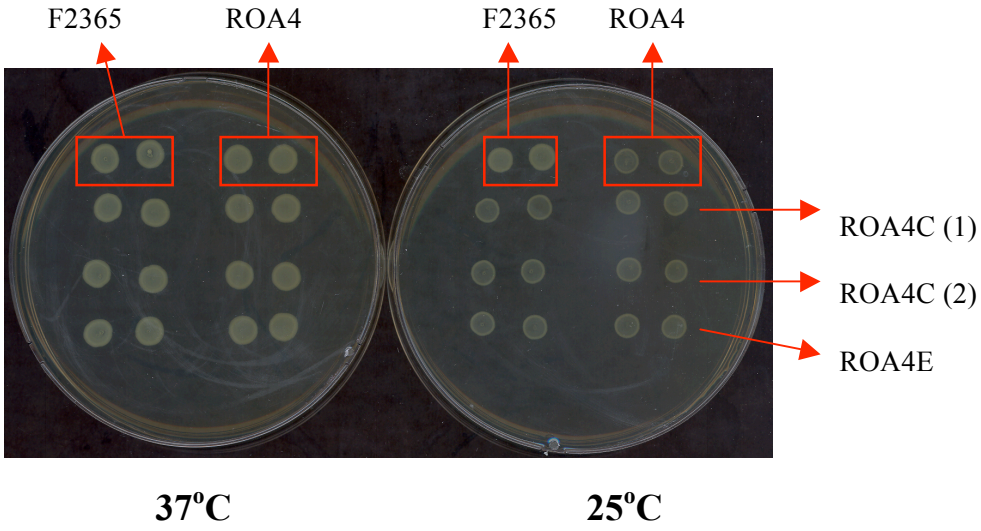


Fig. 7. 1: PCR confirmation for the integration of the pPL2AP::LMOf2365_1746 into chromosomal DNA of complementation constructs in ROA4. (A) shows the confirmation of integration by primers NC16 and PL95. Lanes 1-4 ROA4 with integrated pPL2AP:: LMOf2365_1746 , lanes 5-8 ROA4::pPL2AP, lane 7 ROA4 and lane 8 F2365. (B) primers NC16 and ROA4R.; lanes 1 and 2 ROA4 with integrated pPL2AP:: LMOf2365_1746, lane 3 and 4 ROA4::pPL2AP, lane 5 and 6 ROA4, and lane 7 and 8 F2365.

(A)



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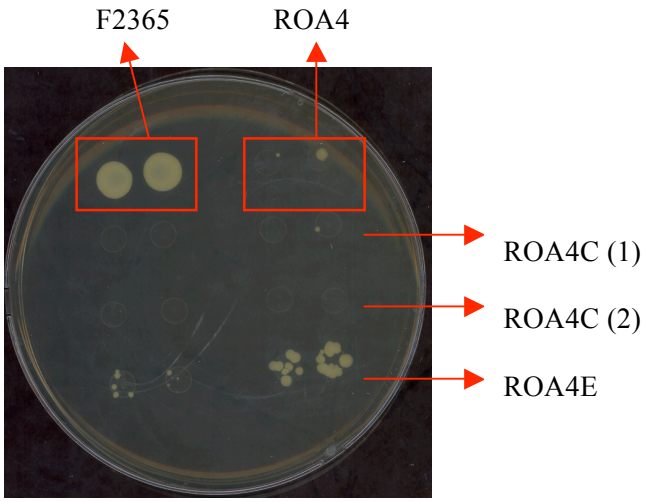
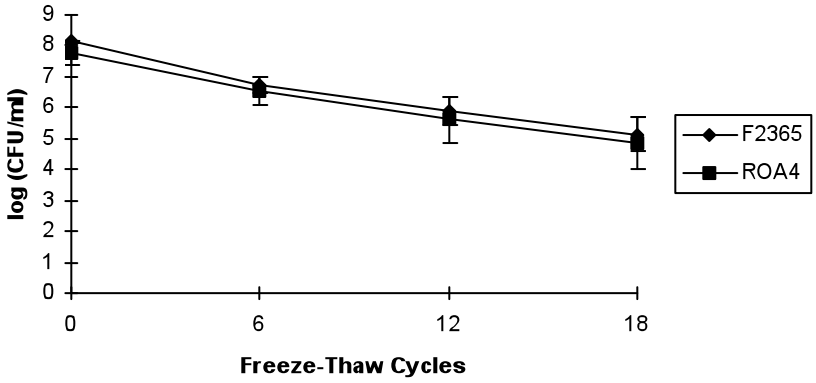


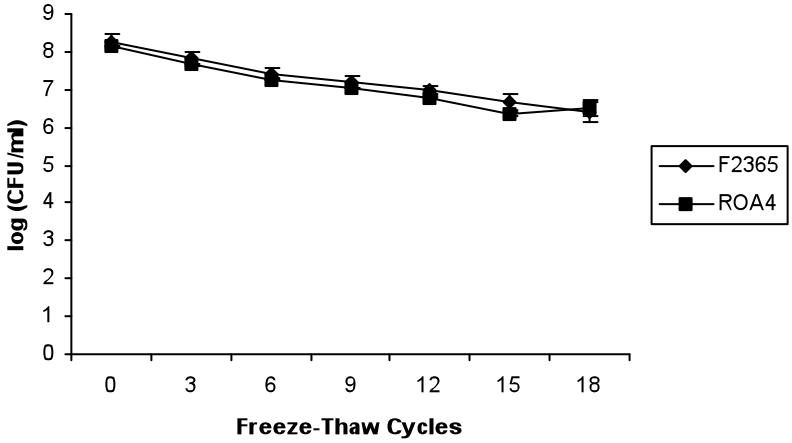
Fig. 7. 2: Spotting results for F2365, ROA4, ROA4C, and ROA4E following (A) 48 h incubation at 37°C and 25°C, (B) 10 days incubation at 10°C. ROA4C (1) and ROA4C (2) represent cultures derived from two independent colonies of complemented ROA4.

Fig. 7. 3: Freeze-thaw tolerance of (A) ROA4 grown at 25^{o}C (i) and 37 ^{o}C (ii), (B) ROA13 compared to F2365 after growth at 37^{o}C. Freeze-thaw tests were conducted over one represented experiment done in duplicate.

(A) ***(i)***


log (CFU/ml)
9
8
7
6
5
4
3
2
1
0
0
6
12
18
Freeze-Thaw Cycles
F2365
ROA4

(ii)


log (CFU/ml)
9
8
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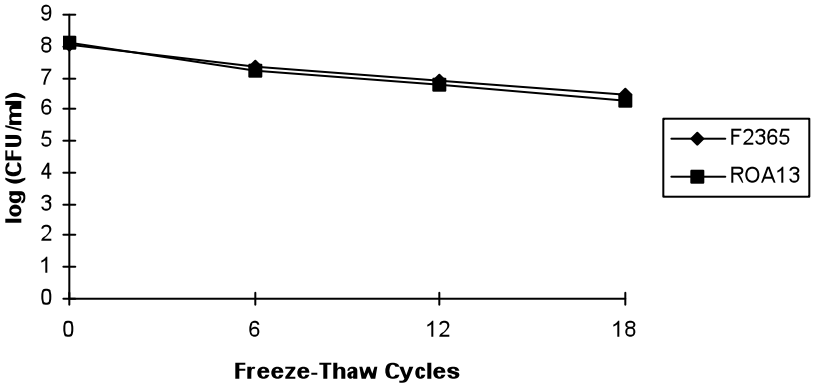
12
15
18
Freeze-Thaw Cycles
F2365
ROA4

(B)



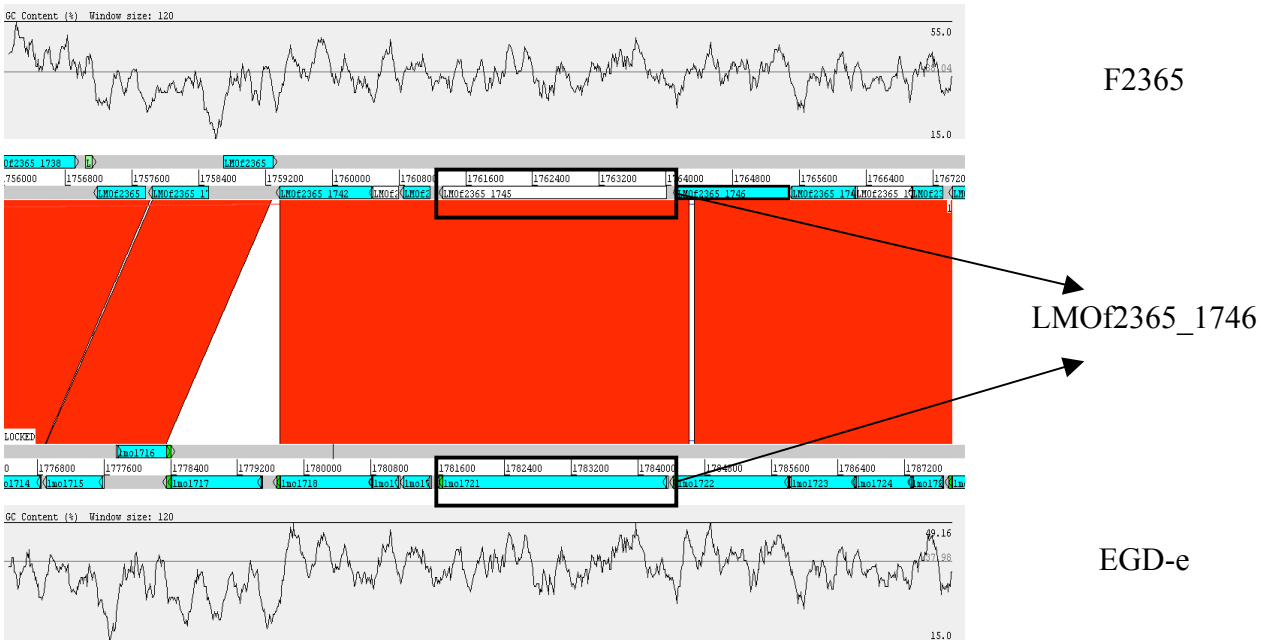


Fig. 7. 4: The comparison of LMOf2365_1746 gene region of F2365 and EGD-e.

CHAPTER VIII: Characterization of Freeze-Thaw Tolerance of Selected *L. monocytogenes* Mutants of Special Interest

8. 1. ABSTRACT

L. monocytogenes is a gram-positive, facultative intracellular organism that has the ability to cause severe illness (listeriosis). Previously, we described the impact of growth temperature on the freeze-thaw tolerance of *L. monocytogenes*. However, the genetic basis of this phenomenon is not known. Therefore, in this study we address the freeze-thaw tolerance of a panel of mutants characterized previously in our laboratory along with some of the mutants identified in this study. The mutants studied include non-hemolytic mutants, a mutant with altered phage susceptibility profile, mutants in a restriction-modification system, and a mutant in a putative biosynthesis locus, that exhibited impaired growth on blood agar. With the exception of the *purA* mutant of strain F2365 that had moderate reduction in cryotolerance, the other mutants were as tolerant to repeated freezing and thawing as their respective parental strains, following growth at 37^{o}C. The possible impact of *purA* in cryotolerance needs to be further investigated under conditions where cryotolerance is compromised, e.g. with cells grown at low temperature.

8. 2. INTRODUCTION

Listeria monocytogenes is a gram-positive, non-sporeforming, facultative anaerobic, and facultative intracellular rod (Farber et al., 1991). *L. monocytogenes* can cause serious invasive illness (listeriosis) in both humans and animals. People at high risk for the infection by *L. monocytogenes* includes; neonates, pregnant women, elderly and immunocompromised people (Farber et al., 1991, Schlech, 2000). The majority of the listeriosis cases are caused by *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b (Farber et al., 1991).

It is known that the genes involved in the pathogenicity of *L. monocytogenes* are clustered together on a pathogenicity island, and they are regulated by a transcriptional regulator encoded by *prfA*. The virulence genes present in this island include *prfA, plcA, hly, mpl, actA,* and *plcB* (Vazquez-Boland et al. 2001). The expression of *prfA* is regulated by temperature being high at 37^{o}C and impaired below 30^{o}C. Thermoregulated expression is mediated by formation of secondary structure in untranslated mRNA preceding the *prfA* start codon, which blocks the ribosome-binding region (Johansson et al. 2002).

Previously, we showed that freeze-thaw tolerance of *L. monocytogenes* exhibited a temperature-dependent pattern similar to that of the expression of the virulence genes. *L. monocytogenes* showed increased freeze-thaw tolerance when grown at 37^{o}C, than when grown at 25oC or 4^{o}C. However, the genetic basis for this temperature-dependent cryotolerance phenotype is currently unknown. To elucidate this area, we characterized mutants identified based on their susceptibility to oxidative and other stresses. We also

characterized freeze-thaw tolerance of previously identified mutants of special interest, including mutants harboring insertions in *hly* (encoding listeriolysin O), mutants with a blood agar sensitive phenotype and harboring insertions in *purA* and *purB*, and mutants lacking a GATC-specific restriction modification cassette. Here we describe the findings from the characterization of freeze-thaw tolerance of this panel of selected mutants.

8. 3. MATERIALS AND METHODS

8. 3. 1. Bacterial strains and culture conditions

Listeria monocytogenes strains and mutants used in this study were from the *L. monocytogenes* strain and mutant collection of our laboratory. The bacterial strains used in this study are listed in Table 1.

Initially, bacteria were grown on trypticase soy agar with 5% sheep blood (Remel, Lenexa, KS) at 37^{o}C for 36 h. Liquid cultures were started by transferring a single colony into 5 ml of tryptic soy broth (TSB) (BBL, Cockeysville, MD) supplemented with 0.7% Yeast Extract (YE) (Becton, Dickinson & Co., Sparks, MD) (TSBYE) and incubating at 37^{o}C overnight. A sample (30 µl) of this culture was added to 30 ml of TSBYE and incubated at 37^{o}C. Cell enumerations were conducted by plating in duplicate on TSAYE after serial dilution in TSBYE, and incubation at 37^{o}C for 36 h.

Two non-hemolytic strains of *L. monocytogenes* were screened for their freeze-thaw tolerance. The non-hemolytic mutant of *L. monocytogenes* serotype 4b strain F2365 (ROA2) was found previously in this study during the screening of mutant library for mutants with increased susceptibility to cadmium and paraquat. The other non-hemolytic

mutant (J29H) was isolated in our laboratory by J.-W. Kim during the screening of a pMC39 mutant library of *L. monocytogenes* serotype H7550 (serotype 4b). Mutant J46C was a bacteriophage sensitive mutant of *L. monocytogenes* H7550, also isolated by J.-W. Kim following screening of a pMC39 mutant library of this strain.

During our screening of the mutant library of F2365 one mutant (ROA14) had markedly impaired growth on blood agar (trypticase soy agar with 5% blood, Remel) at 37^{o}C, whereas growth was normal on trypticase soy agar without blood. Mutant ROA14 harbored a single transposon insertion in *purA*. A mutant with identical phenotype (mutant J22F) was isolated earlier by J. -W. Kim during the screening of a mutant library of strain H7550, and harbored a single transposon insertion in *purB*. Mutant A7RM and F2365 RM were derivatives of strain A7 (serotype 1/2a) and strain F2365 (serotype 4b), respectively, harboring a deletion of a restriction- modification gene cassette responsible for cytosine methylation at GATC sites, and was constructed by D. Elhanafi and S. Yildirim.

8. 3. 2. Freeze-thaw tolerance assays

Stationary phase bacteria grown at 37^{o}C were transferred (1.5 ml) into sterile cryovials (Nalgene, Rochester, NY) and frozen at -20^{o}C. Thawing was performed at room temperature for 10 min in a water bath. Freezing and thawing cycles were repeated every 24 h for 18 cycles. Every three or six cycles, cell enumerations were done in duplicate, as described previously.

8. 4. RESULTS

The survival data following 18 cycles of repeated freezing and thawing of bacteria grown at 37^{o}C showed that both of non-hemolytic *L. monocytogenes* mutants had freeze-thaw tolerance similar to that of their respective wild type parental strains (Fig. 1A, B).

Furthermore, cryotolerance of 37^{o}C –grown cells of the phage susceptibility mutant J46C and the restriction-modification cassette deletion mutant A7RM and F2365RM indicated that the mutations did not confer alterations in cryotolerance of the mutants, in comparison to the respective wild type strains (Fig. 2 and Fig. 3).

Freeze-thaw tolerance assessments with 37^{o}C- grown cultures of the *purA* mutant ROA14 revealed that the mutant had moderate reduction in cryotolerance, in comparison to the wild type parental strain F2365 (Fig. 4A). However, a mutant of strain H7550 with phenotype identical to ROA14, but harboring an insertion in *purB*, appeared to not have detectable impairment in freeze-thaw tolerance, when 37^{o}C-grown cultures were examined (Fig. 4B)

8. 5. DISCUSSION

Early in this study the impact of growth temperature on the freeze-thaw tolerance of *Listeria* was characterized. Interestingly, the role of growth temperature showed similarity with the expression of virulence genes, in which the expression was suppressed below 30^{o}C as a result of the secondary structure of the mRNA inhibiting the translation of *prfA* (Johannson et al. 2002). It was previously reported that oxidative stress contributes highly to the freeze-thaw injury in cells (Stead et al. 2000). Therefore, it

might be possible that *hly* plays role in regulation of the expression of oxidative stress related genes. In order to address this we characterized the freeze-thaw tolerance of two *L. monocytogenes* mutants of two different strains that had transposon insertions in *hly*. No difference in the tolerance of the mutants and the wild type strains suggests that liseriolysin O (LLO) is not required for tolerance of the bacteria to the stresses associated with repeated freezing and thawing at 37^{o}C. These findings are in agreement with the observation that cryotolerance of *L. innocua* and *L. welshimeri*, which lack *hly,* is similar to that exhibited by 37^{o}C-grown *L. monocytogenes* (see Chapter 2). However, it is possible that in *L. monocytogenes* it may contribute to the temperature-dependent response of the bacteria to repeated freezing and thawing. Assessment of cryotolerance of LLO-negative mutants and their parental strains following bacterial growth at 25^{o}C or below would be informative in this regard.

Phage resistance of ECII strains has been shown to be temperature-dependent, with all tested ECII strains failing to form plaques at 30^{o}C or lower (Kim et al. 2008). This temperature dependence showed similarity with the observed freeze-thaw tolerance of *L. monocytogenes* (see Chapter 2). It was, therefore, of interest to test the freeze-thaw tolerance of mutant J46F that had lost temperature-dependent phage susceptibility. However, following growth at 37^{o}C this mutant's freeze-thaw tolerance did not exhibit noticeable differences from that of the wild type parental strain H7550.

The F2365 mutant with the transposon insertion in *purA* provided some evidence of possibly impaired cryotolerance. However, the impact was modest and needs to be further characterized with repeated cryotolerance assays using cells grown at different

temperatures. A mutant of strain H7550 with similar phenotype, and harboring an insertion in *purB*, lacked detectable impact in cryotolerance. The *purA* and *purB* are not present in the same locus in the genome of *L. monocytogenes.* This difference in the location of the genes might impact the transcription profile of the mutants, which might be the reason for the observed difference in the cryotolerance of these mutants.

Epidemic clone I strains of *L. monocytogenes* harbor a number of unique genes and gene cassettes in its genome. Among these is a gene cassette that harbors genes responsible for methylation and restriction of DNA and that makes DNA of strains from this clonal group resistant to digestion by the restriction enzyme, Sau3A (Yildirim et al. 2004, Nelson et al 2004). The deletion of this cassette from strain F365 and from a serotype 1/2a strain that also harbored this cassette (strain A7) was performed previously in our laboratory. Our findings suggest that this restriction-modification cassette does not make noticeable contributions to cryotolerance of the bacteria under the conditions of the assays.

8. 6. REFERENCES

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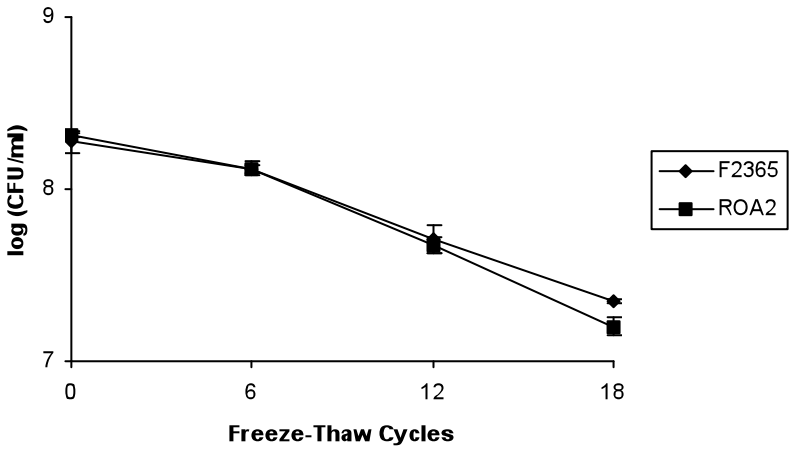
Table 8. 1: *Listeria monocytogenes* strains used in this study.

Strains	Genotype and features	Source and reference
F2365	Cheese isolate from California outbreak	(Swaminathan and Gerner-Smidt 2007)
H7550	Clinical isolate from hot dog outbreak (1998-1999)	(Evans et al. 2004)
A7	Guacomole isolate of *L. monocytogenes* serotype 1/2a (1999)	FDA
ROA2	Transposon mutant of F2365 *hly*::transposon (pMC38), Em^R Km^S, non-hemolytic	This study
ROA14	Transposon mutant of F2365 *purA*::transposon (pMC38), Em^R Km^S, blood agar sensitive.	This study
J22F	Transposon mutant of H7550-Cd^s, *purB*::transposon (pMC39), Em^R Km^S, blood agar sensitive	Jae-Won Kim and S. Kathariou
J29H	Transposon mutant of H7550-Cd^s, *hly*::transposon (pMC39), Em^R Km^S, non-hemolytic	Jae-Won Kim and S. Kathariou
J46C	Transposon mutant of H7550-Cd^s, ORF2753::transposon (pMC39), Em^R Km^S, phage-susceptible at low temperature	Jae-Won Kim and S. Kathariou
F2365RM	Restriction-modification system deleted mutant of F2365	D. Elhanafi and S. Kathariou
A7 RM	Restriction-modification system deleted mutant of A7	D. Elhanafi and S. Kathariou

(A)



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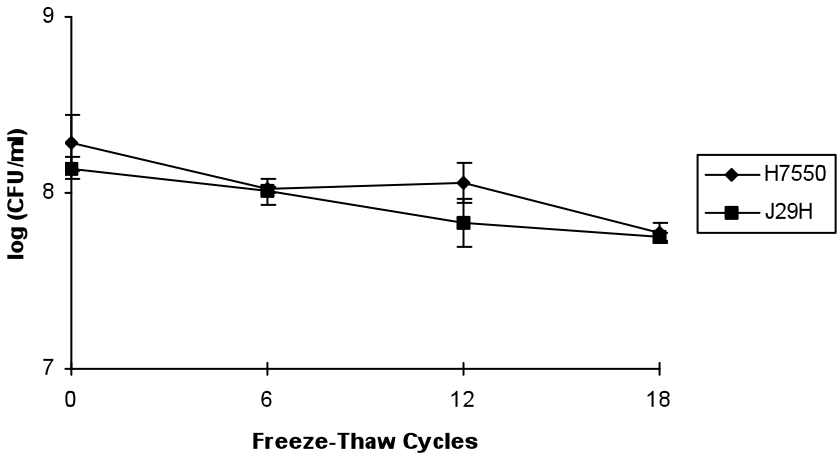


Fig. 8. 1: Freeze-thaw tolerance of (A) F2365 and the non-hemolytic mutant ROA2 and (B) H7550 and the non-hemolytic mutant (J29H) after growth at 37^{o}C. Freeze-thaw test was conducted over one represented experiment done in duplicate.

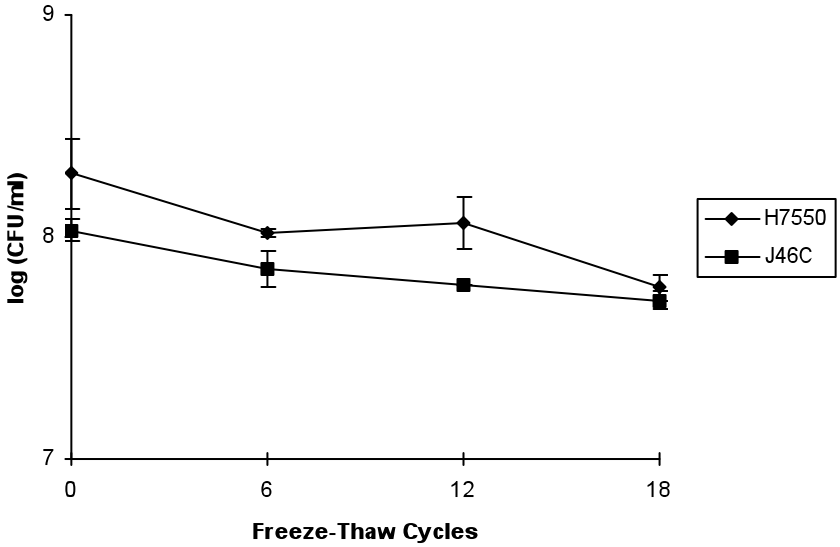
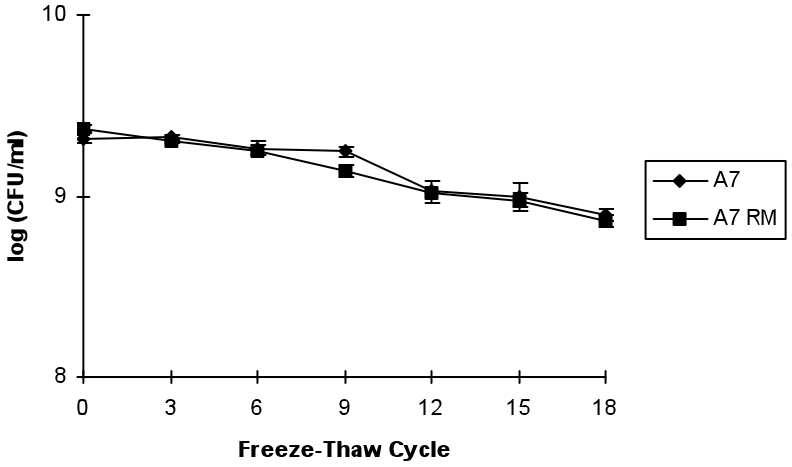


Fig. 8. 2: Freeze-thaw tolerance of H7550 and bacteriophage sensitive mutant, J46C after growth at 37^{o}C. Freeze-thaw test was conducted over one represented experiment done in duplicate.

(A)



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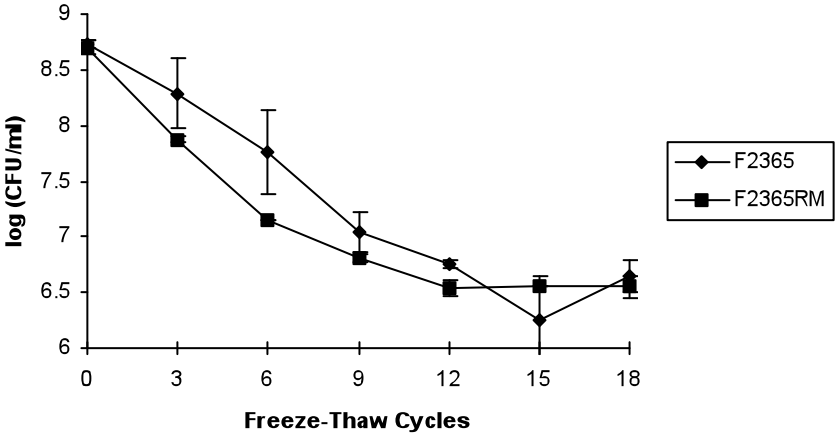
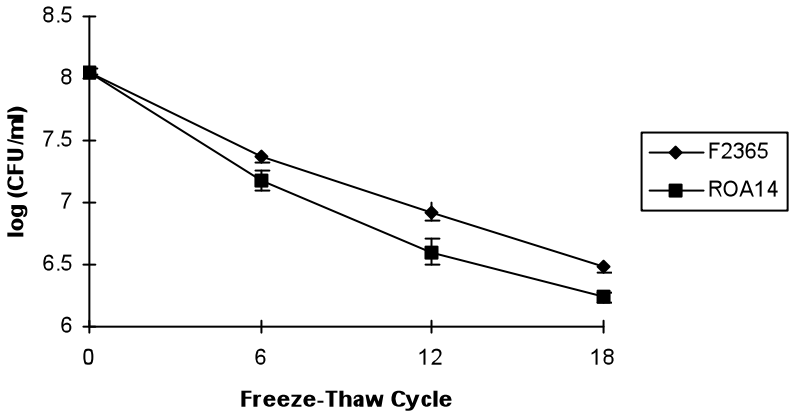


Fig. 8. 3: Freeze-thaw tolerance of strain (A) A7 and mutant A7 RM harboring a deletion of the GATC-specific restriction-modification cassette and (B) F2365 and mutant F2365 RM harboring a deletion of the GATC-specific restriction-modification cassette after growth at 37^{o}C. Freeze-thaw test was conducted over one represented experiment done in duplicate.

(A)



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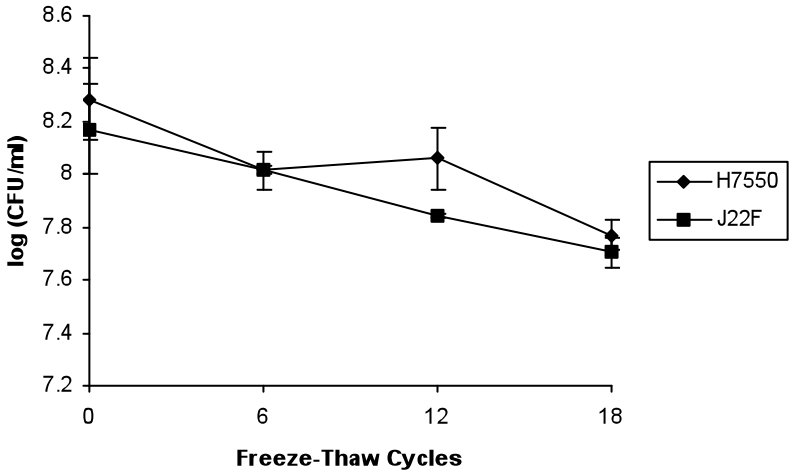
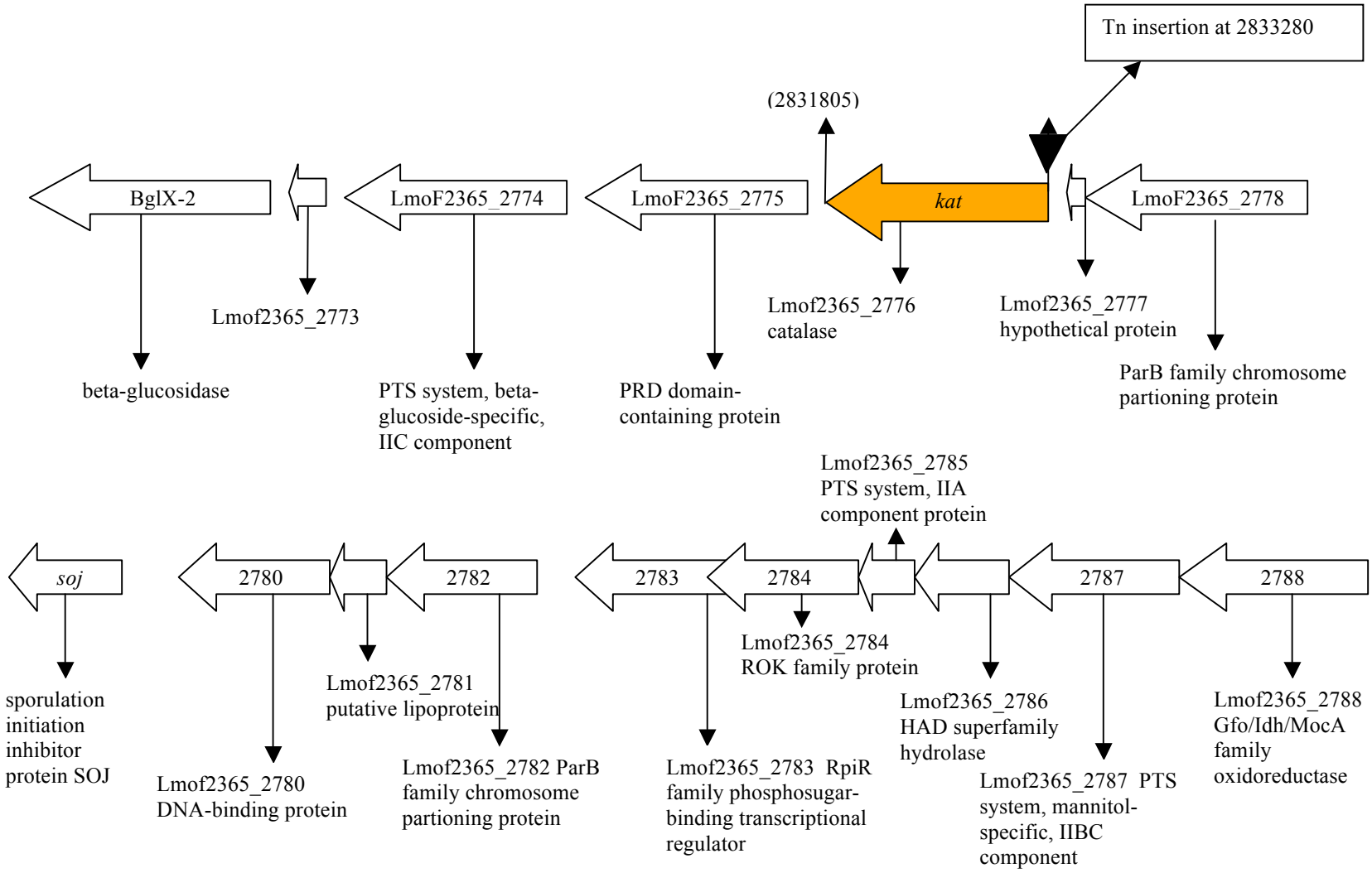


Fig. 8. 4: Freeze-thaw tolerance of (A) F2365 and purine biosynthesis (*purA*) mutant ROA14 and (B) H7550 and purine biosynthesis (*purB*) mutant J22F after growth at 37^{o}C. Freeze-thaw test was conducted over one represented experiment done in duplicate.

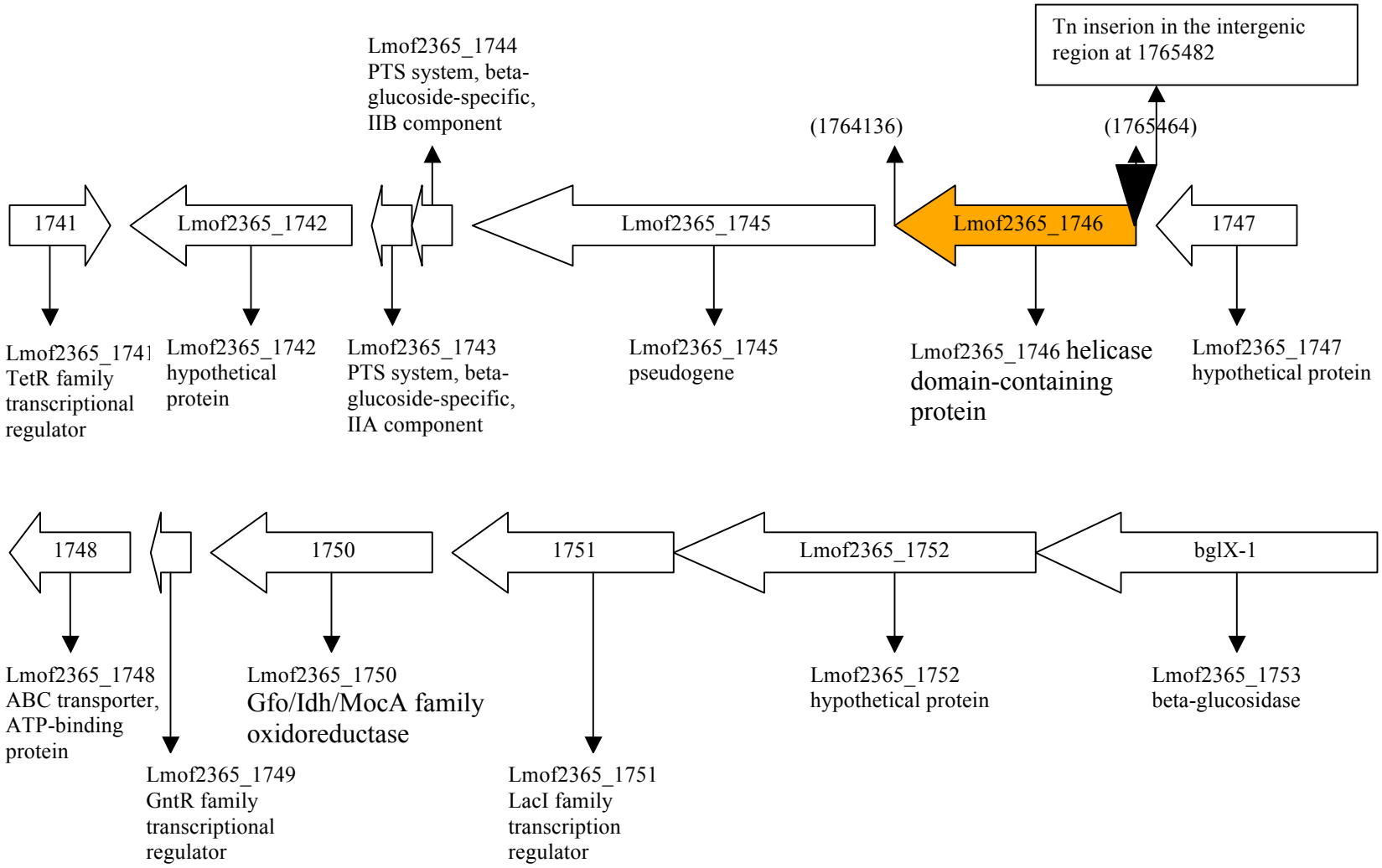
APPENDIX

Appendix A: Genomic regions harboring the transposon insertions in mutants constructed in this study

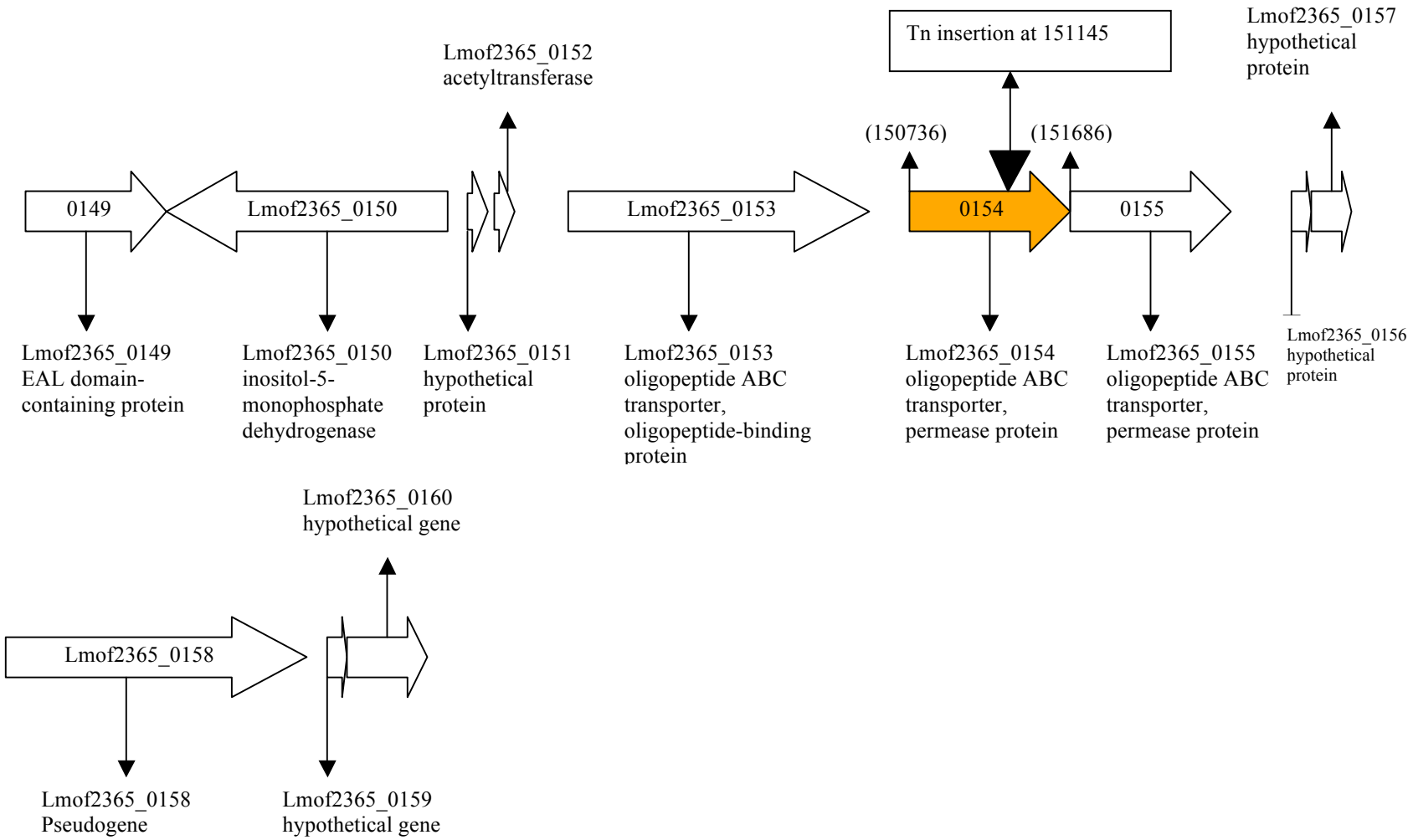
ROA3: Transposon mutant of F2365 *kat*::transposon (pMC38), Em^R Km^S.



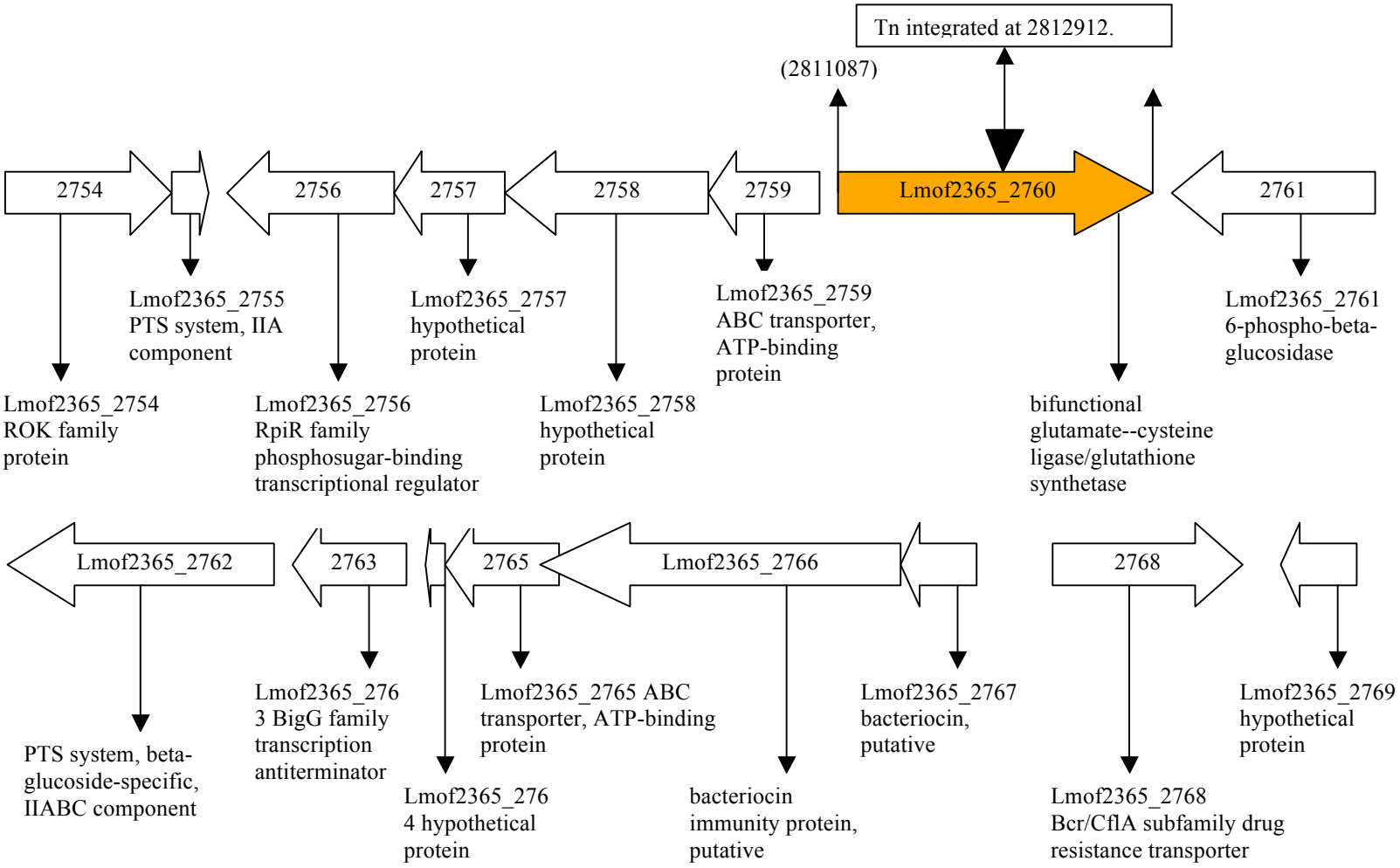
ROA4: Transposon mutant of F2365 LMOf2365_1746 (helicase domain protein)::transposon (pMC38), Em^R Km^S.



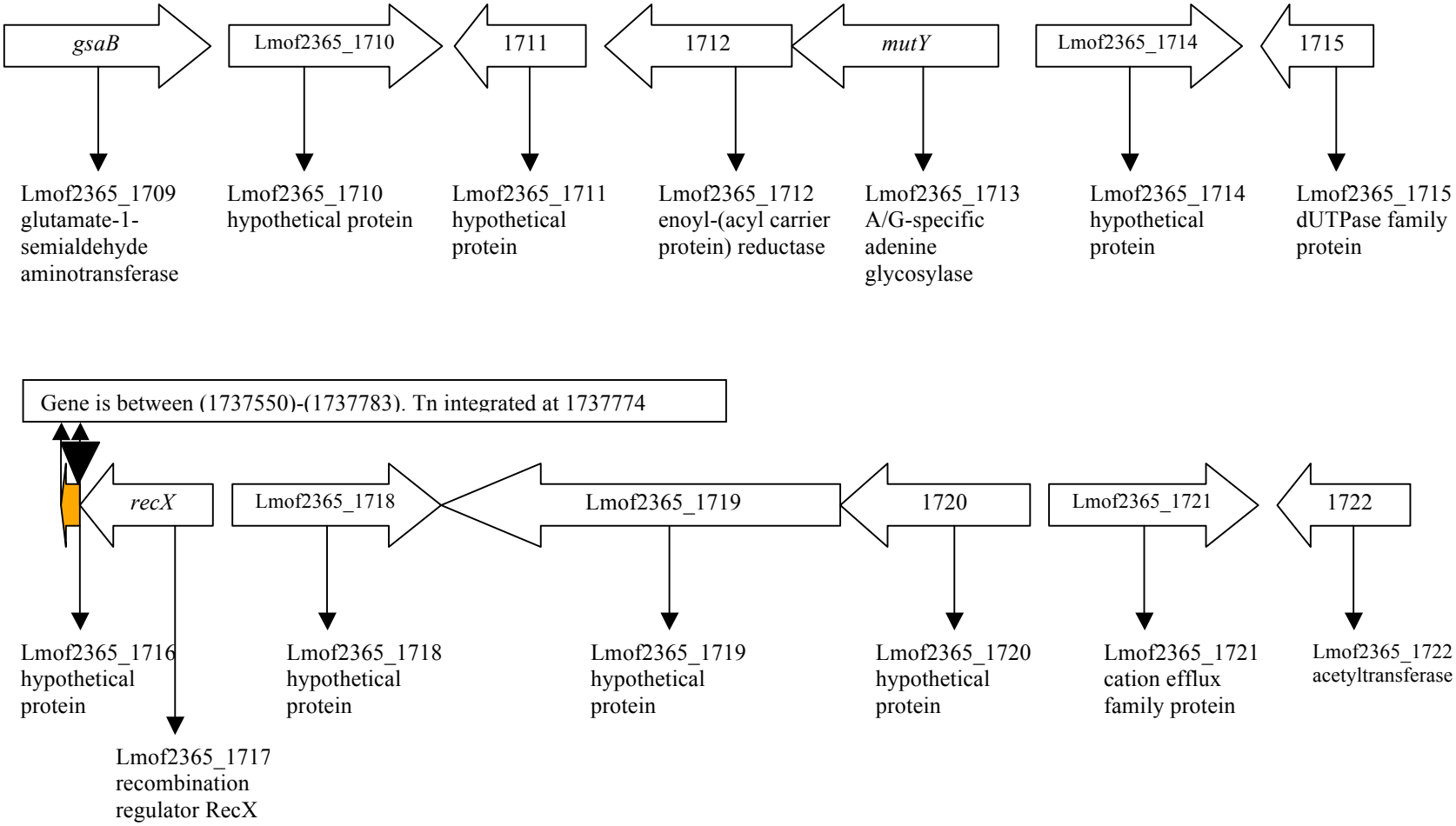
ROA6: Transposon mutant of 10403S (oligopeptide ABC transporter, permease protein)::transposon (pMC38), Em^R Km^S.



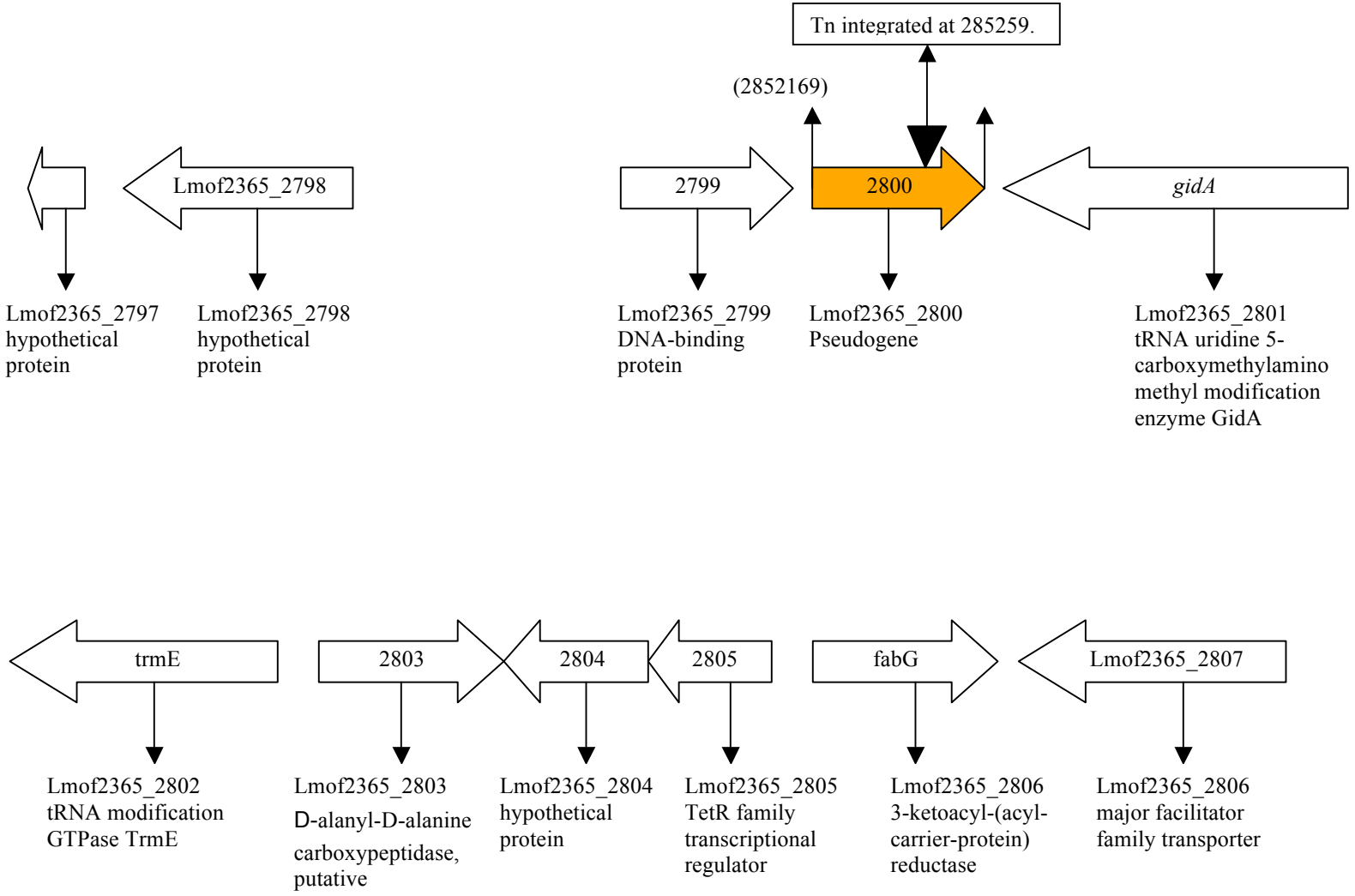
ROA7: Transposon mutant of F2365 LMOf2365_2760 (putative glutamate--cysteine ligase/amino acid ligase)::transposon (pMC38), Em^R Km^S.



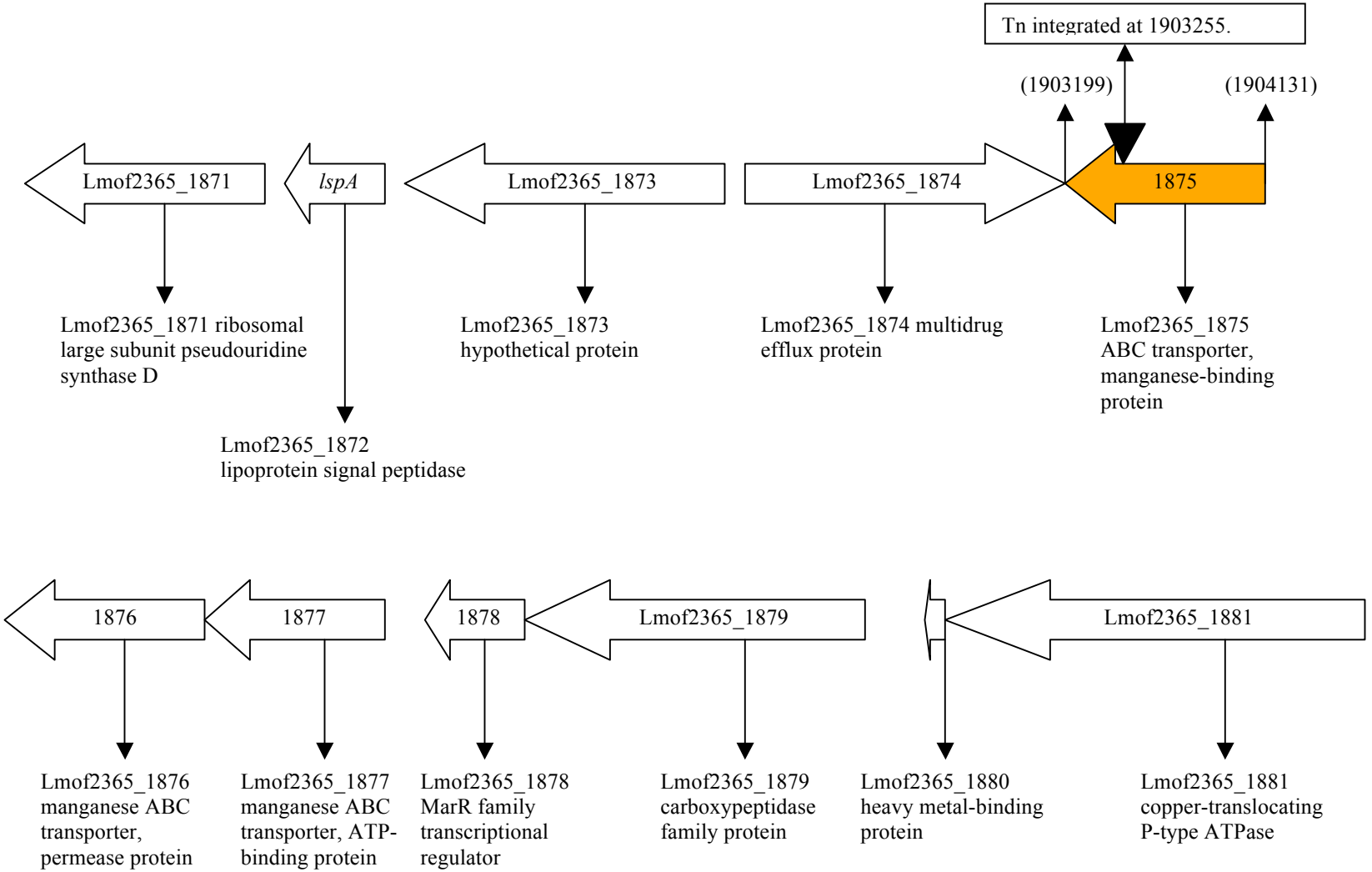
ROA8: Transposon mutant of F2365 LMOf2365_1716 (conserved hypothetical protein)::transposon (pMC38), Em^R Km^S.



ROA9: Transposon mutant of F2365 LMOf2365_2800 (pseudogene)::transposon (pMC38), Em^R Km^S.



ROA13: Transposon mutant of F2365 LMOf2365_1875 (ABC transporter, manganese-binding protein)::transposon (pMC38), Em^R Km^S.



ROA14: Transposon mutant of F2365 LMOf2365_0065 (adenylosuccinate synthetase) *purA*::transposon (pMC38), Em^R Km^S.