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**IMPORTANT FACTORS THAT INFLUENCE THE SURVIVAL OF  
*LISTERIA MONOCYTOGENES* DURING THE MINIMAL THERMAL  
PROCESSING OF FRESHLY SHREDDED CABBAGE**

by

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Submitted  
in partial fulfillment of the requirements  
for the degree of

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## LIST OF ABBREVIATIONS

$\Delta$ T6P	Mutated phosphotrehalase ( <i>treA</i> ) gene
2D-GE	Two-dimensional gel electrophoresis
568 $\Delta$ T6P	<i>L. monocytogenes</i> strain 568 with deleted phosphotrehalase gene
ABC	ATP-binding cassette
Adh	Alcohol-acetaldehyde dehydrogenase
ANOVA	Analysis of variance
AR	Acid resistance
ATR	Acid tolerance response
BHI	Brain heart infusion
bile	Bile salts resistance gene
Bkd	Branched-chain $\alpha$ -keto acid dehydrogenase
CAPS	Cold acclimation proteins
CcpA	Catabolite control protein A
CFU	Colony forming units
CIAP	Calf intestinal alkaline phosphatase
CIPS	Cold induced proteins
CIRCE	Controlling inverted repeat of chaperone expression
ClpC	Caseinolytic Protease C
ComG	Competency protein
CRE	Catabolite responsive element
Crm	Chloramphenicol
CSP	Cold shock proteins
CtsR	Class Three Stress Regulator
dATP	2'-deoxyadenosine 5'-triphosphate
DCCD	<i>N, N'</i> - dicyclohexylcarbodiimide
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DIG	Digoxigenin
D <sub>L</sub>	Logistic Decimal reduction time
DNA	Deoxyribonucleic acid
DnaK	Molecular chaperonin (heat shock protein 70)
dTTP	2'-deoxythymidine 5'-triphosphate
D-value	Decimal reduction time
EDTA	Ethylenediaminetetraacetic acid
EGD-e	<i>L. monocytogenes</i> strain EGD-e
EPS	Extracellular polymeric substances
Erm	Erythromycin
GABA	$\gamma$ -aminobutyric acid
GAD	Glutamate decarboxylase system
Gbu	Glycine betaine uptake
GntR	Gluconate transcriptional regulators

GroESL	Molecular chaperonin complex with GroES (heat shock protein 10) and GroEL (heat shock protein 60) subunits
GUS	$\beta$ -glucuronidase
HGPT	Hypoxanthine-guanine phosphoribosyl transferase
HK	Hexokinase
HlyA	Listeriolysin (hemolysin)
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
Hrc	Heat Regulation at CIRCE
HSP	Heat shock proteins
IPTG	Isopropyl $\beta$ -D-thiogalactoside
Kan	Kanamycin
LB	Luria-Bertani
Li	<i>Listeria innocua</i>
Lm	<i>Listeria monocytogenes</i>
Lm568	<i>Listeria monocytogenes</i> strain 568
LPS	Lipopolysaccharide
LPXTG	Leucine-proline-(x = any amino acid)-threonine-glycine motif
Lw	<i>Listeria welshmeri</i>
MAP	Modified atmosphere packaging
MIC	Minimum inhibitory concentration
mRNA	Messenger RNA
MSG	Monosodium glutamate
MW	Molecular weight
MWB	Modified Welshimer's Broth
NMR	Nuclear Magnetic Resonance
NRAMP	Natural resistance associated macrophage protein
OpuC	Osmoprotectant uptake gene
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonia-lyase
pAM401	Gram-positive/negative DNA shuttle vector
pAM401:TREOP	pAM401 vector carrying intact trehalose operon
pAUL-A	Gram-positive/negative DNA shuttle vector
pAULA: $\Delta$ T6P	pAUL-A vector carrying <i>treA</i> gene (see above) with 462 bp deletion
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PIA	Polysaccharide intercellular adhesin
PNPG	p-nitrophenyl- $\alpha$ -D-glucopyranoside
PrfA	Positive Regulator Factor A
PTS	Phosphoenolpyruvate phosphotransferase system
pTV1-OK	Thermosensitive DNA shuttle vector carrying the transposon Tn917

RNA	Ribonucleic acid
RNAP	RNA polymerase
rRNA	Ribosomal RNA
Rsb	Regulator of Sigma B
RTE	Ready to eat
RT-PCR	Reverse Transcription PCR
SCOTS	Selective capture of transcribed sequences
ScottA	<i>L. monocytogenes</i> outbreak strain ScottA
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
SEM	Scanning electron microscopy
SOE	Splicing by overlap extension
S <sub>R</sub> values	“Binding strength”/ binding efficiency
SSR	Starvation survival response
T6P	Trehalose-6-phosphate
TAE	Tris-acetate EDTA
TBE	Tris-Borate EDTA
TBS	Tris buffered saline
TE	Tris-EDTA
Tet	Tetracycline
Tn917	Transposon 917
TreA	$\alpha$ , $\alpha$ phosphotrehalase
TreB	Trehalose-specific enzyme II <sub>BC</sub> of the phosphotransferase system
TreR	Regulator of trehalose operon (GntR-like family)
tRNA	Transfer RNA
TSA-Tre	Tryptic soya agar with trehalose
TSA-YE	Tryptic soya agar with yeast extract
TSA-YEP	Tryptic soya agar with yeast extract and pyruvate
TyrS	Tyrosyl-tRNA synthetase
UFA	Unsaturated fatty acids
VNBC	Viable but non-culturable
$\sigma^B$ (SigB)	Alternative Sigma Factor B
$\sigma^S$ (SigS)	Alternative Sigma Factor S

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## ABSTRACT

*Listeria monocytogenes* is widespread in nature and is often isolated from the soil or decaying plant debris. Therefore, this pathogen is likely to be found on raw produce with relatively high frequency. Several key factors (attachment, growth and thermal resistance) important for the survival of *Listeria* spp. on minimally processed cabbage have been examined. Attachment of cells to cabbage tissues was rapid (< 5 min) for all 24 tested strains with some strains showing greater proficiencies to attach to both cut and intact cabbage surfaces. In all cases, cut tissues were preferred by all strains with an increase of 1.0 to 1.2 log in colony forming units (CFU) per cm<sup>2</sup> over intact tissues. Scanning electron microscopy analysis confirmed the presence of these cells along cut edges and within crevices. Increasing the contact time resulted in the formation of cell clusters and microcolonies. Attached cells for 24 different *Listeria* strains were shown to survive at relatively high levels for up to 28 days on intact cabbage, albeit with a general decreasing trend in CFUs over time. In contrast, most strains showed moderate growth (1.0 to 1.5 log CFU/cm<sup>2</sup> increase) on cut cabbage tissues stored at 5°C over the same time period. The application of a mild thermal treatment (50°C for 3 min) on shredded cabbage resulted in a significant increase ( $P < 0.05$ ) in CFUs for all tested strains after refrigerated storage at 5°C for 21 days. This increase exceeded 3.0 log CFU/g for two *L. monocytogenes* strains. Cellular changes resulting in heat resistant phenotypes were examined using a transposon library constructed in *Lm568* (serotype 1/2a). Sixteen thermotolerant mutants were isolated with Tn917 insertions in a diverse array of genes. The seven mutants displaying the highest level of thermal resistance at 52°C were also exposed to higher temperatures (56°C and 60°C). Five of these mutants continued to be significantly more ( $P < 0.05$ ) thermal resistant than the wild type. The interrupted genes for the 16 mutants were determined to be involved in; transport, metabolism, replication and repair, general stress, and structural properties such as cell wall and membrane composition, and surface structures. An isogenic mutant with a 462 bp deletion in a putative *treA* gene was constructed and shown to be devoid of phosphotrehalase activity. The mutant showed subsequent intracellular accumulation of high levels (22.8 µg/ mg cell protein) of trehalose-6-phosphate (T6P) when grown in the presence of trehalose. A concomitant build up of trehalose (11.6 µg/ mg protein) was also detected, presumably through the dephosphorylation of T6P. This mutant was also shown to be significantly ( $P < 0.05$ ) more resistant to osmotic stress and desiccation in comparison to *Lm568*. The work presented in this thesis will help to advance our understanding of how *Listeria* contaminate, survive and proliferate in a vegetable processing system implementing a mild thermal treatment. Moreover, these data may help elucidate the mechanisms involved in enhanced thermal resistance in *L. monocytogenes*. As such, this information may lead to new strategies for the implementation of remedial measures to reduce the risk of foodborne illness pertaining to fresh cut, minimally processed fruits and vegetables.

## **1. INTRODUCTION**

The eating habits of Canadians have changed dramatically over the past two decades. This is largely due to the efforts of organizations such as the Canadian Dietetics Association promoting increased dietary intakes of fruit and vegetables. Canada's Food Guide recommends a daily consumption of 5 to 10 servings of fresh or cooked fruit and vegetables to maintain a healthy diet. Other factors also contribute to this trend. For example, the number of immigrants coming to Canada has increased over the last 20 years and the influx of new cultures has introduced diverse culinary tastes that have led to the import of a broad range of fruit and vegetable commodities previously unavailable in Canada. As a result, Canadians now consume more fresh produce than at any other time in the past. This demand has challenged the fruit and vegetable industry to not only increase field crop production and import capabilities to meet these needs, but also processors have added variety by developing new minimally processed, ready-to-eat (RTE) products. Many of these RTE foods also exhibit extended shelf lives thereby benefiting both the retailer and consumer (Laurila and Ahvenainen, 2002). The same trends have also been observed in the United States and nations within the European Union, thus resulting in a marked increase in the global distribution of produce. In the United States from 1970 to 1993, it was estimated that fruit and vegetable consumption increased by 27% (De Roeve, 1999). Table 1.1 shows the amount of fruits and vegetables consumed (per capita) in the United States between 1982 and 1997.

Table 1.1. The consumption of fruit and vegetables per capita per year in the United States from 1982 to 1997.

Year	Fruits (kg)	Vegetables (kg)
1982	38.7	52.9
1983	41.0	50.9
1984	40.2	55.8
1985	39.3	57.5
1986	42.1	57.0
1987	44.1	60.1
1988	44.1	61.5
1989	43.7	64.7
1990	41.6	60.9
1991	40.7	60.9
1992	44.5	64.2
1993	45.3	66.4
1994	45.6	69.6
1995	44.4	67.7
1996	44.8	70.8
1997	46.7	74.4

Source; Report on Foodborne outbreaks associated with fresh and fresh cut produce. Source: U. S. Food and Drug Administration Center for Food Safety and Applied Nutrition. September 30, 2001.

In the past, the greatest concerns pertaining to the microbiological status of fresh produce and their related products were, for the most part, related to overt spoilage. Soft rots, visible mould growth, unsightly surface scabs, blisters, lesions and other manifestations associated with spoilage or deformation were considered the primary problems for consumers and producers. However, in recent years it has become clear that these foods can also serve as potential vehicles for the dissemination of human pathogens (Tauxe et al., 1997; Sewell and Farber, 2001). Numerous outbreaks of foodborne disease involving produce have been documented worldwide. These incidents have involved several different pathogens (bacteria, protozoa, viruses) and various produce commodities. Some primary examples include; *Listeria monocytogenes*, cabbage (1981, NS, CAN); *Salmonella* Stanley, alfalfa sprouts (1995-96, BC and PQ,

CAN); *Salmonella* Hartford, orange juice (1995, FL); *Escherichia coli* O157:H7, radish sprouts (1996, Japan), lettuce and spinach varieties (1991, ME; 1995, MT; 1996, CT; 2006, 26 U.S. States, and ON, CAN) and unpasteurized apple cider (1991, MA; 1996, CA, CO, WA, USA., and BC, CAN); *Shigella sonnei*, imported chopped parsley (1998, ON and AB, CAN); *Shigella flexneri*, scallions (1994, IL); *Cyclospora cayetanensis*, imported Guatemalan raspberries (PQ, ON, CAN and 20 States in USA), mesclun lettuce from U.S. and Peru (1997, FL), and basil products from USA and Mexico (1997, VA, MD and DC; 1999, MO); *Cryptosporidium parvum*, unpasteurized apple cider (1994, ME; 1996, NY); Hepatitis A virus, sliced strawberries from Mexico and processed in U.S. (1997, several U.S. States), and Calicivirus (norovirus), imported Bosnian raspberries (1997, PQ) (De Roever, 1999; Francis et al., 1999a; Sewell and Farber, 2001; Anonymous, 2007b). These represent only a small cross-section of reported incidents. A list of reported foodborne outbreaks from 1981 to 2000 linked to fresh or minimally processed produce in Canada is given in Table 1.2. It should be noted that the majority of foodborne incidents are never reported, as is often the case with less severe symptoms not involving hospitalization. Even when reported, the point of contamination is extremely difficult to track in many cases. This is due to the numerous possibilities along the food continuum where contamination could occur, including; the initial planting of a crop, its growth cycle, harvest, processing, distribution and handling by the consumer (Beuchat, 1996; Beuchat, 2002). Despite significant gains in food safety practices over the past few years, it is clear that much more support is required for research, surveillance and public education regarding foodborne diseases associated with fresh or



minimally processed produce. To accentuate this point, is the recent outbreak of *Escherichia coli* O157:H7 associated with the consumption of contaminated spinach. The spinach was provided by a California producer and the outbreak affected 204 individuals in 26 U.S. States and 1 person in Canada (Anonymous, 2006).

A number of factors may contribute to the recent changes in the epidemiology of foodborne diseases associated with produce. A combination of changing food industry practices coupled with demographics may enhance the risk of pathogens reaching consumers (De Roeve, 1999). The food industry has become dominated by larger, more centralized food production facilities which provide consumers with products over greater distances. Hence, the opportunity for contamination is much greater and the period of time for pathogen growth is increased, especially if temperature abuse occurs. Another contributing factor to the increased risk rests in the fact that we are living in an aging society, thus placing a significant proportion of our population in higher risk groups for pathogen susceptibility (De Roeve, 1999). Public perception that processed foods have a compromised nutritional status has led to a greater demand for fresh produce. Trends have now shifted to convenience type minimally processed foods that have not undergone microbial destructive processes such as thermal processing. Therefore, increased consumption of fresh produce in itself provides an increased opportunity for greater exposure to pathogenic microorganisms (Brackett, 1999).

Table 1.2. List of foodborne outbreaks in Canada from 1981-2000 involving fresh or minimally processed produce.

Pathogen	Year	Food	Province	Canadian Cases
<i>Listeria monocytogenes</i>	1981	coleslaw (cabbage)	NS	41 (17 deaths)
<i>Salmonella</i> Poona	1991	cantaloupe	ON, PQ, NF, SK, MN	78
<i>Salmonella</i> Oranienberg	1998	cantaloupe	ON	22
<i>Salmonella</i> Stanley	1995	alfalfa sprouts	BC	30
<i>Salmonella</i> Newport	1995-1996	alfalfa sprouts	BC, PQ	121
<i>Salmonella</i> Meleagridis	1997	alfalfa sprouts	AB, ON, SK	124
<i>Salmonella</i> Paratyphi B var. Java	1999	alfalfa sprouts	AB, BC, SK	46
<i>Salmonella</i> Enteritidis	2000	alfalfa sprouts	AB, SK	8
<i>Escherichia coli</i> O157:H7	1995	Imported iceberg lettuce	ON	23
<i>Escherichia coli</i> O157:H7	1998	potato salad	NS	194 (1 death)
<i>Shigella sonnei</i>	1998	Imported chopped parsley	ON, AB	40
<i>Staphylococcus aureus</i>	1988	potato salad	SK	49
<i>Cyclospora cayetanensis</i>	1996	Guatemalan raspberries	ON, PQ	195
<i>Cyclospora cayetanensis</i>	1997	Guatemalan raspberries	ON	31
<i>Cyclospora cayetanensis</i>	1998	Guatemalan raspberries	ON	315
<i>Cyclospora cayetanensis</i>	1999	Guatemalan blackberries	ON	104
Calicivirus	1992	Salad	ON	27
Calicivirus	1997	Bosnian raspberries	PQ	>200

Source: Sewell A.M. and J.M. Farber. 2001. J. Food Protection, Vol. 64 (11): 1863-1877.

Many fruits and vegetables are grown in either direct contact with, or in close proximity to, the soil environment such that transfer of microorganisms from the soil is eminent. A number of foodborne pathogens are known to be indigenous to many soils (i.e., *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus cereus* and *L. monocytogenes*) and are likely to be frequently carried on produce in relatively low numbers. However, the greatest source of contamination is thought to be through the fecal-oral route where common farm practices such as the use of manure fertilizers (often improperly composted) and irrigation of crops with water contaminated with fecal matter provides great opportunity for intestinal pathogens to come in contact with produce. Nicholson et al. (2005) found that *E. coli* O157:H7, *Salmonella* and *Campylobacter* were able to survive for up to 3 months in stored slurries prepared from dairy manures (2% dry matter). Moreover, *L. monocytogenes* was able to survive for up to 6 months. Dry solid farmyard manures were more detrimental to all tested pathogens where, for example, maximum survival times for *E. coli* O157:H7 was found be *ca.* 1 month in turned pig manure and only 4 days if the same manure was left unturned. The decline in pathogen numbers obviously depends on several factors (temperature, pH, aeration, dry matter content) pertaining to the management and storage of the manure. Therefore, proper management is crucial to reducing the risk of the carry-over of pathogens since once contaminated, most commodities entering the fresh produce market do not undergo further processes that would aid in the removal of these contaminants (Francis et al., 1999a; Beuchat, 2002).

The intracellular pathogen, *L. monocytogenes* provides unique challenges for the food industry. The widespread occurrence of this organism and its ability to survive harsh environments make *L. monocytogenes* difficult to control in processing facilities (Gravani, 1999). The frequent occurrence of this pathogen on fruits and vegetables guarantees its entry into food processing plants, and once access is gained, it can spread throughout the processing facility and contaminate otherwise “clean” food products. Mostly, contamination of foods is by environmental strains of *L. monocytogenes* that are well adapted to deal with the stresses imposed by the natural and food processing environments (Hill et al, 2002).

In consideration of the continuum for the production and processing of fresh fruits and vegetables, there are many natural and intentional barriers for *L. monocytogenes*, and other pathogens to overcome if they are to survive and proliferate. For example, listeriae on the surface of leafy vegetables must be able to withstand the broad temperature fluctuations of the natural environment, UV radiation, desiccation, nutrient depletion and the actions of competitive resident microflora. Once harvested, *L. monocytogenes* may be subjected to cold shock as produce is often rapidly cooled in the field and then stored under refrigeration. Other processing stress factors may include; shear forces from washing and handling, antimicrobial agents such as chlorine in the wash water, modified atmospheres or mild heat treatments. Therefore, in order to persist in this environment *L. monocytogenes* must be able initiate a number of stress adaptation mechanisms.

The ability of foodborne bacterial pathogens to adapt to food processing environments, presents food scientists and food processors with a difficult dilemma. In light of the frequent association of minimally processed fruit and vegetables with foodborne disease, it is clear that more research is required to enhance our current understanding of how these bacteria enter the food production continuum and survive the inimical barriers imposed by processing. Enhanced knowledge in this area will lead to appropriate remedial action to reduce the risk of outbreaks of foodborne diseases associated with fruit and vegetables.

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **2.1 Ready-to-eat (RTE) fruits and vegetables**

In order to assess the microbiological status of minimally processed RTE fruits and vegetables, one must first understand the nature of the processing and handling practices. Fruits and vegetables can be sold individually or as a mixture of several produce constituents. They may remain whole and intact or they can be peeled, sliced or shredded. Moreover, prepackaged salads have become very popular and in the case of coleslaw, shredded cabbage may be sold already mixed with a dressing. In some instances, fruits or vegetables are mixed with other components such as precooked meats, cheeses and/or pastas. These salads may introduce other potential dangers with regard to the types and numbers of pathogens (Anonymous, 2002b).

The first priority of the processor is to clean the produce of extraneous and residual materials carried over from the growing environment of the plant or those obtained through contact during the handling of materials prior to processing. In general, the overall quality of the final product is a reflection of the quality of the produce at time of harvest. That is to say, given that all other factors are equal throughout the process, the best quality final products stem from the highest quality items in the field. If the produce

is to be cut or shredded, it is imperative that microbial numbers are reduced through removal of contaminated parts or washing. Knives and shredders will spread these microorganisms throughout the product which becomes a more suitable substrate to support microbial growth once tissues are damaged (Wilcox et al., 1994). For plants that grow subterranean or in relative close proximity to the ground (i.e., low-bush blueberries, broccoli, cabbage, carrots, cauliflower, celery, cucumber, lettuce, onions, strawberries, tomatoes, etc.), the removal of soil materials is paramount and may first involve a rinse step followed by the removal of the outer leaves (leafy vegetables) where detritus from the soil and microbial loads may be excessively high (i.e., cabbage and lettuce) (De Roever, 1999; Francis et al., 1999; Bhagwat et al., 2002). Next, one to several washes in clean potable water is usually performed prior to cutting or peeling. It is often common practice to incorporate a disinfectant dip followed by a final wash to remove chemical residues. Chlorine (100-200 ppm), 1% ascorbic acid and 1% citric acid as well as a number of commercial detergents are commonly used for produce. The effectiveness of these agents is largely dependent on the type of produce, initial microbial load, time of exposure, and the type and level of disinfectant (Zhang and Farber, 1996; Delaquis et al., 1999; Li et al., 2001; Francis and O'Beirne, 2002). In general, one can expect only a modest reduction in resident microflora following washing. Alternative methods to increase the effectiveness of washing have also been examined. For example, augmented washes, with and without chlorine, in conjunction with ultra-sonication have demonstrated enhanced reductions in microbial numbers (Seymour et al., 2002). Once dried, produce can be further processed according to the parameters of the desired end

product. This may involve shredding, peeling and slicing prior to mixing (Laurila and Ahvenainen, 2002).

Intact plant surfaces are designed to prevent the entry of invading microorganisms. Although most soil dwelling microbes cannot actively penetrate plant tissues, phytopathogens have evolved intricate mechanisms to circumvent these defenses (Hildebrand et al., 1999; Braun et al., 2001). However, once these barriers are broken many saprophytes can utilize disrupted tissue surfaces as attachment sites and leaked plant juices as growth substrates. It has been shown that pathogens such as *E. coli* O157:H7 and *L. monocytogenes* will attach preferentially to cut surfaces within a very short time frame and the binding strength makes their removal difficult (Takeuchi et al., 2000; Ells and Truelstrup Hansen, 2006). The type of slicing instrument used for cutting also has an influence on creating hospitable surfaces for the attachment and growth of foodborne pathogens. Gleeson and O' Beirne (2005) observed that more rudimentary cuts or tears created better support surfaces for the growth of *E. coli* and *L. innocua* on a variety of vegetables than did cuts with sharp instruments. The cutting process not only affects the microbial quality but also has a major impact on the physiology of produce. It has been reported that hand peeled carrots had an increased respiration rate of *ca.* 15% over unpeeled carrots during storage (Ahvenainen, 1996). When carrots were peeled mechanically using an abrasion peeler, the respiration rate was twice that of unpeeled carrots. The cutting of plant surfaces disrupts cells causing leakage. Cell constituents, that were previous separated within compartments of intact tissues, are mixed and can act as substrates for enzymatic reactions. For example, polyphenol oxidases require oxygen,



copper, and a substrate to cause browning in compromised plant tissues (Ahvenainen, 1996). Lipooxidases are another important group of enzymes contributing to the degradation of produce. These enzymes catalyze a number of peroxidation reactions ultimately forming a myriad of ketones and aldehydes creating foul odours (Varoquaux and Wiley, 1994).

The final stage of the process, prior to storage, is packaging. Careful consideration must be given here since poor packaging decisions and practices will negate the care put into the process prior to packaging. Because fruits and vegetables will continue to respire, they must be packaged in semi-permeable packaging materials (Legnani and Leoni, 2004). It has been reported that oxygen levels will fall from 21% to 2-5% and carbon dioxide will increase from 0.03% to 3-10% during respiration in closed packages (Francis and O'Beirne, 1996). Therefore, modified atmospheres with similar gas regimes have been successfully employed to provide longer shelf stability (Francis et al., 1999). The objective of modified atmosphere packaging (MAP) is to produce a gas balance inside the package that will reduce respiration to minimum levels without negatively influencing the integrity of the product. A general rule of thumb is to create a package environment with a final gas composition of 2-5% CO<sub>2</sub> and 2-5% O<sub>2</sub> with N<sub>2</sub> making up the remaining bulk of gas in the package (Kader et al., 1989). However, the optimal final gas mixture is dependent on the type of produce and whether it is whole or cut. Due to the high respiration rates of cut produce and inadequate permeable packaging films, appropriate conditions are often difficult to achieve (Laurila and Ahvenainen, 2002). A recent study investigating the effect of packaging film for MAP on the quality

of coleslaw mix found that the use of oriented polypropylene films (low CO<sub>2</sub> and O<sub>2</sub> permeability) caused the development of exceptionally high CO<sub>2</sub> levels (25-35%) while O<sub>2</sub> levels dropped to below 1% creating conditions for anaerobic respiration (Cliffe-Byrnes et al., 2003). This resulted in decreased product pH, loss of firmness, production of off-odours, high cell permeability and the production of excessive exudates and surface moisture in the package after 9 days at 4°C. The use of microperforated films (high CO<sub>2</sub> and O<sub>2</sub> permeability) resulted in less atmosphere modification resulting in high O<sub>2</sub> levels and a firm product after storage at 4°C for 9 days. However, the appearance of the coleslaw mix was not optimal as dehydration along cut surfaces and tissue discolouration was evident, therefore emphasizing the difficulty in finding the right conditions and materials for MAP of respiring cut vegetables.

Ultimately, one of the most important factors contributing to the quality of minimally processed fruits and vegetables is the storage temperature. However, it would be more prudent to recognize that temperature not only plays a major role for produce quality during storage but also throughout the processing regime; from immediately after harvest, during processing, storage, distribution and retail (Dallaire et al., 2006). Therefore, the removal of the field heat from freshly harvested produce is an important first step in delaying the onset of both enzymatic and microbial spoilage. As a general rule, during processing, all wash treatments should be at 0-5°C, as should peeling, packaging and storage. Temperature abused products will generally have shortened shelf life expectancy. However, the actual optimum temperature and ultimate shelf life will vary according to product and cultivar (Laurila and Ahvenainen, 2001).

## 2.2 Microbiological Status of RTE Fruits and Vegetables

The soil environment plays host to a huge diversity of microorganisms. In any given sample of agricultural soil one will find numerous species of bacteria, yeasts, moulds, and protozoa. The soil type, agricultural practices and plant cultivars present will impact the microbial community (Atlas, 1984; Øvreas and Torsvik; 1998). Initially, the makeup of soil community will obviously influence the organisms present on the plant material. However, in order to remain viable and compete for a new niche, these microbes must adapt to the new conditions created by the plant tissues/exudates, other resident microflora, as well as surrounding environmental factors. Microbes in direct contact with subterranean plant surfaces have different stresses to confront than those found on aerial surfaces. In general, the rhizosphere is considered a moist, nutrient rich ecosystem supporting a diverse microbial community with high cell numbers, whereas the phyllosphere is a less hospitable arena subjected to rapid temperature and moisture fluctuations, ultraviolet radiation, and limitations in available nutrients (Lindow and Brandl, 2003). Since it is virtually impossible to remove all contaminants from the surfaces of fruits and vegetables, RTE produce can contain fairly high levels of bacteria ( $10^5$ - $10^7$  CFU/g). This is especially true for broad leaf commodities such as lettuce and spinach which provide large surface areas for the capture of debris and microbial contaminants. Often the predominant inhabitants of these surfaces are Gram-negative rods such as *Pseudomonas*, *Enterobacter* and *Erwinia* and, present to a lesser degree, are a broad range of yeasts and moulds (Francis et al., 1999). The sharp contrast between the

ecologies of the phyllosphere and rhizosphere can be epitomized by the fact that dominant root colonizing genera such as *Rhizobium* and *Azospirillum* cannot become established on leaf surfaces (Lindow and Brandl, 2003).

The levels and diversity of microflora on a given surface will ultimately depend on the original organisms present, handling and processing practices, as well as the storage temperature and gas regimes in modified atmosphere packaged (MAP) RTE fruits and vegetables. For example, if storage temperatures were to rise during MAP storage along with the reduction of O<sub>2</sub> levels in the package, a general shift in population from pseudomonads being the most common microflora to lactic acid bacteria could be expected (Garg et al., 1990; Brackett, 1994; Ahvenainen, 1995; Manzano et al., 1995). Furthermore, it is not uncommon for total bacterial counts on some food commodities to increase following processing. Recently, Johnston et al. (2005) conducted a survey of the microbiological quality of fresh produce in the southern United States. This study involved the collection of 398 produce samples (leafy greens, herbs and cantaloupe) from processing lines and packing sheds, and the enumeration of bacterial populations via traditional culturing and rapid identification methods. All leafy greens and herbs sampled gave total aerobic bacteria counts ranging from 4.5 to 6.2 log CFU/g, whereas counts for coliforms and enterococci were more variable (1 to 4.3 log CFU/g). Although the total microbial numbers found on cantaloupe did not increase substantially from the field through to the end of processing (aerobic plate counts were 6.4 and 7.0 log CFU/g at beginning and end, respectively), coliforms increased from 2.1 to 4.3 log CFU/g; *Enterococcus* spp., from 3.5 to 5.2 log CFU/g; and *E. coli* rose from less than 1 to 2.5 log

CFU/g. However, the frequency of pathogen isolation for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. was 0.7, 0 and 0%, respectively, emphasizing the fact that the detection of certain foodborne pathogens is a rare occurrence on these surfaces. The presence of spoilage organisms on minimally processed fruits and vegetables may aid in the suppression of growth of pathogenic strains. Francis and O'Beirne (1998) found that interactions with the resident microbial population decreased the numbers of *L. innocua* on lettuce. Since, it is common to find certain microbial communities associated with specific types of produce, some may be more effective in the deterrence of pathogenic species from establishing dangerous numbers. This may explain the relative infrequency of these pathogens isolated from fruits and vegetables in many surveillance studies (Babic et al., 1996; Lin et al., 1996; Sagoo et al., 2001; Johannessen et al., 2002; Johnston et al., 2005).

Nevertheless, the growing number of incidents of foodborne illnesses implicating minimally processed RTE fruits and vegetables has certainly left no question pertaining to the ability of pathogens to survive and proliferate in these foods. Bacteria belonging to the genera *Aeromonas*, *Listeria*, *Clostridium*, *Bacillus* and *Yersinia* are generally considered to be indigenous environmental microorganisms commonly isolated from water and soil samples (De Roever, 1999). Therefore, it should not come as a surprise that uncooked fruit and vegetables can act as vehicles for their dissemination (Beuchat, 1996; Beuchat, 2002). Consequently, development of any RTE product should give careful consideration to conditions that will allow these bacteria to propagate. For example, the shelf-life extension resulting from use of modified atmospheres can also

promote the growth of pathogens by giving them more time to proliferate. Of particular concern is the risk for growth and/or germination of *Clostridium botulinum* spores. However, Lilly et al. (1996) has reported that the incidence of *C. botulinum* contamination in MAP vegetables is quite rare.

In contrast, some studies have shown that *L. monocytogenes* can be frequently isolated from certain fruits and vegetables. Heisick et al. (1989) reported *L. monocytogenes* to be present in 28 of 132 (21%) and 25 of 68 (37%) intact potato and radish samples, respectively, taken from supermarkets in Minneapolis, MN. In another study involving cabbage farms and processing facilities in Texas, Prazak et al. (2002) isolated *L. monocytogenes* from 26 of 855 samples (3%) taken from produce, wash water and environmental sponges from contact areas. Moreover, the incidence of this bacterium has been reported as high as 44% (11 of 25 samples) for RTE cut vegetables in the Netherlands (Beckers et al., 1989). The level of contamination in this case was less than 200 CFU/g. However, because of the unique growth characteristics possessed by *Listeria* (refer to section 2.3), storage conditions may provide a competitive edge for multiplication (Beuchat, 1996). It should, however, be pointed out that not all strains of *L. monocytogenes* are virulent hence their mere presence does not always pose a definite risk (Kathariou, 2002). This low incidence of strain pathogenesis is not only restricted to *L. monocytogenes*, but is also true for the Gram-negative rod *Yersinia enterocolitica*. Although this organism has been isolated from a wide range of ecological niches, including various produce (3-5% of samples), most of these strains are considered to be avirulent (Francis et al., 1999).

Non-indigenous pathogenic microorganisms have also been found on fruits and vegetables. These microbes are generally considered to be common inhabitants of the gastrointestinal tracts of man and animals. Therefore, their presence indicates that the produce has come into contact with fecal material in the environment or through poor hygiene of food handlers. The non-indigenous pathogenic bacteria that have been found on produce include: the verotoxin producing *Escherichia coli* O157:H7, *Shigella* spp., and various *Salmonella* spp. (Doyle, 1997). Although direct contact through the use of poorly composted manures had always been considered to be the most probable route of contamination, studies have clearly demonstrated that *E. coli* O157:H7 can persist in water for at least 91 days at 8°C (Wang and Doyle, 1998). This certainly brings the question of current irrigation practices to light since many growers utilize natural waterways or dug-out ponds as their untreated source water. Direct defecation by livestock or feral animals into water systems or sewage runoff may result in high levels of contaminants in irrigation water.

High levels of contamination with *Salmonellae* have also been found on various types of fresh vegetables. In one particular Italian study, it was revealed that 72% of the total lettuce samples were contaminated with *Salmonella* spp. (Francis et al., 1999). Moreover, it has been found that this bacterium is capable of surviving on bean sprouts for up to 10 days when stored at refrigeration temperatures (Jaquette et al., 1996). Since *Salmonella* are exclusively associated with fecal matter, sewage and the intestinal tract of numerous animal species, these findings illustrate the importance of safe on farm practices and good hygiene when handling produce. The fact that common intestinal

flora microorganisms such as *Salmonella* and *E. coli* are found on fruits and vegetables imply that presence of other dwellers of the digestive tract is also quite possible. It has only been a relatively recent discovery that *Campylobacter* spp., as a significant foodborne bacterial pathogen, is disseminated through many foodstuffs of animal origin. There have been few reports linking these bacteria to produce; however, the frequent high levels of these bacteria encountered in manure and sewage, as well as their ability to survive in water, suggests a potential risk (Francis et al., 1999; Cools et al., 2003). Others have suggested that *Campylobacter* spp. are too fragile to survive for long periods on plant materials due to their susceptibility to desiccation and narrow temperature range for growth (Solomon et al., 1999). Clearly much more exploration in this area is required since the inability to isolate these bacteria can purely be attributed to the lack of suitable culturing methods or the reversion of organisms to a viable but non-culturable state (VBNC) (Chaveerach et al., 2003).

### **2.3    *Listeria monocytogenes* and RTE fruits and vegetables.**

The bacterial genus *Listeria* currently contains 6 distinct species. However, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic with the former species being the most prominent human pathogen. These short, rod-shaped Gram-positive bacteria are largely saprophytic, often found associated with decaying vegetation (Seeliger and Jones, 1986; Meng and Doyle, 1997). Although their pathogenesis is primarily opportunistic, they are capable of causing severe invasive illnesses in both



humans and animals (Vázquez-Boland, 2001; Kathariou, 2002). The emergence of *L. monocytogenes* as a true foodborne pathogen is a fairly recent event, even though it had been recognized as a human or animal pathogen since the late 1920's (Murray et al., 1926). Although food had been suggested as early as 1927 as a potential vehicle for its dissemination, it wasn't until 1981 that convincing evidence was finally established to make this link. In this outbreak, the deadly potential of *L. monocytogenes* was experienced in a hospital in Halifax, NS, Canada (Schlech et al., 1983). A total of 41 total cases were documented. Specifically, there were 34 perinatal cases resulting in 9 stillbirths, 23 neonatal cases with a 27% mortality rate and 2 births of healthy infants. There was also a 29% mortality rate in the adult cases. The source was later identified as contaminated cabbage from a farm in Prince Edward Island. The farmer had used improperly composted sheep manure to fertilize his crops and listeriosis had previously been identified in the flock. The cabbage was further processed in Nova Scotia to make coleslaw which was eventually served to patients in the hospital.

Although cases of food related illnesses due to *L. monocytogenes* are far fewer than those reported for the leading bacterial foodborne pathogens (i.e., *Campylobacter* spp. and *Salmonella* spp.), it is the high mortality rate for victims infected with this bacterium that has warranted the extensive attention of health regulatory authorities and researchers. It has been reported that *L. monocytogenes* is responsible for approximately one fourth of all food-related-deaths caused by known pathogens in the United States each year (Mead et al., 1999). The individuals most at risk include pregnant women, neonates, the immuno-compromised and the elderly. The serious clinical nature of

listeriosis has prompted the FDA/USDA in the United States to adopt a 'zero tolerance' policy with regard to the detection of this pathogen in their RTE foods (Anonymous, 2002a). Like many other nations, Canada is only slightly less stringent as no foods may contain in excess of 100 CFU/g. However, for those RTE foods with a refrigerated shelf-life greater than 10 days, that have been demonstrated to support the growth of *L. monocytogenes*, the present attitude of regulatory authorities continues to be a 'zero tolerance' (Anonymous, 2001; Anonymous, 2007a). Although, *L. monocytogenes* has been isolated from most food types, the popular ready-to-eat foods with extended shelf life capabilities are at greatest risk. This is due to certain unique growth attributes of the organism such as its ability to grow at refrigeration temperatures, high salt concentrations (up to 10% NaCl), and low oxygen requirements (Beuchat, 1996; Ryser and Marth, 1999). During long term refrigerated storage of certain foods, these bacteria can increase in numbers by several log cycles. Past research has demonstrated that prepackaged ready-to-eat vegetable salads can harbour *L. monocytogenes* and the population can double in as little as four days when stored at 4°C (Beuchat, 1996). Farber et al. (1998) also demonstrated that *L. monocytogenes* can proliferate rapidly in a variety of packaged fresh cut vegetables or salad mixes at different refrigeration temperatures (4 and 10°C). Moreover, the authors also demonstrated the rapid growth when products were temperature abused at 25°C for short time periods (1-2 days) followed by refrigeration.

*Listeria monocytogenes* has been routinely isolated from many environmental samples including water, sewage, soils, and plant vegetation, as well as the feces of many animal species and humans (Botzler et al., 1974; Dowe et al., 1997; Ryser and Marth,

1999). Due to the ubiquity of this bacterium in nature, coupled with common agricultural practices such as the use of manure fertilizers, composted plant materials, and irrigation water from ponds or natural water systems, it is therefore not surprising that the relative frequency with which *L. monocytogenes* has been isolated from many food commodities is significant (Beuchat et al., 1996). The widespread occurrence of *L. monocytogenes* implies that most individuals have likely been exposed through the ingestion of a contaminated food source. With such a high exposure rate, it is intriguing that relatively few outbreaks occur. This suggests that several factors must be involved in order to cause human disease: 1) infective dosage must be very high; 2) most healthy individuals have a natural underlying resistance to this organism; and 3) few strains possess virulence factors for pathogenicity in humans as only 3 of the 13 known serotypes of *L. monocytogenes* (serotypes 1/2a, 1/2b and 4b) are responsible for most human listeriosis cases (Kathariou, 2002). Moreover, recent research has demonstrated that subtypes of *L. monocytogenes* can be classified into one of three lineages based on ribotyping of the rRNA operon and allelic typing of the virulence genes *hlyA* and *actA* (Wiedmann et al., 1997). It has been shown that human outbreak strains of *L. monocytogenes* generally classify into lineage I group, sporadic case isolates fall into both lineage I and II, while non-human strains belong to lineage III. Other, highly discriminatory molecular based typing methods such as pulsed-field gel electrophoresis (PFGE), phage typing and multilocus enzyme electrophoresis have also led to similar results (Nadon et al., 2001; Graves et al., 1994; Graves et al., 1995).

Although *L. monocytogenes* has garnered a significant amount of attention from both clinical and food researchers, the bulk of information from studies pertaining to food safety have come from research on foods of non-plant origin. This may be due in part to the fact that documented outbreaks linking *L. monocytogenes* to produce are rare (Schlech et al., 1983; Aureli et al., 2000). However, *L. monocytogenes* does raise concern among commodity producers since it is frequently found in a wide range of fruits and vegetables (Francis et al., 1999). Moreover, numerous studies have demonstrated that minimally processed vegetables support the proliferation of this psychrotrophic pathogen once it becomes established on the food surface (Carlin and Nguyen-The, 1994; Carlin et al., 1995; Farber et al., 1998; Castillejo-Rodriguez et al., 2000; Li et al., 2004; Corbo et al., 2005). Therefore, research in the area of food safety of produce has focussed mainly on three general themes: 1) attachment to plant surfaces, 2) detection and surveillance, and 3) survival and/or growth on post-harvest produce under various storage conditions. However, a few studies have dealt with the growth of this pathogen on actively growing plant material. For example, Jablasone et al. (2005) recently investigated the behaviour of *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* Typhimurium on several varieties of germinating seeds (i.e., carrot, cress, lettuce, radish, spinach and tomato). Results showed that *L. monocytogenes* was the most persistent of the pathogens establishing the highest numbers of all three pathogens throughout the duration of the growth cycle (9 days). Moreover, although the other two pathogens were able to internalize into plant tissue (plant species dependent), *Listeria* did not appear to have this ability on any of the tested seeds types.

Contamination of produce by *L. monocytogenes* may occur at any point throughout the food continuum through incidental contact with the organism. Therefore, in order to develop effective strategies to minimize the risk of foodborne disease caused by this organism, it is essential to understand the initial stages of bacterial attachment to various plant tissues. In spite of this, there have been only a few studies conducted on the attachment of *L. monocytogenes* to plant surfaces. However, this research has demonstrated that *L. monocytogenes* is capable of binding rapidly to a variety of plant surfaces with preferential attachment to cut surfaces (Takeuchi et al., 2000; Garrood et al., 2004; Ells and Truelstrup Hansen, 2006). Moreover, there is a wide range of attachment ability among *Listeria* strains and attachment appears to be highly dependent on temperature (Gorski et al., 2003; Gorski et al., 2004; Ells and Truelstrup Hansen, 2006). A recent study using differential display analysis of RNA transcripts during the attachment and growth of *L. monocytogenes* on cut cabbage revealed several genes that were up-regulated (Palumbo et al., 2005). Many of these were typed to open reading frames encoding for predicted proteins with no known function. However, several genes with possible roles in the metabolism of carbohydrates, amino acids and nucleic acids were identified. In addition, genes involved in motility, cell division, and transport were also up-regulated. Although the full significance of the results from this study has yet to be established, the authors noted that several classes of the genes identified in their work were also shown to be important for colonization of legumes by typical plant colonizers such as *Pseudomonas* spp. (Lugtenberg et al., 2001). This data may begin to explain the success of *L. monocytogenes* as saprophyte.

Once established on a plant's surface, *Listeria* may increase significantly in numbers if conditions become permissive for growth. Otherwise, these bacteria can remain intact for long periods of time as passengers on produce, hence allowing for their transfer and contact with other surfaces. Therefore, ample opportunity exists for long term persistence in the vegetable processing continuum. Prazak et al. (2002) observed that approximately 3% of samples taken from various areas in the cabbage processing regime were positive for *L. monocytogenes*. These included samples taken from the vegetables themselves as well as conveyors within the plants. Molecular diagnostics suggested a common origin for these strains. Other large scale surveillance studies such as that conducted by the USDA involving 1000 samples from a multitude of vegetable products have demonstrated high incidence of *L. monocytogenes* in selected vegetables (Heisick et al., 1989). The frequency of positive isolates for potatoes and radishes in this study was 26% and 30%, respectively. This may not be surprising since these sub-surface vegetables grow in direct contact with the soil.

Other studies have concentrated on the survival or proliferation of *Listeria* once applied to a plant surface. Such studies have often included different stress parameters important to the preservation of the food product used, or were concentrated on produce that may have inhibitory attributes. Most recently, these studies have concentrated on fruit commodities. For example, Penteado and Leitão (2004) examined the ability of *L. monocytogenes* to survive or grow on melon, watermelon and papaya pulps at several different temperatures in order to determine whether or not these moderately low acid foods could prevent cell multiplication. Results showed that *Listeria* was able to grow in

all pulps at all temperatures tested therefore demonstrating the versatile nature of this bacterium. Similar trends were also published for *L. monocytogenes* inoculated on fresh sliced pears stored with and without modified atmospheres (Corbo et al., 2005). Conversely, Flessa and co-workers (2005) found that *L. monocytogenes* strains used in their study could not grow on fresh whole or cut strawberries. Interestingly, cell numbers decreased by about 3 log cycles on intact berries irrespective of storage temperatures (4 or 24°C). However, numbers on cut berries did not decrease substantially for up to 7 days when stored at 4°C. The low pH (3.6 to 3.8) of strawberries is well below the minimum (pH 4.6) required to support the growth of *L. monocytogenes*. The authors speculated that the reason for the rapid reduction in cell numbers on intact berries may be due to desiccation and the lack of available nutrients required for maintaining vital cell functions. Although, *L. monocytogenes* could not grow on the berries, this study clearly demonstrated that this pathogen could survive for a period that was typical for the expected shelf life of strawberries. *Listeria* cells frozen with the berries also showed minimal reductions further emphasizing this organism's ability to survive.

A number of studies have concentrated on the survival/growth of *Listeria* on vegetables subjected to a stress condition. Often chlorine or mild acid dips, heat or modified atmospheres or other antimicrobials are implemented to deter the growth of this pathogen. However, conflicting data have often resulted making it impossible to generalize about the behaviour of *Listeria* hence deterring predicted outcomes (Koseki and Isobe, 2005). One of the major factors involved in these anomalies is the *Listeria* strain used. Francis and O.Beirne (2005) found significant differences ( $P < 0.05$ ) in

survival and growth among strains when examining the effect of acid, heat and antibiotic treatments prior to their inoculation onto lettuce and coleslaw mix (80% shredded cabbage and 20% carrot). In order to understand the ability of these strains to initiate a different stress response, which ultimately provides a competitive advantage for that strain under a given set of environmental factors, information about the bacterium is required at the molecular level. In recent years the advent of modern molecular techniques has provided researchers with the complete genetic codes for numerous bacterial species including *L. monocytogenes* (Glaser et al., 2001). This information has been crucial for the advancement of knowledge of how bacteria adapt to stress.

## **2.4 Bacterial Stress Response**

### **2.4.1 Overview**

There is a widespread perception that “stress” results from an environmental stimulus that alters the bacterial cell’s “normal” metabolic state. However, there is a general bias as to what this really means since we have become accustomed to benchmarks which too often regard the activities of bacteria under optimal conditions in laboratory growth media as “normal”. Hence, it is easy to conclude that deviations in behaviour outside the realm of optimal are abnormal and place stress on the cell. Booth (1999) has proposed a more generalized definition in that “stress” should be viewed as any alteration in genome, proteome, or environment that results in either a change in



growth rate or survival potential. To elaborate on this point it may be stated that any condition that forces a cell to augment metabolism for survival or growth may be regarded as stress. It should be noted that all conditions of metabolism are considered in this very broad definition including transcription, translation and post-translational modification. Therefore, genetic mutations may be included as stress factors. Moreover, it has been postulated that under persistent stress conditions where the classical stress response is under-expressed, forced adaptive mutations can occur (Moxon et al., 1994).

With this definition in mind, it is obvious that bacteria are remarkably adaptive organisms especially suited to adjust to relatively small changes in their surrounding environment. Minor fluctuations in temperature, pH, osmolarity, redox potential, water activity, pressure, etc., are usually tolerated as being only slightly stressful thus making minor compromises with respect to metabolism or to cell integrity. However, pushing these parameters to more extreme but sub-lethal levels leads to a so-called stress response by the cell. It stands to reason that the cell enters a mode that is focussed on survival rather than proliferation. In considering protective actions taken by the bacterial cell, there are three obvious components that stand out as being vital to cell integrity: 1) an intact cell membrane; 2) maintenance of native state of proteins; and 3) DNA stability. Damage to other cellular components is also important for survival; but, the regeneration of such structures is only possible through conservation of the aforementioned attributes (Booth, 2002).

There has been an escalating interest among scientists over the past 10 years with regard to bacterial stress and adaptation. Initially, the main reason for this intrigue is

likely to have been prompted by the alarming rate at which antibiotic-resistant strains of pathogenic bacteria have emerged in hospitals in every corner of the world. However, many other disciplines have now gained an interest in the area of microbial stress adaptation, including the fields of bioremediation, industrial microbiology and bioengineering. In recent years, it has perhaps been research in the area of food safety that has contributed to our current knowledge base of the bacterial stress response. Much of this work has involved collaborative efforts between food scientists and clinical microbiologists in an attempt to unravel the impact of stress response on the ecology, transmission and pathogenesis of foodborne bacteria (Dodd and Aldsworth, 2002; Hill et al., 2002; Streips, 2002).

The food processing environment provides an intricate stage in which bacteria are exposed to a vast array of stressful conditions purposely implemented to retard cellular activity or result in their complete destruction. In many cases, a single lethal stress (e.g., extreme heat) is often utilized to destroy potential pathogens. However, with public demand for less processed RTE foods, the shift to less rigorous treatments in recent years has left processors with the challenge of implementing multiple minimal “hurdles”, sequentially or in combination to provide a similarly lethal cumulative effect. The theory being that the relative small number of so-called resistant strains that could overcome the first hurdle would be weakened and succumb to subsequent obstacles in the process. However, information contrary to this belief has been mounting with respect to the stress response for many foodborne bacteria. It has been demonstrated that many bacteria can become “conditioned” to resist a certain environmental stress. That is to say, bacteria

exposed to a sub-lethal stress parameter undergo cellular alterations that induce greater survival potential at more extreme, normally lethal, levels of the imposed stress. Gahan et al. (1996) demonstrated the increased tolerance of acid adapted *L. monocytogenes* cells in acid foods where cells preconditioned at pH 5.5 could tolerate extreme acidic conditions (pH 3.5); a level that rapidly deactivates cells cultivated at pH 7.0. The fact that so many foods rely on low pH to curtail the growth of food pathogens brings into question the true effectiveness of such inimical barriers for the control of *L. monocytogenes* in certain food commodities. Other studies showing similar trends include increased thermotolerance in heat shocked *L. monocytogenes* and *Salmonella* Typhimurium (Bunning et al., 1990; Farber and Brown, 1990) as well as increased osmotic tolerance (Galinski, 1995). Interestingly, Lundén et al. (2003) tested stress adaptation of *L. monocytogenes* exposed to sub-lethal doses of several disinfectants (i.e., two quaternary ammonium compounds, one tertiary alkylamine compound, sodium hypochlorite and potassium persulphate). Increased tolerance was observed for all disinfectants ranging from a 3 to 15 fold increase in minimum inhibitory concentration (MIC) depending on the compound and temperature. This implies adaptation by either an increase in detoxifying cellular components or greater activity in the transport systems responsible for the export of deleterious chemicals out of the cell.

The “adaptive stress response” is not just restricted to increased tolerances to the environmental condition responsible for inducing the initial response. Cross-adaptation against other stresses has also been clearly demonstrated (Leyer and Johnson, 1992; Farber and Pagotto, 1992; Rowe and Kirk, 1999). The consequence of such cross

protective responses by bacteria pertinent to the food industry is of great concern. The initial hurdles implemented in minimally processed foods, that are intended to weaken potential pathogenic bacteria, may in fact harden cells affording them the capacity to withstand other hurdles downstream in the process. In light of this information it is evident that the food processor must seek new strategies to provide wholesome and safe products.

#### **2.4.2 Cross-adaptation and virulence**

It is interesting to consider the challenges a bacterial foodborne pathogen must undergo in order to survive rigors imposed by the host organism. Exposure to the extreme acidic conditions of the stomach must be withstood in order to reach the intestinal tract where a foreign bacterial cell is greeted by inhospitable bile salts and the competitive residential intestinal microflora. In addition, the armory of natural host defences comes into play and phagocytosis is possible when encountered by host macrophages. It is indeed remarkable that such organisms can establish the necessary levels to cause disease in susceptible hosts, or that they can compete and survive harmlessly in carrier organisms as does *E. coli* O157:H7 in the bovine intestinal tract (Gansheroff and O'Brien, 2000). Equally astounding is the ability of these bacteria to quickly adapt to a saprophytic lifestyle when shed from their host's intestinal environment. In light of the accumulating information regarding the bacterial stress response, it is perhaps not that surprising that a growing number of studies demonstrate

that stress adapted cells may actually have a greater potential to cause disease in experimental animal models (Archer, 1996; O'Driscoll et al., 1996; Wiedmann et al., 1998). Marron et al. (1996) provided compelling evidence that stress response and virulence are closely linked. This work compared the ability of acid tolerant and acid sensitive mutants of *L. monocytogenes* to infect mice. Their results showed an inability of the latter to cause disease. Certainly one can envisage the possible role acid adaptation may play in conditioning a potential pathogen since these cells are likely better suited to survive the low pH of gastric juices or the acidified phagosome of a macrophage.

The interconnection between known stress genes and virulence has been demonstrated by several researchers. It has been shown that the stress response mediator, ClpC, is negatively controlled at the level of transcription by the central virulence regulator, PrfA in *L. monocytogenes* (Ripio et al., 1998). ClpC is a molecular chaperone that is expressed maximally at elevated temperatures (42°C) but only marginally at 37°C. Using a *prfA* mutant that constitutively over-expresses the virulence regulator (and consequently related virulence genes), the authors found reduced levels of *clpC* expression in exponential cells grown at 42°C. However, stationary cells demonstrated the same high level of *clpC* expression at 42°C indicating the regulation of *clpC* is growth phase-dependent. In addition, the mutant also demonstrated increased *clpC* transcription at 37°C to levels near those for wild-type cells grown at 42°C. In a separate study, it was later demonstrated that a single codon deletion in a region coding for a conserved glycine repeat in the class III stress regulator, CtsR, was responsible for the piezotolerant, thermotolerant, hydrogen peroxide resistant phenotype exhibited by a

spontaneous mutant of *L. monocytogenes* Scott A (Karatzas et al., 2003). The CtsR protein negatively regulates the transcription of genes in the *clp* operon, including *clpC*. Therefore, reduced levels or loss of CtsR activity, result in the increased expression of ClpP and ClpC proteins which may play a role in the degradation of misfolded proteins, resulting in the stress tolerant phenotype. The loss of repressor function exhibited by the mutant also resulted in a reduction in virulence. Other experiments involving virulence factors under the control of PrfA, such as the production of listeriolysin O toxin, have also shown up-regulation after mild heat shock (Sampathkumar et al., 1999). These experiments strongly suggest a relationship between the regulation of virulence genes and environmental stressors. Recently, a homologue of the cytosolic chaperone trigger factor of *B. subtilis* has been described in *L. monocytogenes* (Bigot et al., 2006). By constructing a deletion mutant in the gene for this homologue,  $\Delta tig$ , it was shown that the trigger factor was required for the survival of *L. monocytogenes* when exposed to heat stress (50°C) or for growth in the presence of 3% ethanol. Additionally, the  $\Delta tig$  mutant could not survive in the spleen or liver of infected mice (1000-fold reduction after 7 days post infection), although the cells multiplied when introduced into macrophages and HepG-2 and Caco-2 cell lines. The authors noted that there was no detectable change in protein expression as viewed by 1-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, hence they pondered the possibility that the trigger factor influenced weakly expressed proteins. However, answers to this question may be better provided using 2-dimensional gel electrophoresis (2D-GE) to better resolve such proteins

according to both isoelectric points (isoelectric focussing) and molecular sizes (SDS-PAGE).

The inverse effect of stress on virulence has also been reported. Cappelletti et al. (2004) induced a viable but non-culturable (VBNC) state in four strains of *L. monocytogenes* through prolonged incubation in a water microcosm. VBNC *Listeria* was found not to be infective in mouse models or the cell lines used in the study. Although, metabolically active VBNC cells could be detected in the spleens of mice, *Listeria* could not be cultured from the tissues. This indicates that the extreme starvation conditions induced a state that may not be reversed within a host system.

### **2.4.3 Stress Adaptation Mechanisms**

Under any set of environmental conditions the cell responds with the production of a subset of proteins necessary for growth and/or survival. This can be readily visualized by 2D-GE. Upon changing a stimulus, such as temperature, pH, available nutrients, osmolarity, etc., a different subset of proteins is normally expressed. Some of these proteins may be universally present regardless of the stimuli. However, it has been demonstrated that many others are specific to the type of stimuli. For example, under extreme stress conditions where the focus of the cell has shifted to survival, the protein profile would be quite different than that of cells experiencing optimal growth conditions. Phan-Thanh and Mahouin (1999) used 2D-GE to demonstrate the induction of some 47 proteins in *L. monocytogenes* under acid shock conditions at pH 3.5. When

grown at pH 5.5, 37 proteins were induced, 23 of which were also found at the lower pH.

Other researchers also describe the apparent up or down-regulation of various proteins expressed under high salt conditions (Esvan et al., 2000). Duché and co-workers (2002) also found that some 40 proteins were either induced or repressed when *L.*

*monocytogenes* was grown in a chemically defined medium containing 3.5% NaCl.

Twelve of the highly induced proteins were identified by mass spectrometry or N-terminal sequencing and were found to be similar to the general stress proteins Ctc and DnaK, the transporter proteins, GbuA and IIAB (mannose specific phosphotransferase system enzyme) and general metabolism proteins, alanine dehydrogenase, CcpA, CysK, EF-Tu, Gap, GuaB, PdhA, and PdhD. Similar to the results from this study, many of the proteins in other studies involving other bacterial species have also been identified (Periago et al., 2002). However, for many of these proteins functional analysis is needed to uncover the specific roles that these proteins play in cell survival and stress adaptation. It is possible that some of these proteins may serve as regulators of sensory systems (e.g., two component transduction such as CesRK or LisRK), regulators of specific operons (e.g., RpoS in Gram-negative bacteria, SigB in Gram-positive), molecular chaperones (e.g., GroEL and DnaK), and inducible transporters (e.g., GAD system transporters) to name but a few known stress related protein functions (Kallipolitis et al., 2003; Moorhead and Dykes, 2003; Dodd and Aldsworth, 2002; Hanawa et al., 2002; Gahan et al., 2001).



#### **2.4.4 Two Component Transduction Systems**

In order to adapt to changing environmental conditions bacteria must be able to sense minute changes in their surroundings and respond almost instantaneously with the appropriate signals to initiate the necessary mechanisms to combat the hostile conditions and minimize cellular damage. The most common systems used by prokaryotes are the highly conserved two component signal transduction systems. The function of these systems is to convert an extracellular stimulus into a chemical signal (i.e., phosphoryl group) that the cell can recognize and react to via the chemical modification of proteins. In general, these systems consist of a membrane-associated sensor (histidine kinase) and a corresponding cytoplasmic protein called the response regulator (Fabret et al., 1999). The histidine kinase sensor protein contains two domains; a separable sensor and a histidine phosphotransferase domain. The sensor acts to bind a specific recognized attractant (stimulus) which results in the autophosphorylation of a histidine residue on the phosphotransferase domain. The phosphoryl group is subsequently passed through the cytoplasmic membrane via a cascade of chemical relays until it reaches the response regulator protein where it is retained as an acyl phosphate of an aspartic acid residue. In addition to the phosphorylated aspartate domain, the regulator protein also contains an output domain. Depending on the system, in order to process or regulate a signal, the output domain normally involves conformational changes to molecules or charge interactions (phosphoryl group). The end result of the process is often the transcription activation of a subset of genes (Fabret et al., 1999). At least 30 and 34 two-component

regulatory systems have been found in *E. coli* and *B. subtilis*, respectively, each system demonstrating remarkable specificity for an applied stimulus (Bayles and Fujimoto, 2002). Two component systems important for virulence in pathogenic bacteria have also been identified and include: BvgAS in *Bordetella pertussis* (Uhl and Miller, 1996); FlgS/FlgR in *Campylobacter jejuni* (Wösten et al., 2004); MprAB in *Mycobacterium tuberculosis* (Zahrt et al., 2003); PhoP/PhoQ in *Salmonella Typhimurium* (Garcia-Vescovi et al., 1994); and RssA/RssB in *Serratia marcescens* (Wei et al., 2005), to name but a few characterized systems. Still, more of these systems are likely to be characterized since the data bases of whole genomes for many important microorganisms are constantly growing, along with the development of more sophisticated molecular techniques and powerful computer software for analyzing data. This is evident in view of the work of Kallipolitis and Ingmer (2001) where a number of putative response regulators were identified in *L. monocytogenes* and their importance to virulence was also substantiated.

One of the best characterized two component systems is CheA/CheY, first shown to be involved in chemotaxis of *E. coli* and *Bacillus subtilis*. CheA possesses autophosphorylating kinase activity which increases due to the binding of target attractants to receptors that span the cytoplasmic membrane. The phosphate residues are transferred through the membrane where the CheA sensor passes them on to the CheY response regulator (CheY-P) which interacts with a complex that acts as a switch to activate a “motor” responsible for counter clockwise rotation of flagella. Hence, once induced cells exhibit a swimming motion as opposed to tumbling which is the result of

the clockwise “default” rotation of the flagellum (Bischoff and Ordal, 1991). Insertional inactivation of the homologue of this operon has been shown to reduce the number of flagella displayed by *L. monocytogenes* (Flanary et al., 1999). Moreover, loss of flagella and CheYA sensing also rendered the cells incapable of responding to oxygen gradients and impaired their ability to attach to mouse fibroblast cell lines. Recently, the importance of the *Listeria* CheA/CheY homologue in virulence was elucidated (Dons et al., 2004). Results in this study suggested that flagella and CheA/CheY regulated polypeptides play a role in the initiation of tumour necrosis secretion factor which ultimately provides protection against infection.

The most thoroughly studied two component system in *L. monocytogenes* is the LisRK system, containing the LisR (regulator) and LisK (sensor). Cotter et al. (1999) first identified the two genes, *lisR* and *lisK*, that encode the two component proteins and subsequently demonstrated the important role this system plays in virulence of *L. monocytogenes* since a *lisK* mutant was *ca.* 10-fold less virulent than the wild type LO28. Moreover, key observations were also made in terms of sensitivity to acid stress. It was demonstrated that acid resistance/sensitivity in the  $\Delta lisK$  mutant of strain LO28 was dependent on growth phase. During stationary phase growth, the mutant was much more resistant to acid stress (pH 3.5) than the parent strain. However, when early exponential phase cells were used the opposite result was observed. The authors speculated that early stage logarithmic cells lack the signal to induce the acid response. But in light of the resistance of stationary phase cells, this assumption would mean a separate system, which overcompensates for acid tolerance, is activated at a latter stage

(Cotter et al., 1999). This study also showed that the mutant grew in the presence of ethanol at levels that were bacteriostatic to the wild type. Since ethanol is thought to weaken membrane integrity, it is thought that the action of products regulated by LisRK must be on the cell envelope (Cotter et al., 1999). The absence of LisRK regulation of certain products may result in changes to the composition of the cell membrane, hence making it less susceptible to ethanol. For example LisRK may be involved in signalling for increasing and lowering the content of membrane unsaturated fatty acids (UFA). Cells growing in the presence of sub-lethal concentrations of ethanol have been shown to have significant increases in membrane UFA (Dombek and Ingram, 1984). Therefore disrupting the histidine kinase sensor component of the LisRK transduction system may result in a phenotype with a higher UFA membrane content than the wild type strain. It would be interesting to evaluate UFA levels in this mutant. A later study, using this same  $\Delta lisK$  mutant also suggested a membrane-related role for LisRK since it was shown that this system influenced the sensitivity of *L. monocytogenes* LO28 to the lantibiotic, nisin, and antibiotics belonging to the cephalosporin family (Cotter et al., 2002). The mutant exhibited an enhanced resistance to nisin but was much more sensitive to cephalosporins. The antimicrobial activity of these agents is manifested through interactions with the cytoplasmic membrane.

Interestingly, the importance of LisRK for osmotolerance in *L. monocytogenes* was also revealed in a recent study. Sleator and Hill (2005) observed a highly reduced rate of growth in BHI supplement with 8% NaCl for the LO28 $\Delta lisK$  mutant relative to the parent strain. This system was shown to function independently of the well defined

primary ( $K^+$  uptake) and secondary systems (accumulation of osmolytes) that normally confer osmotolerance in *L. monocytogenes* (see section 2.4.8). Moreover, it was determined that the transcription of the Class V heat shock protein, HtrA (see section 2.4.6) was down-regulated in LO28 $\Delta$ *lisK*. When the *htrA* promoter elements were replaced with the constitutively expressed P44 lactococcal promoter in the expression vector pNZ44, *htrA* transcription resumed. However, osmotolerance in the complemented strain was not completely restored to the level of LO28. The authors recognized that other cell components besides the regulation of HtrA were being controlled by LisRK and appeared to play a role in osmotolerance (Sleator and Hill, 2005).

CesRK is another two component signal transduction system important for virulence and stress response of *L. monocytogenes*; CesR being the response regulator and CesK the histidine kinase sensor. Kallipolitis et al. (2003) provided evidence that this system was necessary for increased tolerance to ethanol as well as antibiotics of the  $\beta$ -lactam family which act on the cell wall. The *cesR* and *cesK* genes are found as part of a five gene cluster (positions 3 and 4, respectively) in *L. monocytogenes* EGD-e and LO28 (Glaser et al., 2001; Kallipolitis et al., 2003). The two preceding genes (*lmo2424* and *lmo2423*) encode a 94 amino acid protein similar to a thioredoxin and a 291 amino acid protein homologous to the  $Co^{2+}$ ,  $Zn^{2+}$  and  $Cd^{2+}$  transporters of the cation diffusion facilitator family, respectively. The last gene of the cluster (*orf2420*) encodes a predicted 62 amino acid protein of unknown function. Through the use of deletion mutants for all genes in the cluster, deletions in *cesR*, *cesK*, or *orf2420* were demonstrated to provide

increased tolerance to ethanol (Kallipolitis et al., 2003). However, the opposite effect was observed for these mutants when subjected to a variety of antibiotics. Although there was no notable difference in response between wild type and the mutants for most antibiotics tested, there was a marked difference in sensitivity to  $\beta$ -lactam cell wall-acting antibiotics (e.g., penicillin G, ampicillin, and several cephalosporins) where  $\Delta cesR$  and  $\Delta cesK$  were more sensitive than the wild type. Nonetheless, it is obvious that the CesRK system plays a role in the sensing and response to these stimuli. Furthermore, the  $\Delta cesR$ ,  $\Delta cesK$ , and  $\Delta orf2420$  mutants were all less virulent than the parent strain. These results were strikingly similar to findings in the Cotter et al. (1999) study with a  $\Delta lisK$  mutant. Therefore, one can speculate that the CesRK system also acts at the membrane level in *L. monocytogenes*.

## 2.4.5 Global Regulatory Proteins

### 2.4.5.1 Sigma S: alternative stress sigma factor for enteric Gram-negative bacteria

Sigma S ( $\sigma^S$ ) is an alternative sigma factor to sigma A ( $\sigma^A$ ), the general sigma factor required to complete the RNA polymerase holoenzyme complex. The  $\sigma^S$  protein is encoded by the *rpoS* gene in enteric Gram-negative bacteria and is found at relative low levels during exponential growth; however, upon entry into the stationary phase the gene becomes maximally transcribed. In doing so,  $\sigma^S$  controls a regulon of at least 50 different genes that provide the necessary protection from the stresses imposed during this growth phase (Streips, 2002). Stationary phase cells exhibit increased tolerances to many

environmental conditions including osmotic shock, acid shock and starvation. Mutated cells unable to produce  $\sigma^S$  have characteristic elongated pleiotropic phenotypes with reduced growth rates and display sensitivity to UV light and osmotic stress (Nogueira and Springer, 2000). The importance of *rpoS* with regard to its expression in *Salmonella enterica* Typhimurium in various food systems has been elucidated (Dodd and Aldsworth, 2002). It has been shown, using *rpoS* gene fusions with a *lux* reporter system, that the activity of this system is immediately induced in foods imposing stress conditions (high sodium chloride or sucrose) on the cells. Conversely, in certain food systems that better mimic a laboratory growth medium (i.e., skimmed milk), induction only occurs after an initial exponential growth period (Aldsworth et al., 1998).

#### 2.4.5.2 Sigma B: the global stress sigma factor for Gram-positive bacteria

The equivalent alternative sigma factor to  $\sigma^S$  in Gram positive bacteria is  $\sigma^B$  (sigma B), the product of *sigB*. This alternative factor was originally identified in *B. subtilis*; however, it is now known that it is a general stationary phase transcription factor in Gram-positive bacteria including important foodborne pathogens such as *L. monocytogenes* and *S. aureus* (Haldenwang, 1995). Like  $\sigma^S$ ,  $\sigma^B$  is controlled at the transcriptional, translational and post-translational levels and its presence is induced at the end of logarithmic growth, during stress or when ATP levels are low.

The  $\sigma^B$  operon of *B. subtilis* consists of eight genes and its regulation is highly complex (Schumann et al., 2003). The entire octacistronic operon is transcribed at a low level during normal growth from a  $\sigma^A$ -type promoter,  $P_A$ ;  $\sigma^A$  also being the sigma factor

engaging the RNA polymerase core enzyme to form a holoenzyme for non-stress conditions. However, during stress  $\sigma^B$  itself, encoded by the seventh gene of the operon, *sigB*, influences the increased transcription of the last 4 genes in the operon from a second promoter site,  $P_B$ . The product of *rsbW* (6<sup>th</sup> gene in operon) has dual functions; firstly, it is an antisigma factor that sequesters  $\sigma^B$ , therefore preventing its interaction with the RNA polymerase core enzyme under non stress conditions. Secondly, the serine kinase activity of the RsbW protein phosphorylates RsbV, the product of *rsbV* (5<sup>th</sup> gene in operon), which acts as an anti-antisigma factor. Therefore, when phosphorylated, this RsbV-P product is inactivated allowing for the sequestering of  $\sigma^B$  by RsbW. However, during heat stress (or other stresses) the phosphate is cleaved from the RsbV by a phosphatase, encoded by either, *rsbP* or *rsbU*. The regulator RsbP is thought to be associated with energy related stress conditions (e.g., carbon, oxygen, phosphate starvation), whereas RsbU is responsive to environmental stresses (e.g., heat, acid, salt, desiccation, etc.). This frees RsbV to compete with RsbW for  $\sigma^B$ , thus releasing the latter to interact with the core RNA polymerase (RNAP) to form a holoenzyme and subsequently bind  $\sigma^B$ -dependent promoter sites (Schumann et al., 2003). Figure 1 shows a diagrammatic representation of the regulation of  $\sigma^B$ . The organization of the *sigB*



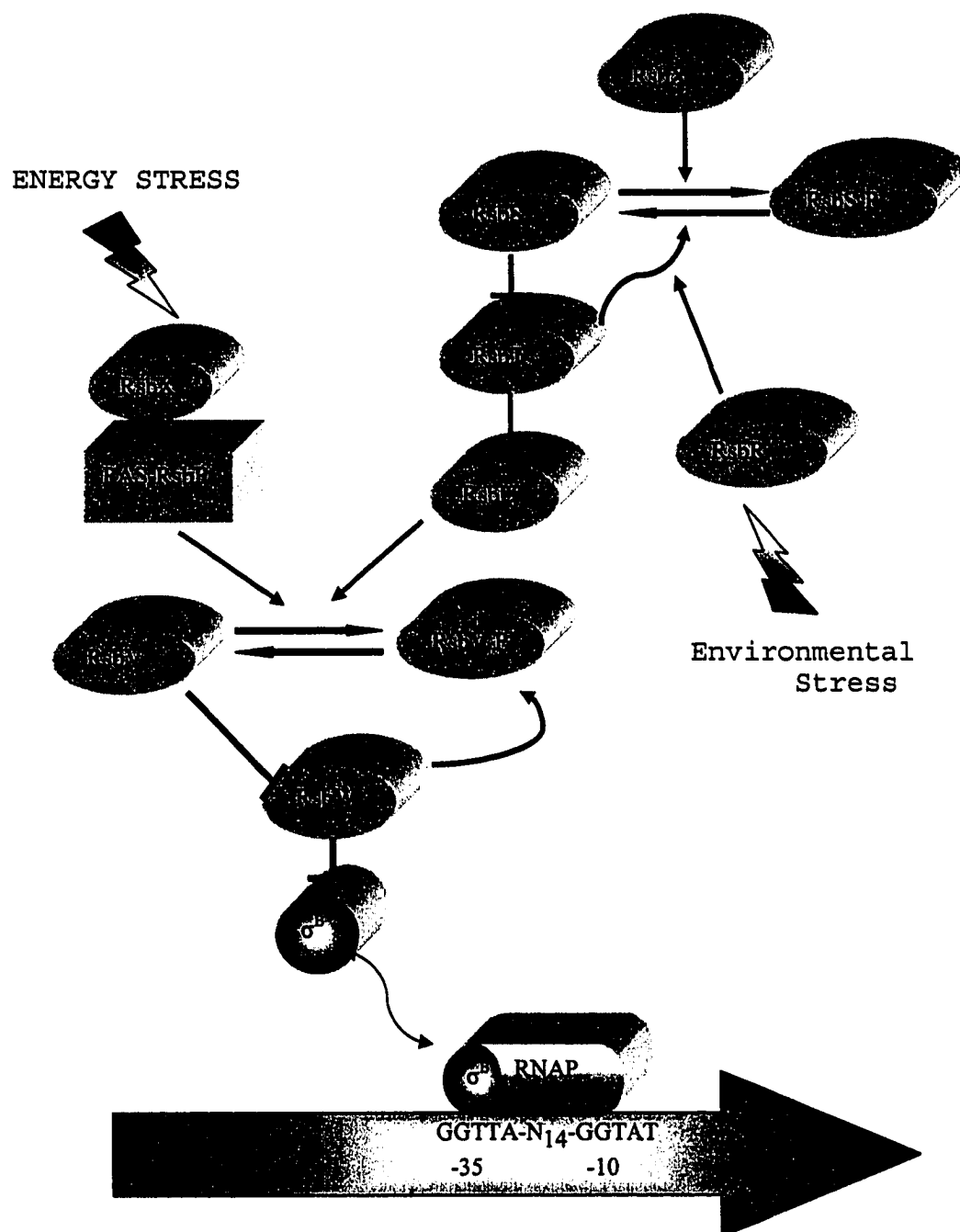


Figure 2.1. Diagram representing stress induced activation and regulation of the Sigma B ( $\sigma^B$ ) operon. See text for details. Rsb are regulators of  $\sigma^B$ ; RNAP is RNA polymerase; PAS is Per-Arnt-Sim domain. Adapted from Schumann, W., 2003. Cell Stress Chaperones. 8:207-217.

operon of *L. monocytogenes* was mapped by two separate groups of investigators and it was determined that the organization of the last five genes of the was identical to that of *B. subtilis* (Becker et al., 1998; Wiedmann et al., 1998). The subsequent publication of the first *L. monocytogenes* whole genome showed that the entire octacistronic operon resembled the *B. subtilis* counterpart (Glaser et al., 2001). Although, the *sigB* gene itself along with the regulatory genes *RsbS* and *RsbT* are highly similar among Gram-positive species, other *rsb* (regulators of *sigB*) genes are less conserved, and the organization of the operon is highly variable depending on bacterial species (Ferreira et al., 2004).

#### 2.4.5.3 The importance of $\sigma^B$ -dependent genes and virulence

Wiedmann et al. (1999) found that stationary phase cells from a *sigB* mutant displayed *ca.* 3 log reduction in the number of survivors relative to the wild type (strain 10403S) after 2 hours in BHI at pH 2.5. However, they found no significant difference ( $P > 0.05$ ) in virulence between the same mutant and wild type strain when assaying the number of *Listeria* present in the livers and spleens of infected mice. They concluded that although  $\sigma^B$ -dependent genes were required for optimal acid tolerance, they were not necessarily essential for host invasion. However, it was more recently demonstrated that known virulence genes are indeed dependent on  $\sigma^B$ -type promoters (Kazmierczak et al., 2003). In this study, a combination of computer and microarray design/analysis showed that 54 genes preceded by  $\sigma^B$ -promoter consensus sequences were transcribed at higher levels (>2-fold) in the parent EGD-e strain over the levels exhibited by its *SigB* mutant when cultures entered stationary phase growth or when subjected to osmotic stress (0.5M

KCl). In addition to known stress genes important for virulence (i.e., *gadB*), several internalin genes (*inlA*, *inlB*, *inlC2*, *inlD* and *inlE*) were transcribed at higher levels and were shown to harbour  $\sigma^B$ -promoter sequences. In particular, *inlC2* was transcribed at 11-fold greater levels in stationary phase cells of the wild type than in the mutant. However, a role in pathogenesis has yet to be determined for this particular internalin. Moreover, the difference in expression levels for the *bsh* gene (bile salt hydrolase) was *ca.* 11 and 20-fold for stationary phase and osmotically shocked cells, respectively. Although these results clearly demonstrate that the  $\sigma^B$  regulon encompasses genes important to virulence functions, their effect on survival or pathogenesis in the host must be more clearly elucidated.

#### 2.4.5.4 The importance of $\sigma^B$ for survival during acid stress

Ferreira and co-workers (2001) investigated the role of  $\sigma^B$  for acid tolerance in *L. monocytogenes* (stationary phase cells), as well as its part in resistance to heat, exposure to ethanol, oxidative stress and carbon starvation. Their results revealed no significant difference in the number of viable cells for wild type strain 10403S and a *SigB* mutant when exposed to lethal temperatures (50°C) or ethanol levels (16.5%). These observations also held true when the cultures were preconditioned by exposure to sublethal stress treatments (i.e., 45°C, 5% ethanol, or pH 4.5) prior to the lethal regimen. However, the *SigB* mutant was more sensitive to both oxidative stress and carbon starvation. Moreover, it also displayed extreme sensitivity to low pH (2.5 for 3h) with 10<sup>4</sup>-fold reduction in the number of survivors (CFU/mL). However, this difference was

only about 1000-fold if the cells were preconditioned for 1 h at pH 4.5 prior to the pH 2.5 treatment. Since the difference in sensitivity was reduced 10-fold following acid adaptation, this suggests that other factors independent of  $\sigma^B$  are involved in the adaptive acid tolerance response (ATR); the phenomenon whereby bacteria exhibit higher tolerance to lethal pH levels if previously conditioned by exposure to a low but non-lethal pH (see section 2.4.7). Continued work in this area examined growth phase dependency of  $\sigma^B$  and acid resistance as well as its role in ATR (Ferreira et al., 2003). Results showed growth phase dependency for both acid resistance (AR) and adaptive acid tolerance response in both the wild type 10403S and the *SigB* mutant. In comparison to early exponential phase cells, the number of surviving acid adapted cells, for both the wild type and the mutant, increased by 6 log orders of magnitude when stationary phase cells were exposed to pH 2.5. This result infers that a  $\sigma^B$ -independent and growth phase-dependent acid resistance mechanism is at play. However, since survivor counts were consistently lower for the mutant relative to the wild type, it can be concluded that the overall acid resistant mechanism is dependent on  $\sigma^B$ . Moreover, it was also discovered that the adaptive acid tolerance response in *L. monocytogenes* is most prevalent for early log-phase cells and that this response is partially dependent on  $\sigma^B$ . The authors also determined that  $\sigma^B$ 's ability to rescue the cells depended on the type of acid used to lower the pH, since the number of viable cells for the wild type was significantly greater than those for the *sigB* mutant after exposure to acetic acid but not HCl. In addition, the net proton flux and the glutamate decarboxylase (GAD) systems appeared to be unaffected by the *sigB* mutation (see section 2.5.7). However, in a recent study employing a more

sensitive method (reverse transcriptase or RT-PCR), it was demonstrated that expression of *gadB*, *gadC* and *gadD* were indeed affected in a *sigB* mutant, whereas, *gadA* and *gadE* appear to operate independently of  $\sigma^B$  (Wemekamp-Kamphuis et al., 2004). Moreover, proteomic analysis revealed that other prominent stress proteins (Pfk, GalE and ClpC) were present in the EGD-e wild type but not the *sigB* mutant. These results clearly indicate the importance of  $\sigma^B$  for the regulation of one of the hallmark stress attributes of *L. monocytogenes*.

#### 2.4.5.5 Osmotolerance and $\sigma^B$ -dependent genes.

The role of the  $\sigma^B$  regulon was also shown to be important for osmotolerance. It was demonstrated that *sigB* mutants were sensitive to high salt concentrations (6% NaCl) (Becker et al., 1998). Furthermore, using Northern blot analysis the authors also revealed that the transcription of the *sigB* gene was induced by an osmotic up-shift. They also monitored the uptake of the compatible solute glycine betaine and found a substantial decrease in the amount of the osmolyte in the mutant as compared to the parent strain. A similar study conducted by Sleator and co-workers (1999) also provided support for these findings. These studies represented the first where compatible solute transport was shown to be regulated by  $\sigma^B$ . As an extension of this work, Beckers et al. (2000) also investigated the contribution of  $\sigma^B$  to the growth of *L. monocytogenes* at low temperatures. Adaptation of stationary phase cells to growth in minimal media containing either glycine betaine or L-carnitine at 8°C was greatly impaired for the *sigB* mutant. While the lag phase for the wild type 10403S strain was about 4 days (cells were

downshifted from 37°C to 8°C), the mutant took approximately 8 days to begin exponential growth. Moreover, the addition of glycine betaine was found to reduce the lag phase of the wild type but not the mutant, whereas the addition of L-carnitine was found to reduce lag phase equally for both cultures. However, cold adapted logarithmic phase cells for the mutant and wild type were not different with respect to the length of their lag phases after the temperature down shift. These results suggest that two pathways exist for cold adaptation and the pathway for stationary phase cells is dependent on  $\sigma^B$  regulation. Interestingly, although studies using [ $^{14}\text{C}$ ]betaine or [ $^3\text{H}$ ]carnitine demonstrated that the uptake of both osmolytes were greatly diminished in the mutant, only the loss of betaine accumulation appeared to retard growth at 8°C. This may imply that carnitine is a more effective cryoprotectant in *L. monocytogenes* since the low levels accumulated by the mutant continued to allow growth at 8°C. The authors speculated that carnitine may serve only as a cofactor for reactions involved in fatty acid modifications to the membrane and is therefore required at very low concentrations. Nonetheless, the results of this study suggested that both glycine betaine and L-carnitine uptake systems are  $\sigma^B$  dependent (Becker et al., 2000).

The *betL* and the *opuC* operons are responsible for a sodium-dependent secondary transporter of glycine betaine and ATP-dependent transporter of L-carnitine, respectively (see section 2.4.8 below). DNA sequence analysis has shown that both of these operons are preceded by consensus  $\sigma^B$ -type promoter motifs (Sleator et al., 1999; Fraser et al., 2000). A study using Gus ( $\beta$ -glucuronidase) transcriptional reporter fusions to *betL* and *opuC* determined that  $\sigma^B$  was involved in the regulation of expression of

*opuC* but not *betL*. The level of Gus activity was determined to be seven times greater for the *opuC-gus* fusion in the wild type in comparison to the same fusion in a *sigB* mutant. However, no difference was observed for the *betL-gus* fusions (Fraser et al., 2004). RT-PCR also showed that although *betL* was induced by the presence of NaCl (0.5M), the level of transcription was not altered in the *sigB* mutant. This also held true for the primary glycine betaine transporter *gbuA*. However, *opuC* transcription in the mutant was lower than in the wild type even at high osmolarity. It was also discovered that an osmotically inducible unknown gene, *lmo1421*, exhibited reduced expression in the *sigB* mutant. Cetin et al. (2004) also demonstrated that *betL* expression was transcribed from a  $\sigma^B$ -independent promoter, while *opuC* transcription is exclusively dependent on  $\sigma^B$ . This  $\sigma^B$ -independent promoter showed some similarity to the  $\sigma^A$ -like promoter site found in the *B. subtilis* ortholog, but for the most part varied substantially from consensus  $\sigma^A$ -promoters. Interestingly, *gbuA* was found to be transcribed from two promoters, one of which was  $\sigma^B$ -dependent and the other independent; the latter promoter, displaying striking similarity to the  $\sigma^B$ -independent promoter of *betL*. The presence of two promoters including what appears to be a specialized  $\sigma^A$ -promoter for these genes assures expression under a wide array of osmotic conditions. Using both primer extension analysis and RT-PCR, the transcripts from *sigB* and the  $\sigma^B$ -dependent promoter site of *gbuA* was shown to continuously increase 40 minutes after osmotic shock whereas the transcription from the other *gbuA* promoter was weak and constant over all sample times. These results suggest that the *gbuA* promoter is required for long term osmotic stress rather than short periods of osmotic shock. The results taken from this study clearly

shows that  $\sigma^B$  plays a primary role in the regulation of osmotic stress response in *L. monocytogenes*.

#### 2.4.5.6 The $\sigma^B$ - regulon and survival in food systems

It is obvious that the alternative sigma factor  $\sigma^B$  is essential for the survival of *L. monocytogenes* when confronted with a broad array of stress conditions. Hence, there has been widespread interest in elucidating the role of  $\sigma^B$ -dependent expression stress genes. The current trend in the food industry is bent toward the minimal processing of foods. Therefore, it stands to reason that bacteria capable of rapid responses to these “minimal stresses” will be the most successful at survival. Hence, from a food safety perspective, it may be necessary to implement new techniques that counter bacterial stress responses. Since the  $\sigma^B$  regulon encompasses such a large number of genes with a wide range of stress roles, this regulon may be exploited as a potential target for future technologies designed to inhibit pathogen survival or growth (van Schaik and Abee, 2005). However, one must bear in mind the complexity of this regulon;  $\sigma^B$  not only directly regulates certain genes in response to stress, but also it regulates regulators of operons (i.e., Hfq in *L. monocytogenes*) (Christiansen et al., 2004). Moreover, it cannot be assumed that all bacterial species or strains will behave in the exact same manner since there appears to be considerable variability in the  $\sigma^B$  regulon.

Moorhead and Dykes (2003 & 2004) found differences in the contribution of  $\sigma^B$  to the survival of two *L. monocytogenes* serotypes (serotype 1/2a and 4c). *SigB* deletion mutants were constructed in each of the two serotypes and then subjected to a wide array



of stress conditions including; bacteriocins (nisin and sakacin), high osmolarity (in the presence/absence of glycine betaine or L-carnitine), oxidative stress (exposure to cumene hydroperoxide), carbon starvation (0.06% w/v glucose in a defined medium), resistance to ethanol (16.5%), acid (pH 2.5) and heat (50°C) with or without preadaptation (5% ethanol, pH 4.5 or 45°C). The authors found considerable variation in behaviour between the wild type strains. Serotype 1/2a was more resistant to heat, acid and ethanol stress, as well as carbon starvation, while the 4c serotype was more resistant to nisin. No difference was observed for oxidative stress. Variations were observed for osmotic stress depending on the conditions. Results showed that the serotype 1/2a strain behaved in a similar manner to another 1/2a serotype (10403S) in earlier work (Becker et al., 1998). However, the 4c strain was considerably different in that osmolyte uptake and osmotolerance appeared to be independent of  $\sigma^B$  control. In general, the role of  $\sigma^B$  in the stress response of the serotype 4c strain was minimal in response to heat or acid stress. However, mechanisms involved combating oxidative stress, carbon starvation or exposure to lethal ethanol concentrations and bacteriocins were part of the  $\sigma^B$  regulon (Moorhead and Dykes, 2003). In a separate study, the same authors also evaluated the ability of these mutants to survive on different substrates during long term cold storage. The mutants and their respective wild type strains were inoculated into phosphate buffered saline (PBS pH 5.5) and fresh sirloin beef steaks and then placed in long term storage at 4°C. Samples were taken regularly over an eight week period and survivors were assessed on TSA or TSA with 4% NaCl to estimate sublethal damage. There was no difference between wild types or their *sigB* mutants after 8 weeks in PBS (*ca.* 1 log

reduction). However, both mutants showed substantially lower survivor numbers on the beef steaks throughout the sample period as compared to their wild type strains. The trends observed for sublethally injured cells also did not appear to be influenced by the *sigB* mutation after a 3 week sample period (Moorhead and Dykes, 2003). These results suggest that caution should be exercised in making generalizations about the control of the  $\sigma^B$  regulon for a specific bacterial species.

#### **2.4.6 Heat Shock Proteins**

Heat shock proteins (HSP) are induced by a variety of physicochemical and environmental conditions. Of course as the name implies, it was initially those proteins induced in response to a rapid temperature up-shift that garnered this label (Streips, 2002). HSPs are found in all living organisms and the highly conserved structure of many of these proteins across species is evidence of their importance to evolutionary success (Rosen and Ron, 2002). Many HSPs function as molecular chaperones that prevent aggregation or assist in the refolding of misfolded proteins, or as ATP-dependent proteases that degrade denatured proteins incapable of refolding into their native conformations. Therefore, the activity of many of the HSP proteins is closely linked, with chaperones and proteases acting in concert during stress situations. In addition to the damage-limiting roles of HSPs, many of these highly conserved proteins are also thought to perform general housekeeping functions since all known heat shock proteins

are constitutively expressed at low levels even in non-stress conditions (Schumann, 2003).

Some well characterized chaperones include GroEL, GroES, DnaK, DnaJ, GrpE, and several proteases. During heat stress (or other stresses), native proteins have the propensity to unfold and seek more stable conformations under the given set of conditions (Booth, 2002). Moreover, these partially unfolded forms may interact with other unfolded entities resulting in aggregation largely through hydrophobic interactions. As the complex grows in physical size, insolubility becomes inevitable. Studies conducted with purified *E. coli* chaperones such as DnaK, DnaJ, GroEL, HtpG, SecB, DegP, HslU and Lon have demonstrated that protein aggregation is prevented by the selective binding of these chaperones to partially unfolded polypeptides (Ben-Zvi and Goloubinoff, 2001). Furthermore, ATPase chaperones (i.e., DnaK, GroEL, and ClpB) have also been shown *in vitro* to assist in the refolding of proteins that are bound by other molecular chaperones (Ben-Zvi and Goloubinoff, 2001). Although laboratory studies using purified protein constituents have demonstrated refolding, the efficiency of these reactions is quite slow. Hence it is believed that other cellular cofactors must also be involved in the process. This theory is reinforced as an ordered addition of these ATPase chaperones results in varying efficiencies (Ben-Zvi and Goloubinoff, 2001). Regardless, of the biochemistry of these mechanisms, important to the food industry is the fact that the heat shock response enhances survival of cells not only under further heat stress but also cross-protects for other stress conditions (Hill et al., 2002). Furthermore, these proteins may also play an important role in the pathogenesis process. Hanawa and co-

workers (2002) used a *Listeria* DnaK mutant to demonstrate reduced transcription levels in the pathogenicity markers, *flaA* (flagellin) and *lmaA* (encodes hypersensitivity antigen). This may imply that DnaK is involved in either the binding or unfolding of these proteins or other proteins vital for their expression. In a series of studies by Rouquette et al. (1996 and 1998), *L. monocytogenes clpC* mutants were severely crippled in their ability to cause infection in both mice and cell-cultured macrophages. In support of this evidence, Gahan et al. (2001) demonstrated the increased expression of both DnaK and GroESL in mouse macrophages indicating the requirement for the expression of these proteins in order to infect host cells. This study contradicted earlier work that concluded that expression of DnaK and GroE was not increased during macrophage infection (Hevin et al., 1993). Expressed HSPs have also been shown to reside on the outer surface of the bacterial cell and act as adhesion factors for cell binding which has obvious applicability for successful pathogenesis (Lewthwaite et al., 1998).

Presently, heat shock proteins are classified based on how their activity is regulated. For *Escherichia coli*, the heat shock regulon is largely controlled by  $\sigma^{32}$  and  $\sigma^E$  regulators and also includes the *psp* operon which is regulated by  $\sigma^{54}$  and PspF. In contrast, the heat shock regulon in Gram-positive bacteria appears to be somewhat more complex and is most thoroughly studied in *B. subtilis*. A review on the heat shock stimulon in this model organism has been presented (Schumann, 2003). To date, at least six classes of HSPs have been identified in *B. subtilis*.

Class I HSPs are transcribed by genes that are under the control of HrcA-CIRCE repressor. This group of heat shock genes contains only 2 operons encoding for the well

characterized molecular chaperones, *dnaK* and *groE*. The GroE operon contains only 2 genes *groES* and *groEL* while the DnaK operon is more complex enlisting seven open reading frames (*hrcA*, *grpE*, *dnaK*, *dnaJ*, *orf35*, *orf28* and *orf50*). Each of these operons is preceded by a  $\sigma^A$ -promoter and negative *cis* CIRCE element (controlling inverted repeat of chaperone expression), a perfect 9 bp inverted repeat separated by a 9 bp spacer region (Zuber and Schumann, 1994). The consensus sequence for the CIRCE element is TTAGCACTC-N<sub>9</sub>-GAGTGCTAA (Schumann, 2003). This system also incorporates a *trans* protein-repressor encoded by the *hrcA* gene (Hrc = heat regulation at CIRCE). Several studies have provided evidence that the CIRCE element acts as a binding site for the HrcA repressor (Rosen and Ron, 2002). The role of *hrcA*, formerly known as *orf39*, as a repressor was elucidated since: 1) a deletion in this ORF resulted in increased transcription of *groE* (Schultz et al., 1995); 2) mutants deficient in regulating *dnaK* and *groE* were mapped to the *orf39* gene; and 3) the expression of *orf39* from a plasmid resulted in the restoration of regulation of *dnaK* and *groE* in *hrcA* mutants (Yuan and Wong, 1995).

Class II HSPs are under the positive control of the alternative sigma factor,  $\sigma^B$  and make up the largest group of HSPs consisting of well over 100 proteins. The  $\sigma^B$ -dependent genes are not specifically induced by heat shock since induction is also initiated by other stresses such as ethanol, acid, osmotic, desiccation, and starvation for carbon, oxygen and phosphate. Hence, the transcription of *sigB* is a general stress response. According to Price (2002),  $\sigma^B$ -dependent genes can be organized into six groups based on predicted function: 1) genes that encode subunits of inducible proteases,

catalases, reductases that offer direct protection to the cell; 2) genes that are involved in the modulation of  $\sigma^B$ -activity, (RsbU, RsbW, phosphatases, etc.); 3) regulators for genes downstream of *sigB*; 4) genes involved in transport of materials in and out of cells (antiporters, symporters, permeases); 5) genes encoding for products involved in metabolism (dehydrogenases, glucosidases, etc.); and 6) proteins involved in turnover of cell components (cysteine protease, ribonuclease R). In *L. monocytogenes*, conflicting results have been reported pertaining to the role of  $\sigma^B$  during heat stress. Ferreira et al. (2001) reported that  $\sigma^B$  played no major role in survival of strain 10403S at an elevated lethal temperature (50°C) since no difference in survivor curves were observed between a *sigB* deletion mutant and the parent strain. However, Moorhead and Dykes (2003) reported that their L61 (serotype 1/2a) strain exhibited  $\sigma^B$ -dependent behaviour when exposed to a similar temperature regime. In light of the variability observed between strains in the latter study, it appears that the  $\sigma^B$ -regulon encompasses different genes in a strain specific manner.

Class III HSPs are those genes under the negative control of CtsR (for class three stress repressor). In *B. subtilis*, there are three operons under the control of CtsR; the *clpC* operon consisting of four genes (*ctsR*, *mcsA*, *mcsB* and *clpC*); and the two monocistronic *clpE* and *clpP* operons (Schumann, 2003). These operons have the same fundamental organization in *L. monocytogenes* (Glaser et al., 2001). Moreover, CtsR was indeed found to regulate the *clpC*, *clpE* and *clpP* homologues in *L. monocytogenes* since a *ctsR* deletion mutant constitutively expressed the stress proteins and displayed enhanced tolerances to heat and salt (Nair et al., 2000). Additionally, it was shown

through the use of a series of experiments involving transcriptional fusions in a *B. subtilis* host, that CtsR negatively regulates the *clpC*, *clpE* and *clpP* of *L. monocytogenes*. Briefly, the promoter regions of *clpC*, *clpE* and *clpP* from *L. monocytogenes* were fused with the *bgaB* gene of *B. stearothermophilus*, which encodes a thermostable  $\beta$ -galactosidase. Each of these transcriptional fusions was separately integrated into the *amyE* locus of *B. subtilis* host strain QB4991, which lacked a functional *ctsR* gene. The intact *ctsR* from *L. monocytogenes* was fused to a *P<sub>xyIA</sub>* inducible promoter (xylose) and the construct was inserted in the *thrC* locus downstream of the deleted *ctsR* gene and the inserted *clpC*, *clpE* or *clpP* transcriptional promoters. By assaying the  $\beta$ -galactosidase activity in the presence or absence of xylose for each of the three strains carrying the gene fusions the level transcription could be quantified in the presence or absence of heat stress. In the presence of xylose, cells grown at 37°C displayed weak expression of *clpC* (i.e., *ctsR* expressed). However, in the absence of xylose the level of  $\beta$ -galactosidase activity was 12 times higher. If the cells were treated at 48°C, the activity was 26 times greater even in the presence of xylose indicating that under heat stress the repressor activity of CtsR is blocked. Similar results were found for the *clpE-bgaB* and *clpP-bgaB* gene fusions providing strong evidence supporting *ctsR* as the regulator of these genes (Nair et al., 2000).

Class IV HSPs contain only one gene, *hspG*. Schulz et al. (1997) observed that this gene was induced *ca.* 10-fold in *B. subtilis* following heat shock and it was proposed that this was a chaperone type HSP belonging to Class III. However, the gene possesses a unique regulatory site with the sequence GAAAGG adjacent to a  $\sigma^A$ -type promoter,

which if deleted, results in the loss of transcriptional control of HtpG (Versteeg et al., 2003). In this study, the authors also determined that the accumulation of denatured proteins in the cytoplasm (through exposure to ethanol or puromycin) did not activate transcription of *htpG*. Moreover, the induction of *htpG* was also found to be a function of absolute temperature rather than abrupt temperature upshifts since cultures upshifted from 25 to 37°C showed marginal increases in the expression of *htpG* whereas a temperature upshift from 37 to 49°C resulted in high levels of transcription. A putative homologue of this protein is predicted from the sequence of *L. monocytogenes* EGD-e at position *lmo0942* (Glaser et al., 2001). This gene is adjacent to the *fri* gene encoding a ferritin-like protein that was previously shown to be the main protein expressed during cold temperature downshifts (Hébraud and Guzzo, 2000). More recently, the importance of Fri in tolerance to oxidative stress and proliferation in host macrophage-like cells was investigated (Olsen et al., 2005). Presently, a link between HtpG and Fri has yet to be established; however, their close proximity in the genome and importance to environmental stresses implies a relationship. However, it does appear that the two genes possess quite different regulatory mechanisms (Versteeg et al., 2003; Olsen et al., 2005).

Class V HSPs are membrane bound serine proteases belonging to the HtrA family whose expression is negatively autoregulated by a two-component signal transduction system known as the CsrRS (control of secretion stress regulator and sensor) (Schumann, 2003). This system is highly similar to the CpxR/CpxA two-component system in *E. coli* that participates in the response to stresses associated with the cell membrane (Pogliano et al., 1997). The homologues to the HtrA family of proteases encoded by *B. subtilis* are



YkdA, YvtA and YyxA. These genes have been shown to have a  $\sigma^A$ -like promoter with a single mismatch in the  $\sigma^A$ -10 motif but with no similarity to the  $\sigma^A$ -35 region. Replacing the “normal”-35 sequence is part of a six octamer repeat with a consensus sequence of TTTTCACA. The genes encoding YkdA and YvtA have been shown to be induced by heat and the products offer protection against oxidative and osmotic stress (Noone et al., 2000; Noone et al., 2001). However, *yyxA* was not inducible by heat thus its regulation may not be related to the typical heat stress stimulon (Noone et al., 2001). Recently, the role in stress and virulence of the corresponding HtrA-like serine protease (encoded by *lmo0292*) in *L. monocytogenes* has been investigated. Wonderling et al. (2004) found a salt-sensitive Tn917 insertional mutant had a disruption in the *htrA* (*degP*) gene in *L. monocytogenes* strain 10403S. A deletion mutant, created with a 1019 bp segment excised from *htrA* gene, was subjected to various stress challenges. Although the mutant exhibited similar growth rates to the wild type at 30, 37 and 42°C, its growth rate was substantially reduced when incubated at 44°C. Moreover, in addition to salt sensitivity, it was also demonstrated that the mutant was far more sensitive to heat shock at 52°C (Wonderling et al., 2004). However, another study revealed through transcriptional analysis that *htrA* in *L. monocytogenes* strains EGD-e did not behave like classical *htrA* genes in response to heat shock (Stack et al., 2005). Although the gene was confirmed to play a role during growth at elevated temperatures (45°C) the results from this study showed no significant change in the level of transcription of *htrA* when the cells were exposed to 45°C for 30 minutes. However, the gene was inducible by other stressors such as low pH and the presence of the antibiotic penicillin G. Therefore, the authors surmised

that *htrA* in *L. monocytogenes* EGD-e is more likely to encode a homologue of *yyxA* rather than *ykdA* or *yvtA*. It was also noted that the sequence of the HtrA homologue in the EGD-e strain differed from that of the 10403S strain used in the earlier study. The former strain lacked a threonine residue at amino acid position 140 (relative to 10403S). Furthermore, the authors also examined the virulence of *htrA* mutants in a murine model. Using both full deletion and frame-shift mutants, a reduced number of *L. monocytogenes* cells recovered from the spleens of infected mice was observed (1.0 and 0.5 log lower than the wild type, respectively). These results suggest that this gene may also act as another virulence factor in addition to its role against certain stresses.

Class VI HSPs are generally regarded as those proteins that do not fall under the other previously described classes. However, this group encompasses the second largest number of heat shock proteins next to the  $\sigma^B$  regulon. According to Schumann (2003), this class can be differentiated based on: 1) type of promoter; 2) heat shock induction; and 3) stress factors in addition to heat shock that initiates higher levels of transcription. However, the type of regulation involved is still a mystery. Proteins in *B. subtilis* noted to be affected by heat shock that would fit into this category include FtsH, ClpX, YkoZ and SacB; all being monocistronic. However, there is now evidence to suggest that FtsH is not induced by heat only, but by other stresses such as exposure to ethanol (Periago et al., 2002). Additionally, six proteins forming three bicistronic operons have been shown to be upregulated during heat stress. These include *lonA/orfX*, *ahpC/ahpF* and *nfrA/ywcH*. Previously, a microarray approach to analyze transcripts was used to uncover 66 other genes that were induced by heat shock with unknown regulators

(Helmann et al., 2001). The authors assigned these genes to Type U heat shock proteins. Included in this list were several genes that encode transport functions (i.e., *appDFABC* operon, one of two oligopeptide uptake systems in *B. subtilis*; a choline ABC transporter (*opuB* operon); a putative Na<sup>+</sup>/nucleoside cotransporter (*yutK*), and; a multidrug efflux homolog (*yuxJ*). Due to the close relationship to *B. subtilis*, homologues for many of these genes can be identified in *L. monocytogenes* (Glaser et al., 2001). However, their role in heat shock or response to other stresses has yet to be examined.

#### **2.4.7 Acid Resistance and Acid Tolerance Response (ATR)**

Lowering the pH in food is one of the oldest preservation methods known to man. Therefore, foodborne pathogens must have efficient mechanisms to allow survival during extended periods in acidic environments. Moreover, to be successful at manifesting foodborne disease, these microorganisms must be also able to survive the low pH of gastric juices in the host's intestinal environment. This important relationship has inspired numerous studies directed at elucidating the systems involved in acid resistance in bacteria. A review article on the survival of enteric bacteria at low pH has been published (Audia et al., 2001). Relevant to *L. monocytogenes*, Cotter and Hill (2003) also reviewed acid tolerance but specifically focused on mechanisms in Gram-positive bacteria. The authors detailed the changes that occur in the cell that ultimately dictate its chances to survive during exposure to acidic environments. These changes may be constitutive or inducible depending upon the organism and external conditions. Survival

strategies may include: 1) the activation of alternate metabolic pathways; 2) rapid activation of two component sensing systems; 3) initiation of DNA repair mechanisms; 4) the production of stress proteins such as molecular chaperones to prevent the denaturation of acid sensitive molecules; 5) augmentation of the chemical composition of the cell membrane; 6) adjustment of cell densities and biofilm formation; 7) increasing the intracellular pH by the removal of protons; 8) alkalinization of the extracellular environment; and 9) expression of transcriptional regulators required to mediate the process (Cotter and Hill, 2003).

When subjected to low pH environments, two main inhibitory mechanisms are thought to suppress bacterial growth; 1) intracellular acidification resulting in lost homeostasis, and 2) interaction of cellular components with non-dissociated acid forms which interferes with metabolic processes. In *L. monocytogenes*, Ita and Huskins (1991) concluded that the primary inhibitory mechanism of acid stress is not likely due to acidification of the cytoplasm since cells subjected to pH 3.5 (adjusted with a variety of inorganic and organic acids) maintained an intracellular pH of 5.0. Therefore, it was surmised that interactions of non-dissociated acid fractions is likely the main mechanism of inhibition. However, cellular enzymes function optimally within narrow pH ranges and the “normal” state of these processes is near neutral pH. More recent findings have suggested that intracellular pH is indeed a major factor in the ATR (Hill et al., 2002). To date, two major mechanisms have been described for ATR in *L. monocytogenes*. Both of these systems are effective proton pumps and have been found to raise intracellular pH in acid adapted or naturally acid tolerant strains. Specifically, these systems are; 1) a

multisubunit  $F_0F_1$ -ATPase enzyme system and 2) a glutamate decarboxylase system (GAD).

The  $F_0F_1$ -ATPase multisubunit complex has been described in many Gram-positive bacteria. The majority of the described bacteria belong to the lactic acid bacteria group which lacks a proton-linked electron transport system. However, it was later discovered that the Gram-negative enteric bacterium, *Salmonella* spp. also possesses the genes for the  $F_0F_1$ -ATPase operon (Portillo et al., 1993). The system is comprised of the  $F_1$  catalytic portion that consists of five subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ ) and a three subunit (a, b and c) membranous proton translocation port called the  $F_0$  (Cotter and Hill, 2003). Therefore, the system evokes a mechanistic link between the transmembrane proton motive force (PMF) and ATP generation. The  $F_1$  portion is thought to be involved in either the synthesis and/or hydrolysis of ATP depending on the electron transport system in place for the bacterium in question. In bacteria capable of oxidative respiration, ATP can be synthesized from uptake of protons via the  $F_0F_1$ -ATPase system. Under anaerobic conditions this same system is used to generate a proton motive force by the translocation of protons out of the cell; the net result being an increase in intracellular pH (Hill et al., 2002). Therefore, since lactic acid bacteria lack a respiratory chain and are incapable of generating ATP via this system, it appears that the sole purpose of the  $F_0F_1$ -ATPase in these bacteria is for pH homeostasis (Harold et al., 1970; Harold, 1972). Mutations in the *atp* gene retards the activity of the  $F_0F_1$ -ATPase system and as a result, acid sensitizes the cells (Foster and Hall, 1991). Cotter et al. (2000) investigated the possibility of a  $F_0F_1$ -ATPase system in *L. monocytogenes* strain LO28. Initially, they

observed that acid adapted cells treated with the ATPase inhibitor, *N, N'*-dicyclohexylcarbodiimide (DCCD) were less acid tolerant than non-treated acid adapted cells. According to Sebald et al. (1982) the target of DCCD is the Glu54 residue of the c subunit in the  $F_o$ . Since this portion of the complex is responsible for the formation of the transmembrane canal, modifications would alter the cells ability to pump protons out of the cell thence sensitizing the cells to acid treatments. Since there appears to be significant homology between  $F_oF_1$ -ATPase even in distantly related bacteria, Cotter and co-workers (2000) designed degenerate primers based on published sequences in an attempt to isolate the genes in the  $F_oF_1$ -ATPase operon. The highest degree of homology displayed by one of their cloned regions was *ca.* 76% identity and 83% similarity with the  $\alpha$  subunit of the  $F_oF_1$ -ATPase of *B. megaterium*. Although the authors were unable to generate a non-polar deletion in the  $F_oF_1$ -ATPase operon their data nonetheless provided strong evidence for the existence of a functional  $F_oF_1$ -ATPase system.

The second system described for pH homeostasis in *L. monocytogenes* is the glutamate decarboxylase system (GAD). Amino acid decarboxylases have been proposed to function as control mechanisms to maintain internal pH since the 1940s. In addition to glutamate, the decarboxylation of lysine, arginine, and threonine have also been shown to contribute to pH homeostasis (Cotter and Hill, 2003). Small and Waterman (1998) have proposed the mode of action of a GAD system for maintenance of internal pH. They theorized that a specialized transport system is used for the uptake of glutamate. Within the cytoplasm an intracellular proton is used up in the decarboxylation of glutamate to form  $\gamma$ -aminobutyric acid (GABA) which is then transported out of the cell in exchange

for another extracellular glutamate molecule. The lost proton results in an increase in intracellular pH. Because GABA has a slightly higher pKa than glutamate the extracellular surroundings experience slight increases in pH as well. Therefore, it should be obvious that since the GAD system relies on the uptake of glutamate, acid adaptation via this system will not occur in foods where the amino acid is not present. However, glutamates such as monosodium glutamate (MSG) are often used as additives to enhance food flavour. Thence one can speculate that there may be an increased risk for the survival of bacteria possessing functional GAD systems in acid foods containing such additives. Acid adaptation of *L. monocytogenes* in foods high in glutamate has been demonstrated (Cotter et al., 2001b). Construction of deletion mutants with no GAD activity resulted in increased sensitivity of *L. monocytogenes* strain LO28 when subjected to acidified skim milk, yoghurt, fruit juices (apple, orange, tomato) or dressings (salad or mayonnaise) (Cotter et al., 2001b). Moreover, the level of GAD activity in these cells was found to be directly proportional to its survival rate in gastric acids and three listerial GAD genes were identified (*gadA*, *gadB* and *gadC*) (Cotter et al., 2001a). The *gadA* and *gadB* genes encode for decarboxylases, and *gadC* encodes for the glutamate/GABA antiporter. The *gadB* gene was found to be co-transcribed along with the *gadC* antiporter which is positioned just upstream of the decarboxylase and increased levels of transcription were associated with exposure to mild acid treatments. Transcription of the monocistronic *gadA* was much weaker than that of *gadB* and its role in acid tolerance was determined to be less significant. For example, acid tolerance in the  $\Delta$ *gadA* mutant was found to be greater than that of a  $\Delta$ *gadB* mutant. Moreover, the

authors also revealed that there were significant strain differences with respect to GAD expression and that strains that generally expressed lower levels of these genes were naturally more sensitive to acidic conditions (Cotter et al., 2001a). Oiler and co-workers (2004) also found that strains isolated from food or food-processing environments showed lower levels of GAD activity than clinical isolates or those isolated from asymptomatic human carriers.

More recently, it was discovered that some strains of *L. monocytogenes* also possessed an additional decarboxylase gene along with a second transporter (Cotter et al., 2005). Therefore, the authors proposed changes to the nomenclature. The genes encoding for the decarboxylase proteins were designated *gadD1*, *gadD2* and *gadD3*, whereas the antiporter proteins were labeled *gadD1T1* and *gadD2T2*, to indicate the decarboxylase to which the transport protein is paired within the genome. The new *gad* genes were identified by searching the complete genome sequence of *L. monocytogenes* strain EGD-e (Glaser et al., 2000) for putative *gad* sequences. The position of these genes in the EGD-e genome correspond to *lmo447*, *lmo448*, *lmo2362*, *lmo2363* and *lmo2434*, for *gadD1*, *gadD1T1*, *gadD2*, *gadD2T2* and *gadD3*, respectively. Once identified, PCR primers were designed to screen for these genes in other strains of *L. monocytogenes*. The authors determined that the new *gad* genes (*gadD1*, *gadD1T1*) were absent in serotype 4 strains. It was also revealed through the use of deletion mutants that the *gadD2/gadD2T2* pair dictates the overall resistance of the cells at bactericidal pH. However, in terms of growth at low pH (5.1), the new *gadD1/gadD1T1* genes were found to play an important role since deletions in these genes severely reduces the



specific growth rate. Moreover, as a group, strains possessing these genes (i.e., non-serotype 4) also were able to reach higher growth rates at pH 5.1 than strains lacking these genes. These results suggested that different selective pressures have resulted in the divergence of mechanisms dealing with acid stress resulting in at least two lineages of the bacterium.

## 2.4.8 Osmotolerance

### 2.4.8.1 *Primary response to osmotic stress*

*L. monocytogenes* are highly resistant to salt concentrations that would normally be lethal to the vegetative cells of most other foodborne pathogens. In fact, growth has been observed in media with salt concentrations as high as 10% (McClure et al., 1989). In general, there are two mechanisms that influence a cell's ability to survive high osmolarity: 1) a cell can increase levels of salt in its cytoplasm, a strategy that is restricted to halophilic bacteria; and 2) cells may take up small organic molecules deemed compatible solutes to counter the stress (Galinski, 1995). The second strategy has been best described, and for *Listeria* the earliest work recognized three main compatible solutes; glycine betaine (*N, N, N*-trimethylglycine), L-carnitine ( $\beta$ -hydroxytrimethyl aminobutyrate) and proline. However, more recently, other compatible solutes, mainly derivatives of the aforementioned, have also been added to this list. These include proline betaine, acetyl carnitine,  $\gamma\gamma$ -butyrobetaine and 3-dimethylsulfoniopropionate (Bayles and Wilkinson, 2000). These highly soluble

substances carry no net charge at physiological pH and are inert in terms of interactions with cellular proteins. Thence they are ideal osmotic regulators assisting in the restoration or maintenance of cell volume and stabilization of proteins (Sleator et al., 2003).

When subjected to high osmolarity, the initial response observed in most bacteria is the intracellular accumulation of potassium ions along with its counter ion, glutamate. In a study conducted by Patchett et al. (1992), it was found that the intracellular concentration of  $K^+$  doubled and glutamate concentrations increased 5 times for cells grown in the presence of 7.5% NaCl as compared to cells grown with no NaCl added to the medium. Interestingly, no significant increase in  $K^+$  was observed when 5% NaCl was used in the medium. The authors also noted increases in intracellular glycine betaine, glycine, alanine and proline with only a very small increase in overall cell volume. Moreover, the addition of glycine betaine, choline or glycine increased the growth rate of *L. monocytogenes* on chemically defined agar medium containing 4% NaCl. However, the addition of proline did not stimulate cell growth (Patchett et al., 1992). This study represented the earliest work regarding compatible solute uptake by *L. monocytogenes*.

#### 2.4.8.2 Accumulation of compatible solutes

The accumulation of osmolytes constitutes the secondary osmotic response. Beumer and co-workers (1994) observed that the addition of 10 mM proline, 1 mM glycine betaine or 1 mM L-carnitine to a minimal medium (Premaratne et al., 1991) significantly increased growth under high osmolarity at both 10 and 37°C. In the

presence of betaine, growth was observed in media containing 5% NaCl, whereas exclusion of the supplement resulted in no growth. Moreover, proline concentrations had to be 10-fold higher than betaine or carnitine to stimulate growth in the minimal medium containing 3% NaCl. Therefore, insufficient proline levels (1 mM) added to the medium in the Patchett et al. (1992) study may have been the reason for the lack of growth in the presence of high NaCl concentrations. The authors concluded that the uptake of proline was less efficient than either of the other two osmolytes (Beumer et al., 1994). Ko et al. (1994) also found that the exogenously added glycine betaine stimulated growth in osmotic or cold stressed but not in non-stressed *L. monocytogenes* cells. Compared to cells grown in BHI with no additional NaCl, the amount of intracellular glycine betaine accumulated was 20-fold greater in BHI containing 8% NaCl when cells were grown at 30°C (intracellular concentrations of 0.065 and 1.3 M, respectively). For cells grown in BHI without the addition of NaCl, lowering the growth temperature to 4°C resulted in approximately 5-fold more glycine betaine being accumulated than in cells grown at 30°C. The highest levels of glycine betaine (1.8 M) were observed for cells grown at 4°C in the presence of high salt, indicating that both cold stress and osmotic stress induced the uptake of the osmolyte. The authors also demonstrated that the disruption of protein synthesis by the addition of chloramphenicol had no effect on the activation of glycine betaine uptake system indicating a constitutive process. Moreover, carnitine levels did not increase when cells were stressed indicating the preferential use of the glycine betaine system under their test conditions.

It was later demonstrated through the use of membrane vesicles prepared from *L. monocytogenes* that the uptake of betaine occurred via a sodium dependent transport system (Gerhardt et al. 1996). The system was functional in the presence of high concentrations of  $\text{Na}^+$  and requires a hypertonic solute gradient. Additionally, membranes prepared from osmotically stressed cells were 94% more effective in transporting glycine betaine than those prepared from non-stressed cells. Furthermore, the high dependency of  $\text{Na}^+$  was realized since membranes prepared from cells osmotically stressed by KCl only exhibited 35% of the efficacy of NaCl-stressed cells. These results strongly indicated that a strong proton motive force was required to translocate glycine betaine across the membrane. Also observed in this study was the specificity for glycine betaine since other tested osmolytes showed no inhibitory effect on the passage of the molecule (Gerhardt et al., 1996).

In contrast to the sodium-driven glycine betaine uptake system, Verheul et al. (1995) provided evidence of an ATP-dependent L-carnitine transporter in *L. monocytogenes*. Their study showed that L-[ $^{14}\text{C}$ ]-carnitine was transported into the cells in the absence of a proton motive force and the osmolyte was accumulated and not metabolized. The authors used nigericin, a potassium proton exchanger, and the ionophore valinomycin to reduce the transmembrane pH gradient and potential, but uptake of the L-carnitine was not deterred. Furthermore, a strong inhibition of the uptake of the osmolyte was observed when the phosphate analogs vanadate and arsenate were present thus indicative of an ATP-dependent system. They also revealed that the system had a high affinity for its substrate and increasing concentrations of L-carnitine (and like

compounds) by 10-fold had a strong inhibitory effect on uptake. In contrast, there was minimal inhibition when proline or glycine betaine concentrations were increased by 100-fold. It was later revealed that a second transport system for betaine also existed in *L. monocytogenes*, and along with L-carnitine transport, it was not influenced by osmotic up-shock, but instead the osmotic strength of the extracellular environment (Verheul et al., 1997). Regulation of these systems was determined to be governed by the level of pre-accumulated osmolytes (i.e., either pre-accumulated betaine or carnitine inhibited the uptake of both osmolytes).

#### 2.4.8.3 A molecular basis for understanding osmolyte uptake

The use of molecular-based techniques has provided the tools to further advance the knowledge of these transport systems and a review on pre- and post-genomic research in this area was published by Sleator et al. (2003). To date, genes that encode for the three transport systems for the two main osmolytes, glycine betaine and L-carnitine have been identified. The primary osmolyte is glycine betaine and two transporters have been characterized, the Na<sup>+</sup>-dependent symporter (glycine betaine porter I), and the ATP-driven transporter (glycine betaine porter II). Ko and Smith (1999) isolated a Tn917-LTV3 mutant which showed osmotic and cold sensitivities as well as deficiencies in its ability to accumulate glycine betaine. Isolation of the DNA flanking the Tn917 and subsequent DNA-sequencing, demonstrated that the transposon had inserted into a region containing three open reading frames (*gbuA*, *gbuB* and *gbuC*) with high similarity to the *opuA* and *proU* operons of *B. subtilis* and *E. coli*, respectively

(Gowrishankar, 1989; Kempf and Beumer, 1995). These operons encode for ATP-dependent transport systems hence providing genomic evidence to support the physiological data previously indicated (Verheul et al., 1997). The predicted product of *gbuA* has 68.2 and 48.1% similarities to OpuAA and ProV, the proteins encoded by *opuA* and *proU*, respectively. These proteins are predicted to form the ATPase subunits of the transporters. Furthermore, it was demonstrated that replacing NaCl with KCl as the osmotic stressor resulted in a reduction in the uptake of glycine betaine in the mutant to about 1% of the rate of the wild type strain, thus indicating the requirement of Na<sup>+</sup> to drive the system (Ko and Smith, 1999).

Physiological studies carried out with a mutant lacking the ATP-dependent transporter (Gbu Porter) showed that the mutant accumulated 5 times less glycine betaine than the wild type when grown in the presence of 4% NaCl, demonstrating the primary role of porter II (Mendum and Smith, 2002a). The authors also reported in a second study using *opuC* and *gbu* mutants, that the primary Gbu porter for glycine betaine may also act as a low affinity secondary transport system for L-carnitine with a *K<sub>m</sub>* of 4mM (*K<sub>m</sub>* for glycine betaine is 6 μM) (Mendum and Smith, 2002b). In comparison, the primary carnitine porter OpuC, has a *K<sub>m</sub>* of 1-3 μM. Therefore, in the presence of high concentrations of carnitine the Gbu porter can be utilized to transport this osmolyte.

Sleator et al. (1999) used functional complementation in *E. coli* MKH13, a mutant unable to accumulate glycine betaine, to identify the gene encoding for the secondary transporter in *L. monocytogenes* LO28. It was predicted by sequence homology that the gene, *betL*, would encode a 55 kDa protein with 12 transmembrane

regions. Also a consensus  $\sigma^B$ -dependent promoter region was identified just upstream of the *betL* gene signifying its regulation, full or in part, by the alternative stress induced RNA polymerase. The authors also created a knockout mutant in the *betL* gene in *L. monocytogenes* LO28. The ability of this mutant to accumulate [ $^{14}\text{C}$ ]-glycine betaine was significantly reduced in the presence of 3% NaCl (Sleator et. al, 1999). Continued studies involved the construction of a deletion mutant in *L. monocytogenes* LO28 by gene splicing by overlap extension (SOE) (Horton et al., 1990) and allelic exchange (Sleator et al., 2000). The mutant contained an internal 681 bp deletion in the *betL* gene thus producing a truncated protein that lacked the 12 transmembrane domains. The mutant grew significantly slower than the wild type strain at physiological temperatures and increased osmolarity. However, there was no difference between the wild type and mutant when grown at 4°C indicating that this gene is activated by osmotic stress and not cold stress. Further experiments also revealed that this mutant did not differ from the parental strain in terms of virulence using infected mice (Sleator, 2000).

The inhibitory effect of other compounds that mimic glycine betaine or L-carnitine has also been demonstrated. Results have showed that these compounds could be taken up with specificity by either of the transport systems and could therefore inhibit growth when cells were grown under osmotic stress (Mendum and Smith, 2002b). For example, triethylglycine or trigonelline were inhibitory in the presence of glycine betaine or carnitine, respectively. The former compound was also found to inhibit carnitine uptake through the Gbu porter. Both compounds also prevented glycine betaine uptake through the *betL* porter. These results suggest that if such analog compounds were used

as food additives, they may be able to deter the growth of *L. monocytogenes* in high salt or chilled conditions.

The identification of two open reading frames with high levels of sequence homology to the *opuC* and *opuB* operons of *B. subtilis* were identified in *L. monocytogenes* (Fraser et al., 2000). The operon, designated *OpuC* because of its close similarity to the *B. subtilis* operon, was found to contain four ORFs and was preceded by a putative  $\sigma^B$ -dependent promoter region. This first gene in the operon designated *opuCA* was predicted to encode for an ATP-binding protein. The second and fourth genes, *opuCB* and *opuCD*, are predicted to encode two membrane associated proteins, and the final gene, *opuCC* would transcribe an extracellular substrate-binding protein. Construction of an insertional mutation into the *opuCA* in strain EGD-e produced a mutant that grew at about half the specific growth rate of the wild type strain in BHI supplemented with 4% NaCl. Moreover, the EDG-e parent strain grown in a minimal medium was found to transport L-carnitine at a rate of 10 nmol/ min/ mg of cell protein in the absence of added salt and 5 times this rate when salt was present (0.5 M). On the other hand, no transport of L-carnitine into the mutant could be detected in either medium. In contrast, when glycine betaine uptake was assayed, both the mutant and wild type accumulated the osmolyte at *ca.* 15 and 35 nmol/ min/ mg of cell protein for no salt and salt supplemented media, respectively. Continued research on this operon demonstrated that a high affinity carnitine transport system was indeed encoded by these genes. A Tn917-LTV3 insertion mutant that could not transport L-carnitine was isolated and the point of transposon integration was determined to be the *OpuC* operon (Angelidis



et al., 2002). In addition to osmotic sensitivity, the mutant was also found to be cold sensitive. These results clearly demonstrated that the genes in this operon were responsible for specific carnitine transport and the importance of this osmolyte for osmotic and chill tolerance in *L. monocytogenes*.

Two separate studies have examined the impact on osmotolerance and specificity of the three major transporters, BetL, Gbu, and OpuC, by use of multiple deletion mutants. Wemekamp-Kamphuis et al. (2002) prepared single, double and triple mutants in these operons in *L. monocytogenes* strain LO28. The single mutant with a *gbu* deletion appeared to have the least tolerance of all single mutants as cells growing in BHI supplemented with 6% NaCl grew significantly slower. The mutant containing deletions of all three transporters was still able to grow in the presence of 6% NaCl but its rate of growth was *ca.* one sixth that of the wild type (0.0010 and 0.0059/ h, respectively). The double *betL-gbu* mutant and the triple mutant were found to be devoid of any accumulated glycine betaine, but L-carnitine continued to be transported in the triple mutant when cells were grown in BHI containing NaCl. Moreover, the total amount of L-carnitine detected was influenced by NaCl concentration. For example, when no additional salt was added to the medium, no L-carnitine was accumulated. However, in the presence of 3 and 6% NaCl, L-carnitine levels were determined to be *ca.* 350 and 600  $\mu\text{mol/ g}$  of cells (dry wt.), respectively. These results strongly suggested the existence of another carnitine transport system. Another study was carried out using a different strain of *L. monocytogenes* that yielded contrasting results (Angelidis and Smith, 2003). In this study, single, double and triple mutants for *betL*, *gbu* and *opuC* were generated in *L.*

*monocytogenes* strain 10403S by SOE and allelic exchange. Their results showed that glycine betaine uptake via the secondary BetL- $\text{Na}^+$  porter was solely initiated under NaCl-induced stress and weak transport of the osmolyte was also possible through the OpuC system. L-carnitine could be taken up efficiently through both OpuC and Gbu porters and weakly by BetL in the presence of  $\text{Na}^+$ . However, in contrast to the Wemekamp-Kamphuis et al. (2002) study, no L-carnitine uptake was observed in their triple mutant suggesting that strain differences may exist pertaining to the dedicated roles of these transport systems.

#### **2.4.9 Cold stress response**

One of the hallmark characteristics that make *L. monocytogenes* an especially dangerous foodborne pathogen is its ability to grow at refrigeration temperatures (Juntilla et al., 1988); the lowest growth temperatures reported to be slightly below 0°C (Walker et al., 1990). This attribute acts as a selective mechanism for *Listeria* in a diverse background of resident mesophilic flora. Although cold temperatures reduce the rates of cellular reactions and dramatically change the physical properties of biomolecules, significant numbers of cells can result from low levels if given an extended incubation period. Therefore, for foods that have been shown to support the growth of *L. monocytogenes*, it is important that extended storage times are avoided.

Bacteria such as *L. monocytogenes* are capable of withstanding rapid temperature downshifts and once acclimated, have the ability to grow at reduced temperatures. They

do this by employing a number of molecular and physiological mechanisms that allow for a rapid response to the temperature shift followed by an orchestrated chain of events that allows for growth to resume at the reduced temperature. Membré et al. (1999) found that cells of *L. monocytogenes* pre-cultured at refrigeration temperatures exhibited an enhanced ability to grow at cooler temperatures in comparison to cells pre-cultured at 37°C. Moreover, cold adaptation has been shown to enhance the survival of *L. monocytogenes* under other stress conditions such as low pH (Gay and Cerf, 1997). Recently, a review article on cold stress tolerance of *L. monocytogenes* was published that outlines the molecular mechanisms involved in cold stress adaptation (Tasara and Stephan, 2006). The authors list five mechanisms for adaptation to cold stress: 1) modulation of nucleic acids; 2) alteration of membrane fluidity; 3) accumulation of compatible solutes; 4) production of cold stress proteins; and 5) several general stress response alterations.

#### *2.4.9.1 Altering the fatty acid composition of membranes*

Membrane integrity is vital to the survival of all cells, and to maintain the proper fluid, liquid crystalline state, the gel to liquid phase transition temperature must be lower than the external temperature surrounding the cell (Suutari and Laakso, 1994). The primary mechanism by which bacteria maintain the correct membrane fluidity in response to temperature changes is through alterations in fatty acid composition of membrane lipids (Juneja and Davidson, 1993). This can be accomplished by replacing fatty acids possessing higher melting points with lower melting point fatty acids thus

reducing the phase transition temperature. The fatty acid profile of *L. monocytogenes* is dominated by a very high proportion of iso and anteiso, odd-numbered, branched chain species (Raines et al., 1968). In order to decrease the phase transition temperature of the membrane lipids in response to low temperatures, it has been observed that *L. monocytogenes* alters the chain length and the degree of branching of its membrane fatty acids. Annous et al. (1997) found that the fatty acid profile of cells grown at 37°C predominantly consisted of anteiso-C<sub>15:0</sub>, anteiso-C<sub>17:0</sub>, and iso-C<sub>15:0</sub> forms. However, lowering the growth temperature resulted in less anteiso-C<sub>17:0</sub> type fatty acids. Additionally, reducing the temperature to 5°C resulted in a switch from iso-C<sub>15:0</sub> to anteiso-C<sub>15:0</sub> species of fatty acids. This study also demonstrated that transposon mutants incapable of synthesizing odd-numbered, short-chain branched fatty acids were impaired in their ability to grow at low temperatures. Other studies have also found similar changes in fatty acid composition for cells grown at low temperatures (Puttmann et al., 1993; Jones et al., 1997).

Another mechanism by which bacteria can augment the fluidity of membrane lipids is by increasing the relative proportion of unsaturated straight chain fatty acids. However, many species of bacteria including, *L. monocytogenes*, possess low quantities of straight chain fatty acids. Therefore, degree of saturation is thought to play less of a role in membrane homeostasis than for other species with relatively high levels of unsaturated straight chain fatty acids (Kaneda, 1991; Annous et al., 1997). Changes in membrane fluidity in *B. subtilis* in response to cold temperatures have been shown to be due to increased levels of unsaturated fatty acids. One important mechanism to achieve

these changes involves the *Des* pathway responsible for production of an acyl-lipid desaturase which catalyzes the formation of unsaturated fatty acids from saturated phospholipid precursors (Aguillar et al., 2001). It has been shown through transcriptional analysis that during cold shock the *des* gene in *B. subtilis* demonstrates the strongest inducible response of 46 up-regulated genes (Kaan et al., 2002). However, it has also been demonstrated that this gene is also inducible at 37°C when grown in the absence of the amino acid isoleucine, which acts as a repressor for the operon (Cybulski et al., 2001). Conversely, Zhu and co-workers (2005) recently described the importance of isoleucine precursors for producing anteiso fatty acids in *L. monocytogenes* strain 10403S, and demonstrated the cell's requirement for these precursors through the use of transposon mutants. When isoleucine levels were sufficient, very uniform fatty acid profiles were obtained with anteiso fatty acids making up 95% of the total fatty acids. Failure to supply isoleucine resulted in a complex fatty acid profile and the inability to survive at low temperatures.

To date no pathway for desaturase has been described for *L. monocytogenes*. Liu and co-workers (2002) used selective capture of transcribed sequences (SCOTS) to examine enhanced transcription of genes during growth at 10°C as compared to that of cells grown at 37°C. Several genes involved in regulatory adaptive responses, general stress responses, amino acid metabolism, cell surface alterations, as well as known cold-adaptive responses were shown to be up-regulated, but none of these genes correlated to a desaturase pathway.

#### 2.4.9.2 Accumulation of compatible solutes

Also linked to membrane function and cold adaptation in *Listeria* is their ability to accumulate small peptides (Verheul et al., 1998; Borezee et al., 2000; Wouters et al., 2005) or compatible solutes such glycine betaine and carnitine (Ko et al., 1994; Angelidis et al., 2002). Since *Listeria* do not synthesize either of these osmolytes, accumulation must occur via transport from the external environment via the Gbu, OpuC and BetL transport systems (Gerhardt et al., 200; Angelidis et al., 2002; Mendum and Smith, 2002). It has been demonstrated through the use of deletion mutants that disruption of these transport systems results in an impaired ability to grow at low temperatures and that a hierarchal usage of these porters exists which is dependent on the presence and concentration of the osmolytes in the external environment (Wemekamp-Kamphuis et al., 2004). For cells grown in BHI at 7°C, the order of importance was Gbu > OpuC > BetL. However, for cells grown in minimal media, the addition of betaine altered the hierarchy to Gbu > BetL > OpuC and the addition of carnitine shifted the importance solely to OpuC. The authors also investigated a fourth pathway with significant homology to OpuB, a high affinity choline uptake system in *B. subtilis*, but no significant contribution to cold tolerance could be determined. The mechanism by which these osmolytes impart protection at low temperatures is not exactly known. However, it has been proposed that they act to stabilize the membrane lipid bilayer as well as enzymes systems (Lippert and Galinski, 1992).

#### 2.4.9.3 Cold shock proteins

Much like the heat shock response (section 2.46 above), there is an analogous response to cold temperature downshifts in bacteria. Thus, if the protein profiles are compared for bacteria grown at warm versus cold temperatures, certain proteins will have consistent increased levels of expression at the cold temperatures. Proteins displaying increased synthesis in response to balanced growth at low temperature have been deemed “cold acclimation proteins” or Caps. Whilst other proteins that are expressed at higher rates immediately after an abrupt temperature drop have been labelled, “cold shock” (Csps) or “cold induced” (Cips) proteins (Graumann and Marahiel, 1996). In most mesophilic bacteria exposed to a rapid temperature downshift, there is an initial response where synthesis of most cytosolic proteins are either down regulated or turned off. Thus, an initial lag is observed before growth resumes (Jones et al., 1987). However, psychrotrophic bacteria do not display this decrease or cessation of protein synthesis as levels remain relatively constant (Whyte and Inniss, 1992). Therefore, the initiation of the translation process has been suggested as a limiting step in distinguishing a mesophilic bacterium from a psychrophile (Broeze et al., 1978).

For *B. subtilis*, throughout the cold shock period more than 70 proteins are produced and 36 demonstrate maximum synthesis within 1 hour following the temperature downshift. When the cells are fully acclimated the levels of these up regulated proteins is reduced and new proteins are synthesized when growth resumes (Graumann and Marahiel, 1996). The cold shock response in bacteria has been best studied in *E. coli* and *B. subtilis* and much of this work has been focused on a group of

low molecular weight proteins that have been labelled the CspA family proteins which are highly similar to the N-terminal nucleic acid binding domain of Y-box proteins of eukaryotes (Tasara and Stephan, 2006). In *E. coli*, there are nine proteins in this family (CspA to CspI) with CspA being the first and most dominant protein to be synthesized following cold shock. In fact, levels of this ~7.5 kDa protein may account for approximately 13% of the total proteins synthesized (Goldstein et al., 1990). This protein is a “true” cold shock protein since it is not synthesized at 37°C. Other cold inducible proteins in this family are CspB, CspG and CspI which are detectable at low levels at 37°C but their production is greatly enhanced following cold shock. Conversely, *B. subtilis* produces only CspB to CspD in this family of proteins, the most dominant protein being CspB (Graumann et al., 1996). Characterization of CspA and CspB by N-terminal sequencing, NMR and X-ray crystallography in *E. coli* and *B. subtilis*, respectively, has indicated that these cold shock proteins bind single stranded DNA (Graumann and Marahiel, 1996). It has been suggested that the CspB protein in *B. subtilis* may have a regulatory function since mutagenesis of the *cspB* gene results in the reduced expression or elimination of 15 other cold induced proteins following temperature reduction to 15°C (Graumann et al., 1996). Interesting, the mutant grew at a similar rate as the wild type at this temperature but was highly sensitive to freeze thaw cycles.

Although *L. monocytogenes* demonstrates the ability to grow at refrigeration temperatures, there is relatively little information available on cold shock proteins in this organism. This is surprising since cold growth is perhaps the most perplexing feature that



makes *L. monocytogenes* a particularly frustrating and dangerous foodborne pathogen. The number of reported Csps found in *L. monocytogenes* due to temperature downshifts varies from as many as 38 in strain EDG-e (Phan-Thanh, and Gormon, 1995) to only 12 in strain 10403S (Bayles et al., 1996). These variations likely arise from differences in methodologies as well as how the authors discriminated between Csps and Caps. The main cold shock protein in *L. monocytogenes* has been shown to be a relatively small (~18 kDa) ferritin-like protein (flp) that is transcribed as a 0.8 kb monocistronic mRNA up-regulated following a temperature downshift from 30 to 5°C (Phan-Thanh and Gormon, 1995; Hébraud and Guzzo, 2000). Bozzi and co-workers (1997) have described a non-heme iron-binding ferritin in *L. innocua*. This protein is assembled into a 240 kDa multimer exclusively containing six of the ferritin residues (18 kDa) and *in vitro* analysis revealed that approximately 500 iron atoms could be oxidized and sequestered inside a single multimeric complex. The role of this protein is not known; however, its primary structure suggests that it is similar to the Dps family of DNA binding proteins, a diverse group of stress induced polypeptides.

## **2.5 Attachment of *Listeria monocytogenes* to surfaces and formation of biofilms**

One of the most important features concerning *L. monocytogenes* and the food environment is its ability to attach to surfaces and produce biofilms. If the bacterium is unable to attach to and colonize these surfaces, the chance of survival is greatly diminished. Dolan and Costerton (2002) has described a biofilm as a sessile microbial community that are irreversibly attached to a substratum, interface or to one another, in

which the cells are embedded in a matrix of extracellular polymeric substances (EPS) produced by the community. The composition of the matrix is complex, consisting of polysaccharides, proteins, nucleic acids, teichoic acids, phospholipids, minerals, as well as other complex polymers (Chmielewski and Frank, 2003). The proportion of these constituents varies with the microbial community, nutrient supply and environmental conditions.

Biofilm development occurs in 4 stages: 1) conditioning of the surface by macromolecules; 2) reversible adsorption to a surface; 3) irreversible physical attachment mediated by EPS; and 4) maturation of biofilm involving growth of attached cells and recruitment of planktonic cells, to form microcolonies leading to congregation and spread of cells embedded in the EPS matrix (Chavant et al., 2002). Biofilms are constantly in flux with sloughing off of cell layers and regeneration of new biomass. The shedding of cells in the food processing environment provides a perpetual source of contamination in which these cells may seed food items coming into contact with the biofilm or they may be spread through aerosols or in condensates (reviewed in Chmielewski and Frank, 2003).

The ability of an organism to adhere to any surface is dependent on the physicochemical properties of both the contact surface and its own surface properties. Cell surface properties may change dramatically depending on environmental conditions at the time of contact and composition of the growth medium. Moreover, the growth phase of the organism also appears to influence attachment (Dickson and Siragusa, 1994; Chmielewski and Frank, 2003). Tresse and co-workers (2006) found that all *L.*

*monocytogenes* strains used in their study displayed substantially lower surface hydrophobicity when grown at pH 5 compared to pH 7. As a result, adhesion of the former cells to polystyrene was greatly reduced. The overall surface hydrophobicity can also be altered by the type and availability of nutrients in the growth medium. Briandet et al. (1999b) showed that supplementing the growth medium (TSA-YE) with lactic acid or glucose affected the hydrophobic, electrical, and electron donor and acceptor properties of *L. monocytogenes*, while varying the temperature mainly influenced electron donor and acceptor properties, as well as electrical properties of the cells. Additionally, the influence of growth temperature on the production of flagella and their role in attachment has been elucidated (Vatanyoopaisarn et al., 2000). For example, *L. monocytogenes* grown at high temperatures (37°C) is impaired in the production of flagella and less efficient in seeking out binding sites on the target surface (Peel et al., 1988).

Most research examining the adherence properties of *L. monocytogenes* have been conducted on abiotic surfaces relevant to food processing facilities, such as stainless steel, glass, Teflon, nylon, fluorinated polymers and Buna-N rubber (Mafu et al., 1990; Mafu et al., 1991; Smoot and Pierson, 1998a and 1998b; Kalmokoff et al., 2001; Chae et al., 2006). Smoot and Pierson (1998b) examined the ability of *L. monocytogenes* to attach to stainless steel and Buna-N rubber under different environmental conditions (pH and temperature). For all temperatures (10-45°C) and pH conditions (pH 4 to 9) tested, *L. monocytogenes* attached to stainless steel at faster rates and in greater numbers than they did to the Buna-N rubber. The maximum numbers were achieved for cells that were cultivated at 30°C. However, the strength of attachment was determined to be greater on

the Buna-N rubber surface (Smoot and Pierson, 1998a). In general, surfaces such as stainless steel have high free surface energy and are more hydrophilic allowing for better contact with bacteria and as a result cells may attach more readily (Chmielewski and Frank, 2003). Attachment can be passive or active depending on the motility of the cells, and it has been reported that adsorption of bacterial cells to a surface can occur in as little as 5 seconds, followed by irreversible attachment through dipole-dipole interactions, hydrophobic interactions, hydrogen bonding and ionic covalent bonding within a few minutes and up to 4 h (Lundén et al., 2000; Mafu et al., 1990; Vatanyoopaisarn et al., 2000).

Investigations pertaining to the attachment of *L. monocytogenes* to the surfaces of food commodities, especially of plant origin, are not abundant. Currently, studies on the attachment of *L. monocytogenes* to plant tissues have been conducted on alfalfa sprouts, cabbage, cantaloupe, lettuce, potato and radish (Takeuchi et al., 2000; Ukuku and Fett, 2002; Gorski et al., 2003; Gorski et al., 2004; Garrod et al., 2004; Palumbo et al., 2005; Ells and Truelstrup Hansen, 2006). However, studies have clearly demonstrated that the rate at which *L. monocytogenes* attaches to plant surfaces compares to studies performed with abiotic surfaces. For example, Garrod et al. (2004) investigating the attachment and detachment of *L. monocytogenes* to potato slices, observed that the probability of detachment decreased over the first 2 min of exposure to cut potato surfaces and then remained constant thereafter. Other studies have also shown rapid adhesion to plant tissues especially if intact surfaces are disrupted (Takeuchi et al., 2000; Gorski et al., 2003; Ells and Truelstrup Hansen, 2006).

Following initial attachment, the development of the EPS matrix is enhanced and the maturation of the biofilm is also influenced by the culture medium. In modified Welshimer's broth (MWB), early biofilm development (< 12 days at 21°C) by *L. monocytogenes* Scott A was altered by phosphate and amino acid concentrations (Kim and Frank, 1995). It was also found that the presence of trehalose and mannose in the medium enhanced biofilm formation. Moltz and Martin (2005), found that six of eight strains of *L. monocytogenes* used in their study produced denser biofilms in polyvinyl chloride microtitre plates when grown in a minimal medium (modified Welshimer's broth), whereas the other two strains exhibited thicker biofilms if grown in TSB. Recently, Chae et al. (2006) correlated the proficiency in biofilm formation by *L. monocytogenes* to the organism's overall ability to produce extracellular carbohydrates. Planktonic cells producing higher total carbohydrate levels, ultimately led to increased biofilm formation. However, not all strains are equal in their biofilm capabilities. Kalmokoff et al. (2001) found that despite the fact that all thirty-six *L. monocytogenes* strains used in their study could attach to stainless steel coupons only one strain had the ability to form a biofilm. Furthermore, Borucki et al. (2003) examined eighty strains of *L. monocytogenes* and found that serotypes 1/2a and 1/2c generally produced enhanced biofilms relative to other phylogenic groups. Moreover, strains known to be persistent environmental isolates from bulk milk samples also demonstrated increased biofilm formation over non-persistent strains.

In the laboratory, biofilm experiments have most often been conducted with mono-cultures of *L. monocytogenes*. However, it is unlikely that such purity would exist

in nature. Studies examining the interaction of *L. monocytogenes* with other bacteria such as *Flavobacterium* spp. or *Pseudomonas* spp. indicate an enhancement in biofilm formation (Sassahara and Zottola, 1993; Bremer et al., 2001). Carpentier and Chassaing (2004) examined the ability of three strains of *L. monocytogenes* to form biofilms in binary cultures with other environmental isolates obtained from food processing facilities. Of the twenty-nine isolates, sixteen reduced biofilm development. However, co-cultures with four isolates; identified using APILAB Plus database (bioMérieux, Marcy l'Etoile, FRN) as *Kocuria varians*, *Staphylococcus capitis*, *Stenotrophomonas maltophilia* and *Comamonas testosteroni*, resulted in enhanced biofilm formation. Interestingly, the spatial arrangement of cells within these biofilms, were altered dramatically from that seen in *L. monocytogenes* monoculture biofilms.

Biofilm formation can be initiated in response to stress conditions as well as luxury growth conditions, and the regulatory signals governing these pathways depend on the environment (Stanley and Lazazzera, 2004). Environmental bacteria such as those residing on hard to clean surfaces within food processing facilities (or intact plant surfaces) are nutritionally challenged and stress hardened (Herbert & Foster 2001). Even in situations where a good source of nutrients is available, all regions of the biofilm are not equally exposed. For example, one can imagine that cells near the surface would have the greatest access to the available nutrients and a gradient would form downward depending on channels within the matrix. The starvation stress response (SSR) of *L. monocytogenes* due to carbon deprivation has been shown to vary depending on whether or not cells exist as free living or are part of a biofilm resulting in the up-regulation of

numerous proteins (Helloin et al., 2003). In *E. coli*, a stringent or starvation response is initiated by the transcription of two genes, *spoT* and *relA*, that transcribe two phosphorylated guanosine nucleotide ((p)ppGpp) synthetases. Transcription of these genes results in the accumulation of (p)ppGpp which indirectly lowers protein synthesis through the repression of stable RNA synthesis (Christensen et al., 2001). Taylor et al. (2002) have demonstrated that a similar response involving the accumulation of (p)ppGpp occurs in *L. monocytogenes*. Transposon mutants with Tn917 insertions in the *relA* and *hpt* were found to be impaired in the ability to accumulate (p)ppGpp during amino acid starvation. Although these mutants were able to adhere to the surface of a microtitre plate, they were unable to initiate growth and biofilm formation. These results strongly indicate a link between biofilm formation and starvation stress.

Evidence also suggests that biofilm initiation/formation may be part of the  $\sigma^B$  regulon in other Gram-positive bacteria. For example, several environmental factors have been shown to influence the production of polysaccharide intercellular adhesin (PIA) by *S. aureus*. Exposure, to high osmolarity, iron limitation, anaerobiosis and high temperature have been shown to increase biofilm production. Under osmotic stress,  $\sigma^B$  mutants were shown to be deficient in biofilm capacity (Rachid et al., 2000). However, a later study by Valle et al. (2003) produced conflicting data to these findings. In *L. monocytogenes* 10403S it has been shown that an isogenic  $\sigma^B$  mutant was equally as proficient in attaching to stainless steel as was the parent strain after 5 min and 24 h at 24°C. However, after 72 h at the same temperature the wild type produced significantly higher numbers on the coupons (Schwab et al., 2005). Moreover, in the presence of 6%

NaCl, the  $\sigma^B$  mutant behaved in a similar manner to the wild type. These results suggest that  $\sigma^B$  is not involved in regulation of the initial attachment stage, but under conditions of low osmolarity, may play a role in biofilm development.

Biofilms are thought to play a major role in the persistence of *L. monocytogenes* in food processing facilities (Charlton et al., 1990; Holah et al., 2004). The ability of environmental strains of *L. monocytogenes* to attach to surfaces and produce biofilms, coupled with their ability to grow at refrigerated temperatures, high osmolarity, and relatively low pH, provides food processors with a difficult challenge. Moreover, biofilms are difficult to remove from poorly accessible areas in the processing plant such as conveyor belts, abraded surfaces and drains, and stress hardened cells within the biofilm have been shown to be highly resistant to sanitizers (Frank and Koffi, 1990; Lee and Frank, 1991). The inability to eradicate *L. monocytogenes* from processing facilities leads to a viable source for post processing contamination as substantiated by the frequent isolation of non-heat-injured cells of this pathogen from thermally processed foods (Gravini, 1999). The performance of different sanitizers against *Listeria* biofilms, have been evaluated and the effectiveness of the sanitizer is dependent on the contact time and the application surface. For example, chlorine sanitizers may be less effective on highly soiled areas since it tends to bind to organic matter rendering it inactive. Other sanitizers such as quaternary ammonium compounds or peroxygen sanitizers (i.e., hydrogen peroxide) may be more effective with longer contact times (Chmielewski and Frank, 2003).



Raw produce entering processing facilities may contain bacterial biofilms. Using confocal laser scanning microscopy, Morris et al. (1997) observed natural biofilms on the leaves of all plants sampled in their survey. These included; spinach, lettuce, cabbage, celery, leeks, basil, parsley and broad leaf endive. A more recent study also found evidence of biofilm formation on a variety of raw produce (tomato, carrots, and mushrooms) (Rayner et al., 2004). Based on its efficiency to attach to plant surfaces and evidence of early biofilm formation (microcolonies) on test plant surfaces, it is conceivable that raw produce may naturally contain *L. monocytogenes* as part of a mixed consortium of bacteria residing in a biofilm (Ells and Truelstrup Hansen, 2006). Therefore raw materials entering processing facilities may harbour this pathogen and its removal from plant surfaces could prove difficult. Shedding of these cells to other contact surfaces in the processing plant may lead to a perpetual source of contamination.

### 3. RESEARCH OBJECTIVES

The research component of this thesis was designed to evaluate several key factors important for the success of *Listeria monocytogenes* to survive the chain of events involved in processing of cabbage with the implementation of a mild heat treatment step. *L. monocytogenes* was chosen as the model pathogen due to the historical relevance of this pathogen on cut cabbage as well as its well documented attributes with respect to stress tolerance. Specifically, the areas of investigation included; 1) the attachment of *L. monocytogenes* to cabbage surfaces, 2) survival/growth of *L. monocytogenes* on intact, cut and heat treated shredded cabbage, 3) identification of genes important for the thermal resistance of *L. monocytogenes*, and 4) in depth characterization of novel mechanisms leading to thermotolerance in *L. monocytogenes*.

**Objective 1: Strain and growth temperature influence *Listeria* spp. attachment to intact and cut cabbage.** Prior to the current investigations, the only fruit and vegetable commodities studied as *Listeria* attachment surfaces were alfalfa sprouts, cantaloupe, lettuce, radish and potato. Moreover, these studies were restricted to a few strains of *L. monocytogenes*. The objective of this study was to broaden this commodity and strain base by examining the attachment of *Listeria* spp. to cabbage tissue. **The working hypothesis** of this investigation was that different attachment proficiencies would be exhibited by different *Listeria* species (and strains) and that the condition of the cabbage surface would influence the attachment ability of these strains. The relevance of this

work was to enhance the understanding of how *L. monocytogenes* contaminates fresh produce. Insight into the mechanisms of initial microbe-plant interactions may help in the development of effective strategies to minimize contamination of fresh or minimally processed fruit and vegetables.

**Objective 2: Survival and growth of *Listeria* spp. on intact or cut cabbage tissues:**

**The enhancement of growth on shredded cabbage following a mild thermal treatment.** The implementation of mild heat treatments in the processing of fresh intact or cut fruit and vegetables has been shown to enhance the shelf life of some commodities; presumably through the inactivation of indigenous enzymes. However, the microbiological safety of such treatments has not been fully elucidated. Studies involving lettuce indicate that the growth of *L. monocytogenes* is enhanced following mild thermal processing (Li et al., 2001, Delaquis et. al., 2002). Preliminary work has shown that a mild heat treatment of shredded cabbage can extend its shelf life from about *ca.* 10 days to between 14-21 days. Cabbage (coleslaw) is known to support the growth of *L. monocytogenes*, and coupled with the pathogen's unique ability to proliferate at refrigerated temperatures **the working hypothesis** of this study was that a mild heat treatment of shredded cabbage would enhance the growth of the pathogen. Extension of the shelf life of shredded cabbage beyond 10 days without the implementation of other *anti-listerial* hurdles may prove to be unsafe practices. The information obtained in this study is relevant for assessing food safety concerns of a new processing strategy.

Therefore, informed decisions can be made regarding shelf life recommendations for treated products.

**Objective 3: Insertional mutagenesis of *Listeria monocytogenes* 568 reveals genes that may contribute to enhanced thermotolerance.** The thermal stress response has been thoroughly studied in many organisms important to food processing and safety. In *Listeria*, it has been shown that a subset of proteins are up regulated or initiated in response to heat exposure. Many studies have examined the thermal inactivation of *L. monocytogenes* in a variety of heating media including food. It is clear that there is a wide range of thermal sensitivities depending on strain; however, the reasons for this are not known. In this work, transposon mutagenesis of *L. monocytogenes* 568 was used to prospect for novel mechanisms involved in thermal resistance. **The working hypothesis** of this study was that the insertion of Tn917 into random ORFs would result in mutants displaying “enhanced” thermotolerant phenotypes and that some of these genes would reveal novel mechanisms contributing to heat resistance. Using a single bacterial strain and modifying its ability to resist a lethal heat treatment (or other stressors) through genetic manipulation may provide insight into why some strains are naturally more thermally resistant than others. Advancing our understanding of the mechanisms involved in stress tolerances improves opportunity for processing strategies to eliminate pathogens from foodstuffs.

**Objective 4: Inactivation of *treA*, the gene encoding a phospho-(1,1) glucosidase, causes increased thermal and osmotic resistance in *Listeria monocytogenes* 568 when grown in the presence of trehalose.** Mutants were obtained from Objective 3 displaying enhanced thermotolerance. The position of the Tn917 insert for each mutant was determined. One of the more thermally resistant mutants was found to have the transposon inserted into ORF *lmo1453*. By sequence similarity, this gene is thought to encode a homologue of a phospho-(1,1) glucosidase, previously described in *B. subtilis*. The gene (*treA*) is one of three forming the well characterized trehalose operon in *B. subtilis*; however, the operon has not been described in *L. monocytogenes*. **The working hypothesis** for this study was that the disruption of *lmo1453* would result in the inability of *L. monocytogenes* to utilize the disaccharide trehalose as a source of carbon and energy. The observed heat tolerant phenotype was **hypothesized** to be the result of the accumulation of trehalose which has been shown to act as a compatible solute in other microorganisms. Since trehalose is known to serve as a compatible solute, **a third hypothesis** of this study was that the accumulation of the disaccharide in the *L. monocytogenes* mutant should also offer cross-protection against osmotic related stresses. The information obtained in this study is relevant since trehalose metabolism has not been previously described in *L. monocytogenes* and it describes a novel mechanism to enhance the survival of this pathogen during various stress conditions.

## CHAPTER 4

### **Strain and growth temperature influence *Listeria* spp. attachment to intact and cut cabbage**

(Adapted from the publication: Ells, T.C., and Truelstrup Hansen, L. 2006. Strain and growth temperature influence *Listeria* spp. attachment to intact and cut cabbage, International Journal of Food Microbiology. 111, 34-42)

#### **4.1 Introduction**

The increased consumption of fresh and minimally processed fruits and vegetables has led to an increase in the number of foodborne illnesses linked to these products (Beuchat, 1996; Tauxe et al., 1997; Sewell and Farber, 2001). This is not surprising since manure based fertilizers used in agricultural practices may harbour enteric pathogens if improperly composted (Forshell and Ekesbo, 1993; Olson, 2000). Furthermore, the foodborne pathogens, *Listeria monocytogenes*, *Clostridium perfringens*, *Clostridium botulinum* and *Bacillus cereus*, are ubiquitously distributed in nature and commonly

isolated from soils, sediments and decaying plant debris. Produce commodities may therefore become contaminated through contact with soil and resident insects, or via the splashing of rain, wind action or irrigation water (Portnoy et al., 1976; Beuchat, 2002; Buck et al., 2003). Since these products are often consumed raw or following minimal processing, the potential for ingestion of viable pathogens is a concern. Consequently, the survival and growth of human bacterial pathogens in such products has been investigated extensively. However, relatively little research has examined the initial interactions of bacterial pathogens with a broad range of plant surfaces representative of important commodities (Takeuchi et al., 2000; Barak et al., 2002; Gorski et al., 2003).

The genus *Listeria* consists of Gram-positive, oxidase negative, catalase positive, non-sporulating rods, which can be subdivided into 6 distinct species. *L. monocytogenes* is the only species that has been linked to human foodborne disease (Vazquez-Boland et al., 2001). The first confirmed cases of foodborne listeriosis occurred in 1981, at a maternity hospital in Halifax, Nova Scotia, Canada, where contaminated coleslaw was served to patients (Schlech et al., 1983). This bacterium has been shown to persist for long periods in water systems, various soil types, and in biofilms in packing sheds and food processing facilities (Botzler et al., 1974; Dowe et al., 1997; Prazak et al., 2002; Holah et al., 2004). Recently, *L. monocytogenes* was found to be capable of growth and persistence on several produce commodities following its introduction to germinating seeds (Jablasone et al., 2004). Although the number of documented outbreaks linking this organism to produce are rare, (Schlech et al., 1983; Aureli et al., 2000), *L. monocytogenes* does raise concern among commodity producers. Several studies have

demonstrated that minimally processed vegetables support the proliferation of this psychrotrophic pathogen once it becomes established on the food surface (Carlin & Nguyen-The, 1994; Farber et al., 1998; Li et al., 2004).

Contamination of produce by *L. monocytogenes* may occur at any point throughout the food continuum through incidental contact with the organism. Therefore, in order to develop effective strategies to minimize the risk of foodborne disease caused by this organism, it is essential to examine the initial stages of bacterial attachment to various plant tissues. Using a lettuce model, Takeuchi et al. (2000) demonstrated that *L. monocytogenes* and *Escherichia coli* O157:H7 showed preferential attachment to cut edges of iceberg lettuce compared to intact leaf tissues. In contrast, *Salmonella* Typhimurium attached equally as well to both types of surfaces. Recently, it was reported that particular *L. monocytogenes* strains adhered to and colonized alfalfa sprouts significantly better than other strains by a factor that varied by nearly 5 log CFU per sprout (Gorski et al., 2004). It is interesting that none of the tested strains were defective in motility, since the presence of flagella has been shown to be important in the initial stages of attachment of *Listeria* to both abiotic as well as plant surfaces in other studies (Vatanyoopaisarn et al., 2000; Gorski et al., 2003). The production of flagella is temperature dependent and *Listeria* cultivated at 37°C display significantly lower numbers per cell than those grown at lower temperatures (Peel et al., 1988). In general, growth conditions will impact the physicochemical properties of the bacterial cell surface. For example, *Listeria* grown at different temperatures may differ in hydrophobicity, surface charge, and electron donor and acceptor characteristics, which



ultimately affects its ability to adhere to surfaces (Briandet et al., 1999a; Briandet et al., 1999b). Therefore, prior growth temperature and other conditions encountered by bacteria before entering the food production chain may determine whether or not the cells will attach and remain on a food surface.

In this study, raw cabbage surfaces were used as a template for *L. monocytogenes* attachment. Locally produced cabbage, subsequently processed into coleslaw has previously been implicated in listeriosis outbreaks (Schlech et al., 1983). Although a previous report showed that one single *L. monocytogenes* strain attached preferentially to cut lettuce tissue compared to intact surfaces (Takeuchi et al., 2000), it is not known if this finding may be generalized for all *Listeria* species, serotypes, or strains, is dependent on growth temperature, or transferrable to cabbage. It was hypothesized that attachment of *Listeria* to cabbage would be dependent on the type of cabbage surface, the growth conditions used, and the specific *Listeria* strain-type used. Specifically, the importance of (1) species and strain differences, (2) previous growth temperature and (3) cabbage surface structure (i.e., cut versus intact tissue) on the attachment of *Listeria* spp. to cabbage was investigated.

## **4.2. Materials and Methods**

### ***4.2.1. Bacterial cultures and media***

The *Listeria* strains and their sources are listed in Table 4.1. These consisted of both clinical and food isolates of *L. monocytogenes* (14 strains), as well as food isolates of *L.*

*innocua* (3 strains) and *L. welshimeri* (7 strains). All routine culturing was carried out using Brain Heart Infusion (BHI) broth or agar. All strains were stored frozen in BHI supplemented with 15% glycerol, at -80°C. For experiments, cells were sub-cultured from reconstituted -80°C stocks in BHI broth under the appropriate growth conditions. All media used in this study was supplied by Oxoid Inc. (Nepean, ON, Canada), unless specified otherwise.

#### 4.2.2. General preparation of cabbage surfaces

Stored white cabbage heads (*Brassica oleracea* ssp. *capitata*, L. type Lennox) were obtained locally. For each cabbage head, the two outermost leaf layers were removed using sterile forceps in order to reduce background flora on test surfaces. Preliminary experiments indicated that the internal leaves contained very few bacteria (data not shown) allowing for the direct enumeration of *Listeria* using non-selective media. Two configurations of coupons were prepared for attachment assays. For intact surfaces, disk-shaped cabbage coupons were cut using a sterilized 2.2 cm diameter cork borer. The two faces of the coupons were considered to be undamaged intact tissue. For cut surfaces, strips were cut from the mid vein of each leaf at the thickest point near the base of the cabbage head. The vein was first split longitudinally along the centre with a sterile scalpel, and a 2.2 × 0.7 cm rectangular coupon was cut from each face of the split vein to acquire coupons with both sides having cut surfaces. All coupons were pooled in ziplock bags and placed in Tupperware® containers with water saturated Kimwipes® to maintain high humidity. The containers were stored at 4°C until time of inoculation. Coupons

were used within 48 h of their preparation. For simplification, coupons are referred to throughout this paper as either “disks” or “strips” for intact and cut cabbage surfaces, respectively.

#### *4.2.3. Inoculation of coupons and sampling*

Bacterial inocula were prepared from early stationary phase cultures by harvesting the cells by centrifugation at  $5000 \times g$  for 10 min at 10°C. Pellets were washed twice with sterile phosphate buffered saline (PBS), pH 7.2, and then resuspended to obtain an absorbance of 0.1 at 600 nm in the same buffer. A final density of approximately  $10^6$  CFU/ml was achieved by diluting the suspension in PBS tempered to 10°C. Verification of cell densities was performed by spiral plating (Spiral Systems Instruments, Inc., Bethesda, MD, USA) the suspensions on Tryptone Soy Agar supplemented with 0.6% Yeast Extract (TSA-YE). Cabbage coupons were wedged vertically into the wells of sterile tissue culture trays and completely submerged in bacterial suspensions. For all attachment assays, the trays were incubated at 10°C without agitation. The lower assay temperature was chosen to reduce the effect of bacterial growth (Garrood et al., 2004). Coupons were removed from suspensions at specific time intervals (see below) and trimmed to achieve standardized surface areas. For disk coupons, edges were removed by cutting the original 2.2 cm disks with a sterile 2.0 cm cork borer. The removal of the disk edges eliminated disk thickness as factor contributing to variations in total surface area and provided a surface primarily of intact tissue. Standardization of coupons used for cut surface evaluations was achieved by trimming the rectangular strips on all edges leaving

a rectangular surface of  $2.0 \times 0.5$  cm comprising a standardized cut surface area of  $2.0 \text{ cm}^2$  for each strip.

#### 4.2.4. Effect of growth temperature on attachment of *L. monocytogenes* Scott A

*L. monocytogenes* Scott A was grown to early stationary phase in BHI at three different temperatures (10, 22 and 37°C). Bacterial suspensions and cabbage disk coupons were prepared and inoculations were performed as described above. Trays containing cabbage coupons were incubated at 10°C without agitation. At specific time intervals (0, 1, 4, and 24 h) coupons were removed from their respective suspensions and dipped in two successive changes of PBS to remove residual cells carried over from the inoculum. Since a true “Time 0” obviously would yield no adsorbed or attached cells, “Time 0” for these assays refers to an exposure time of approximately 5 min. The disk coupons were trimmed as described above, then the remaining 2.0 cm diameter disks were placed in sterile 50 ml Falcon® tubes. The coupons were vortexed for 20 s in 25 ml of PBS, pH 7.2, containing 0.1% Tween 20 to assist in the removal of cells adsorbed through weak hydrophobic interactions (Hassan and Frank, 2003). Washing was repeated a second time in a new tube containing fresh PBS/Tween 20. The two wash solutions were combined for the enumeration of adsorbed *Listeria*. For enumeration of attached cells, cabbage coupons were placed in 25 or 100 ml of 0.1% buffered peptone water and homogenized for 20 s on high speed using a PowerGen 700 homogenizer (Fisher Scientific) equipped with a 20 mm generator with knives. The generator was sterilized in 70% ethanol between each sample and rinsed twice in sterile distilled water (SDW) to

remove residual alcohol. Enumeration of *Listeria* was carried out by spiral plating the homogenates and their respective wash solutions on TSA-YE. Plates were incubated at 35°C for 48 h. A total of four coupons were processed for each time per treatment and the entire experiment was repeated three times.

In order to assess the contribution of cell growth to the increasing cell numbers on the cabbage surface, four additional cabbage disks were removed from the cell suspensions after 1 and 4 h of exposure. The disks were washed twice in PBS as described above. Two disks for each exposure were enumerated immediately following washing, while the other disks were placed in Petri dishes containing a few droplets of sterile water to maintain humidity. The plates containing the disks exposed to cell suspensions for 1 h were incubated at 10°C for an additional 3 h whereas disks exposed for 4 h were incubated for 20 h. Subsequently, bacteria on disks were enumerated as described above.

#### 4.2.5. Calculation of “binding strength” ( $S_R$ values)

$S_R$  values were calculated in accordance to the previous description by Dickson and Koohmaraie (1989) as a measurement of “binding strength”.  $S_R$  values essentially express the percentage of cells that are attached within the total population of cells associated with a surface [ $S_R = \text{cells remaining on cabbage coupon} / (\text{cells remaining on coupon} + \text{those removed in wash buffer})$ ]. Therefore,  $S_R$  values are actually a measurement of the binding efficiency within this assay rather than the true strength of the interaction since the force necessary to remove the cells was not measured. However,

to remain consistent with the cited literature,  $S_R$  will be equivalent to “binding strength” in this study.

#### *4.2.6. Attachment of *Listeria* strains to intact and cut cabbage tissues*

Twenty four *Listeria* strains were grown to early stationary phase in BHI broth at 22°C. Inocula and coupons (cut and intact) were prepared as previously described and the attachment assays were performed at 10°C. Samples were pulled from their respective suspensions after 3 h and rinsed by two successive dips in PBS, then washed in PBS/Tween 20 as described above. For this assay, firmly attached cells only were enumerated. The coupons were homogenized, spiral plated on TSA-YE, and enumerated as before.

#### *4.2.7. Scanning Electron Microscopy (SEM) of cabbage tissue samples*

Cabbage coupons removed from the bacterial suspensions after 1, 4 and 24 h were rinsed twice in PBS then immediately fixed overnight at 4°C in 0.1 M cacodylate buffer, pH 7.2, containing 2% glutaraldehyde. The procedure used for sample preparation followed that described by Morris et al. (1997) with some modifications. The coupons were rinsed 3 times in 0.1 M cacodylate buffer supplemented with 3% glucose and then postfixed in 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer for 4 h. Samples were washed 5 times in SDW, then dehydrated through an ethanol gradient series (35, 50, 70, 90 and 100%). All steps in the gradient involved 20 min exposure times with the final 100% ethanol treatment being repeated four times. Drying of samples was achieved through a

hexamethyldisilazane (HMDS)/ethanol gradient (25:75, 50:50, 75:25 and 100:0) for 15 min at each concentration, with the final 100% HMDS step repeated three times.

Samples were briefly vented in a fume hood to remove HMDS vapours and then sputter coated with gold (15 nm). Samples were stored under vacuum until the time of viewing. Samples were viewed using a FEI/Philips, Quanta 200 environmental scanning electron microscope (FEI Company, Peabody, MA, USA). Ten random frames at 1500 × magnification (7600  $\mu\text{m}^2$ / field) were viewed for each sample in order to assess the distribution and general cell densities on the cabbage surfaces.

#### 4.2.8. Statistical Analysis

All treatments for the experiments examining the effect of temperature on the attachment of *L. monocytogenes* Scott A to intact cabbage were carried out using four coupons for each time/temperature combination. The entire experiment was repeated three times. For the *Listeria* strain experiments, four coupons of intact disks and cut strips were sampled for each treatment and the entire experiment was repeated four times. Data were analysed with SYSTAT software conducting two-way Analysis of variance (ANOVA) with a 95% confidence interval. Where appropriate, the post hoc analysis was conducted by the Tukey method ( $\alpha = 0.05$ ) to test for significant differences between individual treatments.

### 4.3. Results

#### 4.3.1. Effect of growth temperature on attachment of *L. monocytogenes* Scott A

Preliminary plating studies on TSA-YE demonstrated that most cabbage coupons were devoid of culturable natural background bacteria or, if present, the numbers were very low and did not interfere with the enumeration of *Listeria* spp. on the non-selective medium (data not shown). Enumeration on a non-selective medium avoids the inherent problems of excluding stressed or injured *Listeria* cells common to a stringent *Listeria* selective medium. For samples with some background flora, only characteristic *Listeria*-type colonies were enumerated and the identity of several of these colonies was confirmed on *Listeria* Selective Media (Oxford formulation, Oxoid).

The results for the adsorption and attachment of *L. monocytogenes* Scott A to intact cabbage over time are shown in Figure 4.1. The rate at which cells attached to the cabbage was very rapid. After only 5 min exposure times (Time 0 h in Figure 4.1), numbers approached 4.3 log CFU/cm<sup>2</sup> for cells grown at 22 and 37°C, and 3.8 log CFU/cm<sup>2</sup> for cells cultivated at 10°C. For all sample times, the cells grown at 10°C attached in significantly decreased numbers in comparison to cells grown at the two higher temperatures ( $P < 0.05$ ). The greatest observed differences in attachment between treatments were for cells grown at 10 and 22°C. Cells grown at 22°C showed increases of 0.41 and 0.55 log CFU/cm<sup>2</sup> above the levels observed for 10°C cells after the 1 h and 4 h intervals, respectively. The attachment levels for cells cultured at 37°C were not



significantly lower ( $P > 0.05$ ) than the numbers obtained for cells grown at 22°C at any sample time.

In general, the number of attached cells on the cabbage increased with longer exposure times for all cultures regardless of cultivation temperature. However, there was a significant decline ( $P < 0.05$ ) in the actual rate of attachment after the initial 5 min of exposure. Levels of attached cells increased by only 0.61, 0.74, and 0.51 log CFU/cm<sup>2</sup> between 5 and 60 min of exposure for cells grown at 10°C, 22°C and 37°C, respectively. A further reduction in attachment rate was observed over the next three hours as the number of attached cells increased by only 0.4 to 0.5 log CFU/cm<sup>2</sup>. Ultimately, the number of attached cells reached between 6.2 and 6.5 CFU/cm<sup>2</sup> for all temperature regimes after 24 h, an increase of 1.0 to 1.3 log CFU/cm<sup>2</sup> over the 4 h samples. However, it is important to note that approximately one half (0.5 CFU/cm<sup>2</sup>) of this increase between the 4 and 24 h sample times was due to attached cells proliferating on the cabbage surface (data not shown).

Enumeration of adsorbed cells removed in the wash buffers resulted in a similar trend over time as that found for attached cells: lower levels were consistently obtained for cells cultivated at 10°C (Figure 4.1). The  $S_R$  values were calculated across all temperatures and ranged from 0.665 to 0.721 for the initial 5 min contact period (Table 4.2). Values obtained at all sample times for cells cultivated 22°C were significantly greater ( $P < 0.001$ ) than those grown at 37°C; whereas, values for only the 1 and 4 h treatments were significantly different between the 10 and 37°C cells. However, following the 24 h exposure period  $S_R$  values for all treatments were similar for all

temperatures. The general trend exhibited for all treatments was an increase in “strength of binding” to the cabbage over time.

#### 4.3.2. Effect of cabbage surface and strain differences

Twenty four strains of *Listeria* representing three species were examined for their ability to attach to intact and cut cabbage surfaces following a 3 h exposure period. Significant differences ( $P < 0.001$ ) were found between numbers of attached cells for the different strains on both intact and cut surfaces (Figure 4.2). Numbers of attached *Listeria* cells ranged from 3.84 to 4.78 log CFU/cm<sup>2</sup> and 4.82 to 5.90 log CFU/cm<sup>2</sup> on intact and cut cabbage, respectively. Attachment appeared to be related to properties of individual strains rather than species or serotype groupings. Strains with the highest numbers of attached cells on intact cabbage were also the most efficient in attaching to the cut surfaces. Two *L. monocytogenes* strains exhibited the highest degree of attachment to both surfaces: strain Lmap8 (serotype 1/2a) reached 4.78 and 5.90 log CFU/cm<sup>2</sup>, while LmK (serotype 4b from frozen peas) achieved 4.70 and 5.88 log CFU/cm<sup>2</sup> for intact and cut surfaces, respectively. *L. welshimeri* strains, Lwap8 and Lwap13, also attached well to both types of surface. In contrast, *L. welshimeri* strain Lwap7 and *L. monocytogenes* 1174 (serotype 4b) reached levels of less than 4.0 log CFU/cm<sup>2</sup> on intact tissue. *L. monocytogenes* 1174 was also the least efficient in attaching to the cut tissues (4.82 log CFU/cm<sup>2</sup>) along with *L. monocytogenes* strains, Scott A and EGD-e (4.98 and 4.90 log CFU/cm<sup>2</sup>, respectively). For all strains tested, colony counts from the cut surfaces were significantly higher in comparison with intact

surfaces ( $P < 0.001$ ) indicating a preference to attach to the former surface (Figure 4.2). Although most strains exhibited a difference of approximately  $1.0 \log \text{CFU}/\text{cm}^2$  for cells attached to the two surfaces, strains EGD-e, Scott A, Lwap6, and Lmap7 showed significantly smaller differences ( $P < 0.001$ ) than these values, with mean differences of 0.63, 0.68, 0.68 and  $0.74 \log \text{CFU}/\text{cm}^2$ , respectively.

#### *4.3.3. Scanning Electron Microscopy (SEM) of cabbage tissue samples and attached cells*

The abaxial (bottom) and adaxial (top) surfaces of the intact leaves were observed to be relatively flat and consistent in areas between the veins with more stomata on the abaxial surface than on the top of the leaf (Figure 4.3a); while cut tissues revealed a network of crevices, folds and protruding structures created from the disruption of leaf cells (Figure 4.3b).

The distribution of *Listeria* cells on the inoculated intact surface appeared to be randomized. No differences were observed for the numbers of *Listeria* cells attached to abaxial or adaxial leaf surfaces. In some cases, *Listeria* cells appeared to congregate around stomata (Figure 4.3c) whereas other stomata contained few or no cells. However, areas on the surface with small abrasions or tears frequently housed numerous cells, indicating a preference for exposed or damaged tissues (Figure 4.3d).

SEM images of cut cabbage leaves showed a greater number of cells observed per field compared to those observed on intact surfaces for the same exposure periods. Although cells were frequently found on protruding structures, they were most often

observed within crevices and folds (Figures 4.4a, 4.4b and 4.4c). Additionally, there appeared to be a tendency for cells to cluster after prolonged exposure times. In general, individual cells were most commonly found after the 1 h exposure period (Figure 4.4a). Although individual cells were also dominant at the 4 h exposure time, cell clusters were also observed for this sample period (Figure 4.4b). Greater numbers of these clusters appeared after 24 h exposure (Figure 4.4c). Additionally, cell clusters often exhibited what appeared to be the initial stages of an extrapolymeric coating (biofilm) connecting cells together. In some cases, stomata became filled with what appeared to be a *L. monocytogenes* biofilm matrix following the 24 h exposure (Figure 4.4d).

#### 4.4. Discussion and conclusions

In the present study it has been demonstrated that *Listeria* species, including the foodborne pathogen, *L. monocytogenes*, has the ability to rapidly attach to both cut and intact cabbage tissues under *in vitro* conditions. Irrespective of the temperature at which the cells were previously cultivated, significant numbers ( $P < 0.05$ ) of *L. monocytogenes* Scott A cells adhered to intact cabbage surfaces within 5 min of contact. The “binding strength” of these leaf associated cells was such that greater than 70% of these cells remained attached after vigorous washing. *L. monocytogenes* has been found to attach efficiently to the surfaces of several other types of produce commodities within short exposure periods. Gorski et al. (2003) reported that 2 to 3 log CFU/g of *L. monocytogenes* 10403 attached to cut radish tissue within the first 5 min during

attachment assays performed at four different temperatures. In another study, *L. monocytogenes* cells were shown to adsorb rapidly to the surface of potato disks during exposure times as short as 5 min. The rate of attachment then decreased over time (Garrood et al., 2004). The results also show that the rate of attachment to intact cabbage tissue decreases significantly ( $P < 0.05$ ) after this initial contact period. However, the relative strength of binding increased with prolonged exposure times.

Since all 24 strains of *Listeria* spp. tested here were able to effectively attach to cabbage tissues, the ability of *Listeria* spp. to attach to cabbage appears to be independent of those attributes that define particular species or serotypes within the genus. However, it was observed that certain strains were consistently most able to attach to cabbage tissues, but no correlation to particular *L. monocytogenes* serotypes or *Listeria* species could be discerned. Gorski et al. (2004) similarly found that there was a strain-specific effect for *L. monocytogenes* in terms of attachment to bean sprouts rather than a correlation to serotypes or lineages. To our knowledge, the present study is the first to compare other *Listeria* species in attachment assays using plant surfaces.

The condition of the cabbage surface is an important factor for attachment. In the present study, all strains, irrespective of species or serotype, exhibited a preference for cut surfaces as compared to intact tissue. These results concur with those of a previous study where attachment to cut and intact lettuce tissues was compared (Takeuchi et al., 2000). Here, SEM revealed an abundance of cells located in the folds and cuticular crevices of the cut surfaces compared to the intact waxy surfaces. Obviously, cutting these plant surfaces effectively increases the surface area available for bacterial

attachment. However, the mechanism by which a bacterial cell attaches to a leaf surface is highly complex and does not solely rely on the topography of the surface. It is also thought to be a function of the physicochemical properties of both bacterium and plant surfaces (Hirano and Upper, 2000; Ukuku and Fett, 2002). The lower numbers of attached *Listeria* observed for intact cabbage tissue may in part be due to increased repulsion of bacterial cells from the intact cabbage leaf surface.

The leaves of higher plants are protected by a polymeric cutin matrix and cuticular waxes which makes the overall surface hydrophobic (Walton, 1990). Important physicochemical properties such as surface charge, hydrophobicity and the electron donor and acceptor properties are instrumental in the initiation of adherence of cells to any surface (Mafu et al. 1991; Briandet et al., 1999a & 1999b; Ukuku et al., 2002). The surface of *Listeria* has been shown to be carrying a large negative charge (Mafu et al., 1991; Ukuku et al., 2002). Therefore, it is likely that the hydrophobic, waxy surface of the cabbage leaf would repel cells thereby reducing the number of collisions and opportunities to become anchored. Cutting these waxy surfaces exposes hydrophilic structures from within allowing for more intimate contact between bacterial cells and the leaf. Ukuku et al. (2002) reported that the attachment to waxy intact plant surfaces is positively correlated to the hydrophobicity of the bacterial cell. In their study, the more hydrophobic *Salmonella* spp. was able bind to the waxy cantaloupe with greater strength than the more hydrophilic *E. coli* and *L. monocytogenes*. This could also explain why *Salmonella* lacked preference between intact and cut lettuce surfaces, as observed by Takeuchi et al. (2000). In the current study, SEM revealed congregations of *Listeria* cells

along edges of natural minute tears on leaf surfaces, suggesting an attraction to these presumably more hydrophilic areas. Therefore, the increased cell counts observed for cut tissues may be not only the result of greater surface area being made available but also may be due to the newly exposed areas being more hydrophilic.

The conditions, such as growth temperature, used to cultivate bacterial cells have been reported to alter their attachment capabilities (Kim and Frank, 1994; Gorski et al., 2003). In the present study, growth temperature was shown to influence the binding strength of *L. monocytogenes*, Scott A to intact cabbage. When comparing the strength of attachment ( $S_R$  values) of cells cultivated at 3 different temperatures (10, 22, and 37°C), cells cultivated at 37°C exhibited significantly lower ( $P > 0.05$ ) binding strengths through the first 4 h of exposure to the cabbage. This may be related to the lack of production of flagella at this temperature which has been reported elsewhere (Vantanyoopaisarn et al., 2000; Gorski et al., 2003). However, after 24 h no significant difference ( $P > 0.05$ ) in binding strength for any of the temperature treatments, suggesting that physical or chemical changes to cells or cabbage coupons had occurred. Palumbo et al. (2005) recently identified numerous genes that were up-regulated in *L. monocytogenes* strain 10403 grown on cabbage as compared to control cells harvested from laboratory media. The majority of the genes identified were related to a multitude of cellular functions including metabolism, cell division, motility, and transport mechanisms. Therefore, it is conceivable that the extended exposure time (24 h) at 10°C provided the necessary time period to invoke metabolic changes that altered the bacterium's capability to attach to the cabbage surface, regardless of the initial cultivation temperature. The increase in binding

strength over time also may be indicative of *Listeria* cells binding to other *Listeria* cells already adhering to the surface coupled with the initiation of production of exopolymeric substances leading to biofilm formation. SEM analysis supports this theory since an increased prevalence of cell clusters over time was observed. Furthermore, what appeared to be an extrapolymeric matrix was formed around cells after extended exposure periods. These observations may explain the difficulty in removing less than 20% of *L. monocytogenes* Scott A cells in the PBS/Tween washes after 4 and 24 h.

In conclusion, this study shows for the first time the preferential attachment of *Listeria* spp. to cut cabbage tissues in comparison to intact surfaces. The attachment efficiency depended on specific strains and was not related to species or serotypes used in this study. Moreover, the binding strength was demonstrated to increase with contact time and showed a temperature dependent response only in the early stages of exposure. Because *Listeria* cells rapidly bind to plant surfaces and the binding strength increases over time, good manufacturing practices must be in place to avoid cross-contamination of other products. For example, in the production of coleslaw, it is important that the soiled outer leaves should never have any contact with surfaces where “clean” inner leaf products are subsequently processed. Since the present study clearly demonstrates that *Listeria* can attach rapidly at refrigeration temperature and initiate biofilm formation, conditions may become permissive to allow the psychrotrophic *L. monocytogenes* to multiply to dangerous levels during processing. It should be noted that plant surfaces are not homogeneous in nature, and they undergo dramatic changes during growth or after harvest and storage. Therefore, generalizations regarding the attachment, growth and



persistence of foodborne pathogens to one commodity may not hold true for another.

Further studies are necessary to enhance our understanding of the establishment of human microbial pathogens on raw or minimally processed fruits and vegetables.

Table 4.1 List of *Listeria* strains used for cabbage attachment and growth studies

Species/Strain Codes	Serotype	Description	Source or Reference
<i>Listeria monocytogenes</i>			
Scott A	4b	human clinical	Fleming et al., 1985
LmK	4b	frozen peas	Canadian Food Inspection Agency, Dartmouth, NS
Lm1174	4b	jelly pork tongue, FRN	de Valk et al. (2001)
Lm4	4b	coleslaw outbreak strain, CAN	Schlech et al. (1983)
ATCC 19115	4b	Human isolate	ATCC
EGD-e	1/2a	rabbit isolate	ATCC BAA-679
Lm568	1/2a	shrimp processing plant	Hefford et al. (2005)
Lmap1	1/2a	cold smoked salmon	Gnanou Besse et al. (2005)
Lmap2	1/2a	cured dry sausage	Gnanou Besse et al. (2005)
Lmap4	1/2a	cold smoked salmon	Gnanou Besse et al. (2005)
Lmap8	1/2a	raw sausage	Gnanou Besse et al. (2005)
ATCC 7644	1/2c	human isolate	ATCC
Lmap3	1/2c	raw pork/beef sausage	Gnanou Besse et al. (2005)
Lmap7	1/2c	raw sausage	Gnanou Besse et al. (2005)
<i>Listeria welshimeri</i>			
Lwap6		raw turkey meat	Gnanou Besse et al. (2005)
Lwap7		raw sausage	Gnanou Besse et al. (2005)
Lwap8		raw turkey meat	Gnanou Besse et al. (2005)
Lwap10		raw turkey meat	Gnanou Besse et al. (2005)
Lwap11		raw turkey meat	Gnanou Besse et al. (2005)
Lwap12		raw turkey meat	Gnanou Besse et al. (2005)
Lwap13		raw turkey meat	Gnanou Besse et al. (2005)
<i>Listeria innocua</i>			
Li228		food isolate	Unknown
Liap5a		cold smoked salmon	Gnanou Besse et al. (2005)
Liap9		raw sausage	Gnanou Besse et al. (2005)

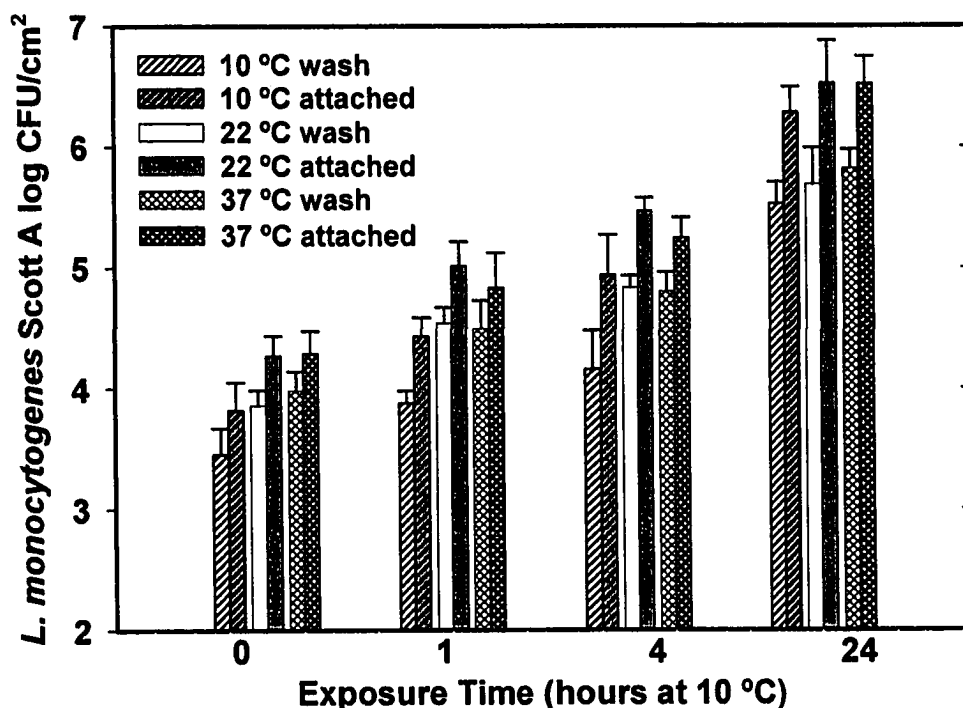
Table 4.2. The relative “strength of binding” ( $S_R$ ) of *Listeria monocytogenes* Scott A to intact cabbage surfaces over time at 10°C as influenced by previous growth temperature.

Cultivation Temperature <sup>a</sup> (°C)	Strength of Attachment ( $S_R$ Values <sup>b</sup> )			
	0 h	1 h	4 h	24 h
10	0.693 ± 0.049	0.770 ± 0.064 A	0.834 ± 0.080 A	0.842 ± 0.053
22	0.718 ± 0.028 A	0.739 ± 0.070 B	0.807 ± 0.047 B	0.863 ± 0.037
37	0.665 ± 0.053 A	0.680 ± 0.061 A, B	0.734 ± 0.028 A, B	0.827 ± 0.066

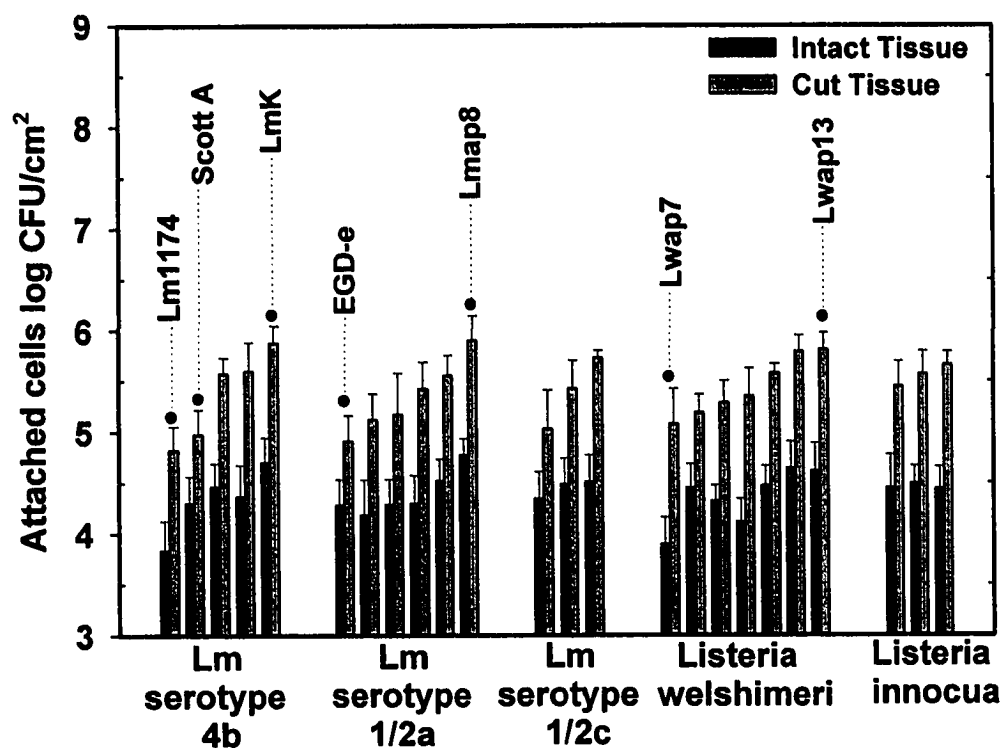
<sup>a</sup>Cells were grown in Brain Heart Infusion broth at the indicated temperature prior to the binding assay.

<sup>b</sup> $S_R$  = (attached cells/attached + adsorbed cells). Higher  $S_R$  values indicate “stronger” attachment. Values represent means ± standard deviations of 12 samples from three independent experiments (four coupons per replicate experiment).

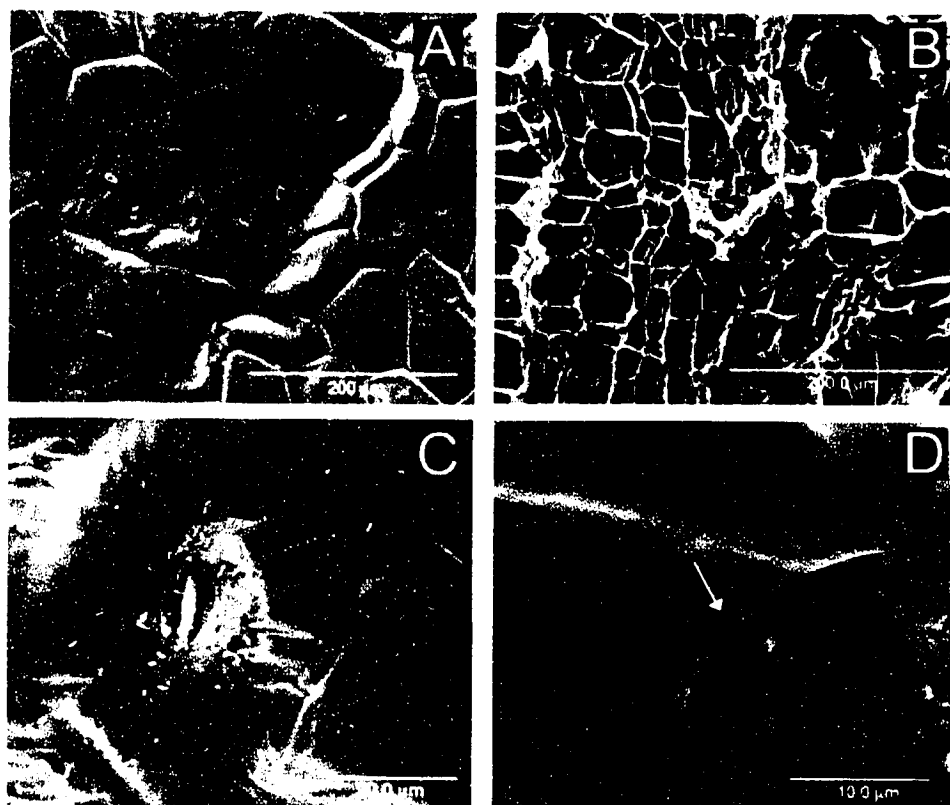
Values in the same column with the same letters are significantly different ( $P < 0.05$ ).



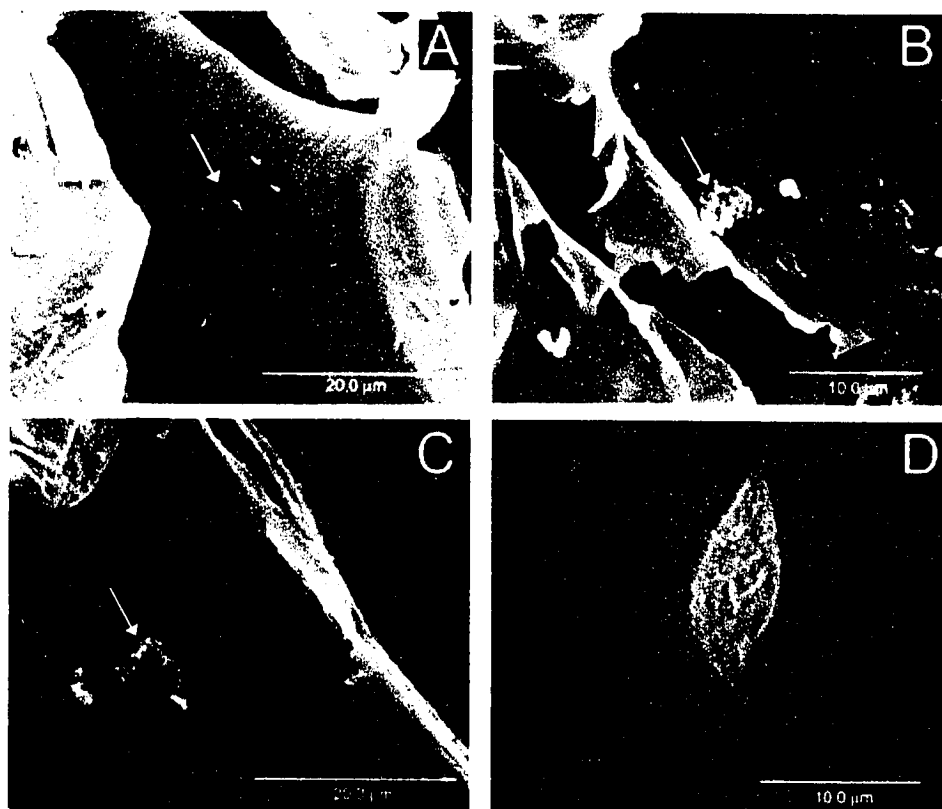
**Figure 4.1.** Attachment to intact cabbage surfaces of *Listeria monocytogenes* Scott A after its cultivation in Brain Heart Infusion broth at three temperatures. Cabbage coupons were immersed in PBS (pH 7.2) containing  $10^6$  CFU/ml. Numbers of adsorbed cells removed by washing in PBS with 0.1% Tween 20 (wash) and numbers of attached cells remaining on cabbage surface (attached) are grouped by growth temperatures and exposure times. Data indicate the means of 12 samples (four coupons sampled for each of three replicate trials). Error bars show the standard deviations.



**Figure 4.2.** Attachment of *Listeria* spp. strains to cut and intact cabbage tissues after 3 h at 10°C. Cabbage coupons were submerged in bacterial suspensions containing  $10^6$  CFU/ml. Some of the strains with high or low attachment efficiencies are named on the graph. Data presented for each strain is the mean for 16 samples (four coupons from each of four replicate trials). Errors bars represent the standard deviations.



**Figure 4.3.** SEM images of cabbage surfaces. A) Abaxial surface showing the topography of the intact leaf and the presence of both open and closed stomata; B) Cut cabbage tissue demonstrating an increase in available surface area through the exposure of internal tissues and the creation of deep crevices and folds; C) Higher magnification showing *L. monocytogenes* EGD-e cells congregated around a stoma on an intact surface after 24 h exposure time; D) Cluster of *L. monocytogenes* Scott A cells along a tear in leaf surface (arrow).



**Figure 4.4.** SEM images of *L. monocytogenes* Lm568 on cut cabbage tissue after various exposure times at 10°C. A) Singular cells (arrow) and small clusters dominate after 1 h exposure; B) after 4 h exposure many large clusters of cells (arrow) are observed; C) small colonies covered with some exo-cellular material (arrow) after 24 h exposure time on the surface of the cabbage; D) cells contained in a stoma appear to have formed a biofilm after 24 h exposure.

## CHAPTER 5

### **Survival and growth of *Listeria* spp. on intact or cut cabbage tissues: The enhancement of growth on shredded cabbage following a mild thermal treatment.**

#### **5.1 Introduction**

*Listeriae* provide unique challenges for the food industry. The widespread occurrence of this organism and its ability to survive harsh environments make *Listeria monocytogenes* difficult to control in processing facilities (Gravani, 1999; Holah et al., 2004). The frequent occurrence of this pathogen on fruits and vegetables enables its entry into food processing plants, and once access is gained, it can spread throughout the processing facility and contaminate otherwise “clean” food products (Hesick et al., 1989; Farber et al., 1989; Beuchat, 1996). Mostly, contamination of foods is by environmental strains of *L. monocytogenes* that are well adapted to deal with the stresses imposed by the natural and food processing environments (Francis and O’Bierne, 2005). In consideration of the continuum for the production and processing of fresh fruits and vegetables, there are many natural and implemented hurdles for *L. monocytogenes*, and other pathogens to overcome if they are to survive and proliferate. For example, *Listeria* residing on the surface of leafy vegetables must be able to withstand the broad temperature fluctuations



of the natural environment, UV radiation, desiccation, nutrient depletion and the actions of competitive resident microflora. Once harvested, *L. monocytogenes* may be subjected to cold shock as produce is often rapidly cooled to remove field heat and stored refrigerated. Other processing stress factors may include; shear forces from washing and handling, antimicrobial agents, such as chlorine in the wash water, and modified package atmospheres.

Recently, the merits of the implementation of a mild heat treatment for the processing of cut vegetables have been recognized (Loaiza-Valarde et al., 1997; Murata et al., 2004). In cut lettuce, the disruption of leaf tissues results in the induction of phenylalanine ammonia-lyase (PAL) activity, promoting the biosynthesis of polyphenols. The subsequent oxidation of these compounds by polyphenol oxidase leads to the formation of brown pigmented end products along the cut edges of the lettuce. Murata et al. (2004) found that 90 second treatments at 50°C followed by refrigerated storage at 4°C resulted in delayed onset of browning and increased the sensory attributes of cut lettuce. The reason for this improved product quality was thought to be due to either the inactivation of PAL or the preferential suppression of the enzyme in favour of the formation of heat shock proteins. Campos-Vargas et al. (2005) has since shown that the level of PAL mRNA and PAL activity was highest 24 h after wounding of lettuce tissue. However, a two minute heat shock (45°C) within five minutes of wounding resulted in a delayed increase in PAL activity but no increase in mRNA transcription. This may imply that heat shock interferes with the translation of PAL mRNA. Alternatively, the exposure

to heat may simply result in conformational changes to the protein thereby lowering its enzymic activity.

Preliminary research into the application of mild heat treatments for the improved quality of other vegetable commodities has also shown promising results. Mild thermal treatments of shredded white cabbage (*Brassica oleracea* ssp. *capitata*, L. type Lennox) resulted in an enhancement of shelf life from *ca.* 10 days to between 14-21 days (AFHRC, unpublished data). Although the results of such studies are encouraging to the fresh cut vegetable industry, there has not been accompanying food safety research to evaluate the impact of such processes on specific pathogens. However, the implementation of mild heat should be cautioned in light of previous studies conducted on lettuce. Li et al. (2001) observed significant increases in populations ( $P < 0.05$ ) of *E. coli* O157:H7 on iceberg lettuce after dipping for 90 s in 50°C water as compared to submerging the lettuce in water tempered to 20°C. The presence of 20 ppm chlorine in the water baths had no impact on the subsequent growth of *E. coli* O157:H7. Subsequently, a separate study conducted by the same authors, demonstrated enhanced growth of *L. monocytogenes* also occurred on cut lettuce following a heat treatment at 50°C (Li et al., 2002). Results showed that during storage there was a significant increase in numbers (1.7 to 2.3 log CFU/g leaf tissue) of the pathogen in the heat treated lettuce in comparison to non-heated samples. The authors speculated that this may have been caused by a reduction in the more thermally sensitive competitive natural flora, as well as the release of nutrients from the heat treated cut tissues. Decreases of 1-2 log CFU/g in the natural microbial inhabitants had been reported earlier when lettuce was subjected to

washes in warm (47°C) chlorinated water as compared to cold chlorinated washes (Delaquis et al., 1999). Previously, Francis and O'Beirne (1998) found that the initial load of co-cultured indigenous microflora had no significant effect ( $P > 0.05$ ) on the survival and growth of *L. innocua* in minimally processed shredded lettuce. However, individual inhabitants such as *Enterobacter* spp. appeared to be inhibitory to the growth of *L. innocua*. Delaquis et al. (2002) later noted no relationship between background microbial population and the increases in populations of *E. coli* O157:H7 or *L. monocytogenes* following treatments in warm chlorinated wash water. More recently, Delaquis and co-workers (2006) turned their attention to natural inhibitory compounds produced in iceberg lettuce. They have shown that wounded lettuce tissue elicits an antilisterial factor resulting in the decline in *L. monocytogenes* populations in lettuce extracts. However, pre-treatment of lettuce in warm chlorinated water (47°C) significantly reduced this effect.

In the previous chapter the ability of *Listeria* spp. to attach to intact and cut cabbage tissues was examined. Here, that work is extended to evaluate the same cabbage surfaces to support the survival and/or growth of *Listeria* spp. It was hypothesized that strains better able to attach to these surfaces would be the most successful in survival or proliferation following long term storage. Moreover, in light of the success of mild heat treatments to potentially extend the shelf life of shredded white cabbage well beyond 10 days, heat treated shredded cabbage was examined as a growth substrate for eight *Listeria* strains. Since enhanced growth has been observed for *L. monocytogenes* and *Escherichia coli* on cut lettuce in previous studies following mild heat treatments (Li et

al., 2001; Delaquis et al., 2002) it was hypothesized that increased growth of *Listeria* spp. would also occur on heat treated cabbage. The findings are reported in this chapter.

## **5.2 Materials and Methods**

### *5.2.1 Bacterial strains and culture media*

All media used in this study were obtained from (Oxoid Canada Inc., Nepean, ON). The bacterial strains used in this study were the same as those listed in Table 4.1 of the study described in the previous chapter. Stock cultures for all strains were stored at -80°C in brain heart infusion broth (BHI) supplemented with 15% glycerol.

Reconstitution of frozen stocks was carried out in BHI overnight at 30°C and culture purity was assessed by streaking on BHI agar. Inocula for growth studies were grown for 16 h in BHI at 30°C. Recovery of *Listeria* following growth on cabbage was conducted on *Listeria* selective media (Oxford formulation) containing the appropriate selective supplements (Oxoid Canada, Inc.).

### *5.2.2 Preparation of cabbage*

Cabbage test coupons were prepared from stored white cabbage heads (*Brassica oleracea* ssp. *capitata*, L. type Lennox) as described in the previous chapter. Briefly, two layers of outer leaves were removed to reduce the load of indigenous microflora associated with the cabbage and coupons were prepared representing both intact (disks) and cut (strips) surfaces. Coupons were stored at 5°C and were inoculated during the day

of preparation. Shredded cabbage was prepared by first removing the outer leaf layers and dipping the heads in chlorinated water. Active chlorine concentration was 100 ppm. Cabbage heads were air dried overnight and then shredded using a commercial slicer (Hobart Manufacturing Co., Troy, OH). Shredded cabbage was collected into plastic touts and stored at 2°C until ready for heat treatments. Heat treatments were conducted on the shredded cabbage in a continuous commercial scale steam system (Agriculture and Agri-Food Canada, Kentville, NS and ABCO Industries Ltd., Lunenburg, NS; Patent pending). Steam temperature was maintained 50°C and the time of exposure for the cabbage was 3 min. Following thermal treatment, the cabbage was rapidly cooled to 4°C by refrigerated forced air.

### *5.2.3 Inoculation and sampling of cabbage coupons*

*Listeria* strains were pre-cultured overnight at 30°C in BHI. An aliquot of 10 µl of pre-culture was used to inoculate 5 ml of BHI in screw-capped culture tubes. The cultures were incubated for 16 h at 30°C without agitation. Following incubation, 1 mL of culture was centrifuged for 1 min at 10,000  $\times$  g and the supernatant was discarded. The cell pellets were washed with 1 ml of phosphate buffered saline, pH 7.2 (PBS) and centrifuged as before. The pellets were diluted in PBS to achieve a final cell density of *ca.* 10<sup>6</sup> CFU/ml. For each *Listeria* strain, sixteen cabbage coupons representing intact cabbage surfaces (disks) were placed vertically in tissue culture trays and submerged in bacterial suspensions for 1 h at room temperature. The coupons were removed from the suspensions and residual loose cells were removed by three successive dips in sterile

distilled water. Inoculated coupons were placed in sterile Petri dishes such that individual coupons were not in contact with each other. The Petri plates were stored in Tupperware™ containers along with water-soaked Kimwipes® to maintain a high level of humidity and the containers were stored at 5°C. The process was repeated for cut cabbage surfaces (strips). At designated time intervals (0, 7, 21, and 28 days) four coupons were removed for each bacterial treatment. The edges of the coupons were trimmed as described in the previous chapter and placed in sterile 50 ml Falcon™ tubes with 20 ml of 0.1% neutralized peptone water. The coupons were homogenized for 20 s with a PowerGen 700 homogenizer (Fisher Scientific) equipped with a 20 mm generator with knives. The generator was sterilized between samples by submerging in 70% ethanol followed by two successive rinses with sterile distilled water. The homogenates were diluted in 0.1% peptone water and spiral plated (Spiral Systems, Bethesda, MD.) onto *Listeria* selective agar (Oxford formulation). The plates were incubated for 48 h at 35°C before colony enumerations. This experiment was repeated twice.

#### *5.2.4 Inoculation and sampling of heat treated shredded cabbage*

Eight *Listeria* strains (Scott A, EGD-e, Lm568, Lm4, Liap 5a, Li228, Lwap7 and Lwap13) were used for the growth studies on heat-treated and non heat-treated shredded cabbage. These strains represented three *Listeria* genera and included three serotypes (1/2a, 1/2c and 4b) of *L. monocytogenes*. Inocula were prepared as described above. Three hundred grams of shredded cabbage (either non-treated and heat treated) was weighed into stomacher bags containing filter inserts and 100 ml of bacterial suspension

( $10^6$  CFU/ml) was added to each bag. The bags were sealed and agitated for 1 h at room temperature to assure even distribution of cells. The mesh-filter inserts containing the cabbage were carefully removed from the stomacher bags and the residual liquid was allowed to drain for 15 min by placing the bag over a large sterile beaker inside a bio-containment hood. The cabbage was rinsed twice by successive dips in sterile water. The shredded cabbage was divided into 50 g aliquots and placed in thin polyethylene pouches, sealed and stored at 5°C. At designated time intervals (0, 7, 14, and 21 days), duplicate pouches were removed and 25 g of cabbage was combined with 225 mL of sterile 0.1% peptone water and homogenized in a stomacher for 1 min on high speed. Homogenates were diluted, spiral plated, and enumerated as before. The entire experiment was repeated 3 times.

#### 5.2.5 Statistical analysis

Growth experiments on cabbage coupons were repeated twice for each *Listeria* strain. Therefore, means for eight separate coupons of each surface type at four time intervals were compared for each strain. Strains were grouped according to *Listeria* species and serotype for *L. monocytogenes*, and means of populations on all group coupons were compared to that of other *Listeria* groups. Experiments for heated cabbage were repeated three times. For each sample time, the mean CFU/g of each inoculated heated cabbage treatment was compared to the levels obtained for their respective non-heat treated experiments. Analysis of variance was conducted using SYSTAT 11

software. The level of significance for all tests was  $\alpha = 0.05$ . Post hoc tests for the separation of means were performed using the Tukey multiple comparison method.

## 5.3 Results

### 5.3.1 Survival and growth on intact and cut cabbage surfaces

Twenty four *Listeria* strains were examined for their ability to survive and/or grow on intact and cut cabbage tissue. The strains used represented three species within the *Listeria* genus which included fourteen strains of *L. monocytogenes*, seven strains of *L. welshimeri* and three *L. innocua* strains. *L. monocytogenes* strains were further subdivided into three serotype groups; six 1/2a, five 4b and three 1/2c serotype strains. Examining the growth trends on intact cabbage tissue for grouped strains showed that there was no significant difference ( $P > 0.05$ ) between groups (Figure 5.1a). In general all groups exhibited a decline from initial counts. The decline ranged from 0.07 to 0.28 log CFU/cm<sup>2</sup> over the first week and increased to 0.14 - 0.31 log CFU/cm<sup>2</sup> after 14 days and finally to 0.65 - 1.03 log CFU/ cm<sup>2</sup> after the final 4 week sample period. This overall decline may be indicative of the lack of cell leakage and the unavailability of nutrients required for supporting the bacterial population. Conversely, on cut tissues there was a general increase in cell numbers through the first 14 days of storage at 5°C followed by a reduction in numbers over the remainder of the storage period (Figure 5.1b). The rate of increase over the first 7 days of incubation was significantly slower ( $P < 0.05$ ) for the serotype 4b group (0.28 log CFU/cm<sup>2</sup>) as compared to the *L. innocua* and serotype 1/2a



groups ( $\sim 0.56 \log \text{CFU/cm}^2$ ). However, after 14 days pair wise comparisons of the net increase from day 0 to day 14 were significantly greater for *L. monocytogenes* serotype 1/2a strains over the other groups ( $P < 0.05$ ). The serotype 1/2a group displayed a  $1.02 \log \text{CFU/cm}^2$  increase, whereas the mean increases for the other groups ranged from 0.68 to  $0.81 \log \text{CFU/cm}^2$ . After 28 days at  $5^\circ\text{C}$ , net decreases of 0.36, 0.38 and  $0.45 \log \text{CFU/cm}^2$  from the original bacterial load were observed for *L. innocua*, *L. welshimeri* and *L. monocytogenes* serotype 1/2c strains. However, the number of recoverable *L. monocytogenes* serotypes 1/2a and 4b continued to be greater than the initial day 0 counts with increases of  $0.12$  and  $0.38 \log \text{CFU/cm}^2$  for 1/2a and 4b serotypes respectively.

The overall trends for the group data are determined by the individual strains in these groups (Figures 5.2-5.6). Therefore, in cases where few individual strains represented a group (i.e., *L. monocytogenes* serotype 1/2c and *L. innocua*) the results of one strain could heavily influence the entire group. For example, the number of surviving cells of strain Li228 on cut cabbage at day 28 relative to the initial levels were significantly lower (*ca.*  $1.50 \log \text{CFU/cm}^2$  decrease) ( $P < 0.001$ ), than for the other two *L. innocua* strains that exhibited levels approximately equal to the initial cell load (Figure 5.5). Similarly, one strain in the serotype 1/2c group was able to proliferate at a much greater rate than the other two strains on cut cabbage (Figure 5.3). Lmap7 increased by  $ca. 1.0 \log \text{CFU/cm}^2$  after the first 7 days. This was significant ( $P < 0.01$ ) compared to  $0.2$  and  $0.04 \log \text{CFU/cm}^2$  increases for strains Lm7644 and Lmap3, respectively. The net increase from day 0 to day 14 was also significantly greater ( $P < 0.05$ ) for Lmap7 over Lm7644 and Lmap3, but this rate was obviously influenced by the increase in

CFU/cm<sup>2</sup> during the first seven days of storage. After 28 days at 5°C the numbers of all three strains dropped below initial levels. However, this decrease was significantly greater ( $P < 0.05$ ) for Lm7644 than for Lmap7 and Lmap3.

Variations among strains were also observed within other groups. Although no significant differences ( $P > 0.05$ ) in log CFU/cm<sup>2</sup> were observed for serotype 1/2a strains through the first 14 days on cut cabbage, strain Lmap8 displayed a significant reduction in log CFU/cm<sup>2</sup> after 28 days ( $P < 0.005$ ) (Figure 5.2). This was interesting since Lmap8 was one of the strains that exhibited a greater ability to attach to cabbage tissues. All other strains maintained cell numbers greater than the initial levels. Similarly, *L. monocytogenes* serotype 4b strains displayed no significant change in CFUs after 7 and 14 days ( $P > 0.05$ ) (Figure 5.4). However, strain Lm1174 had significantly greater levels ( $P < 0.05$ ) after 28 days than all other strains in this group. It was also the only strain to exhibit a continuous increase in CFU/cm<sup>2</sup> throughout the entire incubation period. Interestingly, this strain was also one of weaker strains in terms of attachment ability to cabbage tissues (Ells and Truelstrup Hansen, 2006). The fact that Lmap8 and Lm1174 displayed opposite abilities with respect to attachment and growth on cabbage suggests that an enhanced ability to attach to a plant surface does not necessarily influence its rate of proliferation.

The *L. welshimeri* group contained seven individual strains. Significant differences ( $P < 0.05$ ) in growth on cut cabbage were displayed among strains following incubation for 7 days at 5°C. Specifically, Lwap7 exhibited an increase of only 0.08 log CFU/cm<sup>2</sup> and levels of Lmap11 showed a 0.24 log CFU/cm<sup>2</sup> decrease after this period,

whereas, all other strains increased significantly ( $P < 0.05$ ) (0.27 to 0.48 log CFU/cm<sup>2</sup> increase). After 14 days, levels of Lwap7 and Lwap11 increased to 5.84 and 5.76 log CFU/cm<sup>2</sup>, respectively, but these values remained significantly lower than the other strains ( $P < 0.05$ ). Following the final sample period, levels of Lwap11 and Lwap12 were significantly ( $P < 0.05$ ) lower than the five other *L. welshimeri* strains; log 4.47 and 4.51 log CFU/cm<sup>2</sup>, respectively. All other strains displayed levels from 5.06 to 5.43 log CFU/cm<sup>2</sup>.

The results obtained for *Listeria* on the surface of intact cabbage dramatically differed from those observed on cut tissues. Most strains on intact cabbage displayed reduced numbers after day 0. However, through the first week of incubation at 5°C changes in the populations were subtle with only strain Lwap6 and Lwap11 displaying significant reductions in populations ( $P > 0.05$ ); decreases of 0.46 and 0.51 log CFU/cm<sup>2</sup>, respectively (Figure 5.6). Although no significant increases in CFU/cm<sup>2</sup> were observed ( $P > 0.05$ ), some strains maintained populations near the initial day 0 level after 14 days. After the 28 day sample period, only four strains showed a decrease of more than one log cycle. Specifically, these were Lwap11, Lwap8, Lm7644 and Lmap1 displaying reductions of 1.09, 1.10, 1.13, and 1.18 log CFU/cm<sup>2</sup>, respectively (Figures 5.2, 5.3 and 5.6). Interestingly, two strains, Lm1174 and Liap 5a, maintained levels very close to original day 0 levels (Figures 5.4 and 5.5). The fact that a more rapid decline was not observed over a 4 week period suggests that nutrient leakage on the intact tissue must be occurring.

### 5.3.2 Growth of *Listeria* spp. on heat treated shredded cabbage

Eight *Listeria* strains were examined for their ability to grow on shredded cabbage exposed to a mild thermal treatment. Four strains represented the *L. monocytogenes* genus (two strains from each of the 1/2a and 4b serotypes) and four non-*monocytogenes* genera (two strains each of *L. innocua* and *L. welshimeri*) were used in this study. Both heat treated and non-heat treated cabbage not inoculated with *Listeria* were incubated along with inoculated cabbage. Following 21 days at 5°C the uninoculated heated cabbage more closely resembled its original characteristics. However, the cabbage that did not receive a heat treatment displayed off colouration (greying). All eight *Listeria* strains displayed low level growth on non-heat treated shredded cabbage (Figures 5.7 and 5.8). The pattern of growth closely resembled that observed on the cut cabbage coupons with an initial increase in numbers over the first two week incubation period followed by a plateau or decline thereafter (Figures 5.2-5.6). Contrary to this was the pattern of Li228 that showed maximum increase of nearly 0.90 log CFU/g at 7 days and then declined rapidly during the remaining sample periods (Figure 5.8). The weakest growth observed was for strains Lwap7 and Scott A with maximum increases of only 0.41 and 0.47 log CFU/g, respectively, after 14 days at 5°C. All other strains had increases ranging from 0.82 to 0.97 log CFU/g after this same period (Figures 5.7 and 5.8).

Heat treating the cabbage had a highly significant effect ( $P < 0.001$ ) on the growth of all tested *Listeria* strains. Maximum levels for all strains were in the range of 7.94 to 8.74 log CFU/g of shredded cabbage (Figures 5.7 and 5.8). The *L.*

*monocytogenes* group had an increase in levels for each of the 3 weekly sample periods (Figure 5.7). The greatest increases for all strains were 3.37 and 3.21 log CFU/g at day 21 for *L. monocytogenes* strains EGD-e and Lm568, respectively. *L. monocytogenes* strains, Scott A and Lm4, also showed marked increases with day 21 levels being 2.84 and 2.13 log CFU/g greater than the initial levels. These increases culminated into maximum levels that were 2.36, 2.30, 1.93 and 1.43 log CFU/g greater than maximum levels on non heated cabbage for Scott A, EGD-e, Lm568 and Lm4, respectively. Similarly, the four strains examined in the non-*monocytogenes* group also exhibited enhanced growth, with Liap 5a and Lwap7 showing the greatest increases relative to initial cell loads (Figure 5.8). Lwap7 increased by 2.92 log CFU/g over initial levels by day 21 and Liap 5a increased by 2.73 log CFU/g during the same period. These levels represented a 2.17 and a 1.78 CFU/g increase above the maximum levels recorded for Lwap7 and Liap 5a, respectively, on non-heat treated cabbage. Interestingly, maximum increases for Liap 228 and Lwap13 occurred after 14 days, where increases of 2.05 and 2.53 log<sub>10</sub> CFU/g from the initial levels were recorded for the two strains, respectively. These values translated into increases of *ca.* 1.50 log CFU/g in comparison to the maximum levels obtained on cabbage receiving no thermal treatment. It is interesting to note that the two strains displaying the weakest growth on non-heated cabbage, Scott A and Lwap7, demonstrated the greatest increases in growth after the cabbage was heat treated.

## 5.4 Discussion and Conclusions

In this study the growth trends of several *Listeria* strains on three different cabbage surfaces was examined. These included intact tissue, cut tissue, and intact/cut tissues exposed to a mild thermal treatment. The results showed that the growth of *Listeria* is not supported on intact cabbage surfaces since all strains used here exhibited a decline over the 4 week incubation period. However, this decline occurred very slowly, hence some leakage of nutrients may have contributed to sustaining the population throughout the incubation period. From a practical perspective this information suggests that *Listeria* spp. can remain viable for long periods on pre- and/or post-harvest cabbage. Therefore, cells contaminating cabbage in the field may survive on the surface when cabbage is harvested and transferred to refrigerated storage. Studies have shown that *Listeria* spp. including *L. monocytogenes* have been isolated from field cabbage and on cabbage in packing sheds and the marketplace (Heisick et al., 1989; Prazak et al., 2002). This is important since the cut cabbage tissues in the current study were shown to moderately support growth of *Listeria* spp. Increases of between 1.0 and 1.5 log CFU/cm<sup>2</sup> following inoculation of cut cabbage for some strains of *Listeria* with the maximum CFU levels reaching *ca.* 6.7 log CFU/cm<sup>2</sup> was observed. Lower inoculum levels were not tested but it would be interesting to see if lower initial *Listeria* levels would proliferate at the same rate as was observed in these experiments. Although low dose levels of *L. monocytogenes* have been shown to cause infections in highly susceptible individuals, it is predicted that most cases are the result of ingesting high

numbers of the organism. The USDA risk assessment bureau estimates more than 82% of listeriosis cases are due to the ingestion of at least  $10^7$  cells per serving (Anonymous, 2005). Certainly, the levels obtained here would suggest a high risk factor; although, high initial inoculum levels were used. It is not known if the level achieved is the maximum carrying capacity of cut cabbage as a growth substrate since different cell densities in the inocula were not tested. Cabbage wounded in the field through abrasion, insect damage or microbial pathogens or through handling during harvest may provide surfaces conducive to sustaining *Listeria* spp. for lengthy time periods.

Francis and O'Beirne (2005) examined the growth of different serotypes of *L. monocytogenes* on modified atmosphere packaged dry coleslaw mix consisting of 80% shredded cabbage and 20% shredded carrots. They observed significant differences for the survival of different strains on the coleslaw mix stored at 8°C for 10 days. In general, most strains had an overall slow decline in numbers during the incubation period with no discernable correlation being drawn toward serotypes. However, some strains belonging to serotype 1/2a demonstrated increased growth (0.5 log cycles) or no depreciation in colony counts after 10 days. In this study increases of varying levels were observed over the first 14 days on cut cabbage stored at 5°C for all strains. Differences in the results obtained from these studies may rest in the fact that cut cabbage tissues were used as a substrate whereas the aforementioned study examined coleslaw mix. Carrots have been shown to possess an anti-listerial compound that may have contributed to the inability of *Listeria* to grow in the coleslaw mix (Nguyen-the and Lund, 1992; Beuchat et al., 1994). Moreover, the indigenous microbial population associated with shredded cabbage and

carrots may have influenced the growth of *Listeria* through competition for available nutrients or the production of inhibitory compounds (Francis and O'Beirne, 1998a; Francis and O'Beirne, 1998b). In the current study cabbage coupons carefully excised from the relatively microbial free inner leaves of the cabbage head were used. Previous work revealed that this tissue harboured very few culturable microbes at the onset of the experiment (Ells and Truelstrup Hansen, 2006).

Other studies have also reported significant increases in the populations of *Listeria* spp. inoculated onto shredded cabbage. Omary et al. (1993) observed a 2.5-3.0 log CFU/g increase for the population of *L. innocua* in shredded cabbage packaged in various films after 21 days at 11°C. It has also been shown that storage temperature and the type of packaging film can influence the growth of *Listeria* spp. on shredded cabbage (Farber et al., 1998; Bourke and O' Beirne, 2004). In general, *Listeria* strains packaged in air were able to increase in numbers in dry coleslaw mix when stored at 8°C in air or in packages consisting of microperforated films (Bourke and O' Beirne, 2004). Also, lowering the temperature to 3°C prevented the growth of the *Listeria* strains used in that study. The packaging film used here appeared to have adequate gas exchange (non anaerobic) which in turn was favourable for the growth of *Listeria* spp.

The most striking result obtained in the current study is the profound effect a mild heat treatment on cabbage had on the growth of *Listeria* spp. Significant increases ( $P < 0.001$ ) in colony forming units were observed for all eight *Listeria* strains tested, with *L. monocytogenes* strains achieving the highest levels. Greater than 2.0 log CFU/g increases were observed after the first week of incubation at 5°C. In light of evidence for long



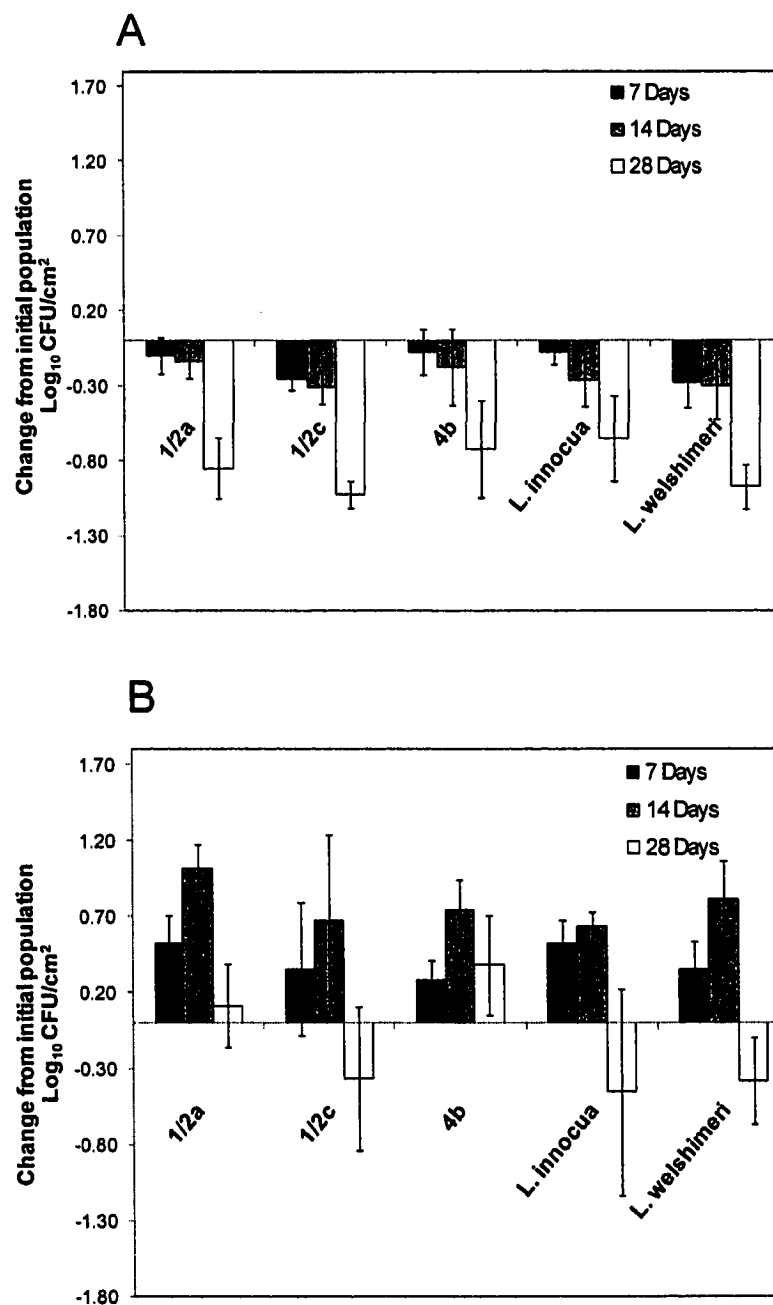
survival periods on intact cabbage, *Listeria* present on intact cabbage may enter the processing regime and be mixed in the shredded product. Further processing of the shredded cabbage may involve a mild thermal treatment since it has been demonstrated that such treatments are beneficial to the retention of sensory attributes and extending the shelf life of leafy vegetables (Loaiza-Valarde et al., 1997; Murata et al., 2004; Campos-Vargas et al., 2006). However, the time/temperature combinations used to enhance these attributes in leafy vegetables provide minimal lethality to *L. monocytogenes* (see following chapters). Therefore, following mild thermal treatments, a suitable growth substrate may be provided for *L. monocytogenes* and prolonged refrigerated storage could allow the pathogen to reach dangerous levels.

The positive influence that mild heat has on cabbage to serve as growth substrate for *Listeria* spp. is interesting. Beuchat et al. (1986) reported that populations of *L. monocytogenes* strains LCDC 81-861 (Nova Scotia coleslaw outbreak strain equivalent to Lm4 in this study) and Scott A rapidly decreased in heat sterilized (autoclaved) shredded cabbage stored at 5°C. However, in the present study both Lm4 and Scott A showed significant ( $P < 0.001$ ) increases in cell densities following mild thermal treatment of the cabbage (Figure 5.7). The severity of autoclaving the cabbage in the earlier study may have led to the formation of toxic compounds that were lethal to *Listeria* spp. Conversely, the mild heat treatment used here may have had a more subtle effect, selectively altering certain cabbage components that are normally inhibitory to *Listeria* spp. One possible explanation is that mild heat may affect the myrosinase-glucosinolate system present in capparales plants (Fenwick et al., 1983).

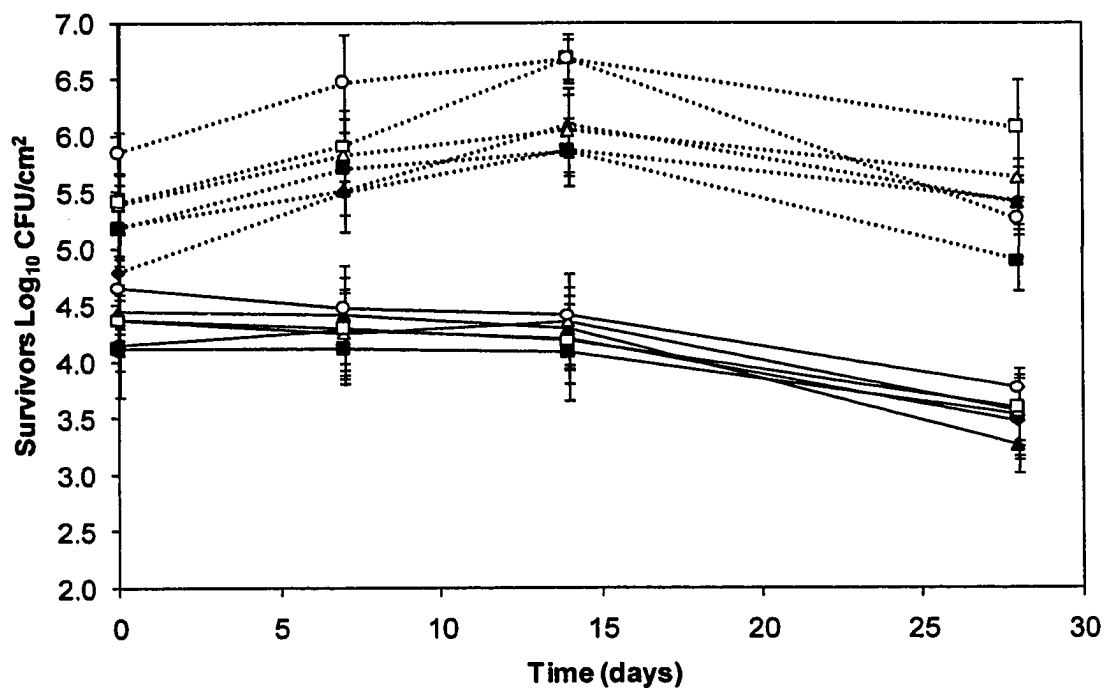
Glucosinolates are secondary plant metabolites occurring exclusively in dicotyledonous plants. More than 120 different glucosinolates are known to exist. The highest concentrations are found in plants belonging to the *Brassicaceae* family which includes broccoli, cabbage, cauliflower and Brussels sprouts (Fenwick et al., 1983). As part of the plant's natural defence system, damaged tissues allow these secondary metabolites to be mixed with the enzyme myrosinase (thioglucoside glucohydrolase). Normally myrosinase is compartmentalized, found in the cytoplasm of specialized myrosin cells that do not contain glucosinolates. However, when glucosinolates come into contact with myrosinase, hydrolysis occurs leading to the formation of various toxic compounds including isothiocyanates, nitriles, indoles, oxazolindinethiones, and thiocyanates (Mithen et al., 2000). These compounds act to inhibit microbial plant pathogens and insects. Heat denaturation of myrosinases could reduce enzymic activity and delay the formation of listerial-inhibitory compounds. The complete inactivation of these enzymes in red cabbage, (*Brassica oleracea* L. var. *Capitata* f. *rubra* DC), have been achieved by microwave heating (Oerlemans et al., 2006). The formation of inhibitory compounds via the myrosinase-glucosinolate system may also explain differences in growth data, from independent studies. One might speculate that inoculating cabbage immediately after shredding may be more conducive for the growth of *Listeria* than using shredded cabbage that has been stored for 24 or 48 hours. Delaquis et al. (2006) noted this effect when working with lettuce. *L. monocytogenes* was able to grow in extracts prepared from fresh lettuce but was inhibited when subjected to one to three day old extracts. Heat treatment of the extracts reduced the inhibitory effect. In a

more recent study, enhanced growth of *L. monocytogenes* and *E. coli* O157:H7 on broccoli and cut green beans was also observed following a hot water wash (52°C for 90 s) of the produce in comparison to produce washed at ambient temperature (20°C for 90 s) (Stringer et al., 2007). Interestingly, this elevated growth effect appeared to be significantly greater on the broccoli compared to the beans. Since broccoli also belongs to the crucifer family this may also suggest the involvement of the glucosinolate pathway.

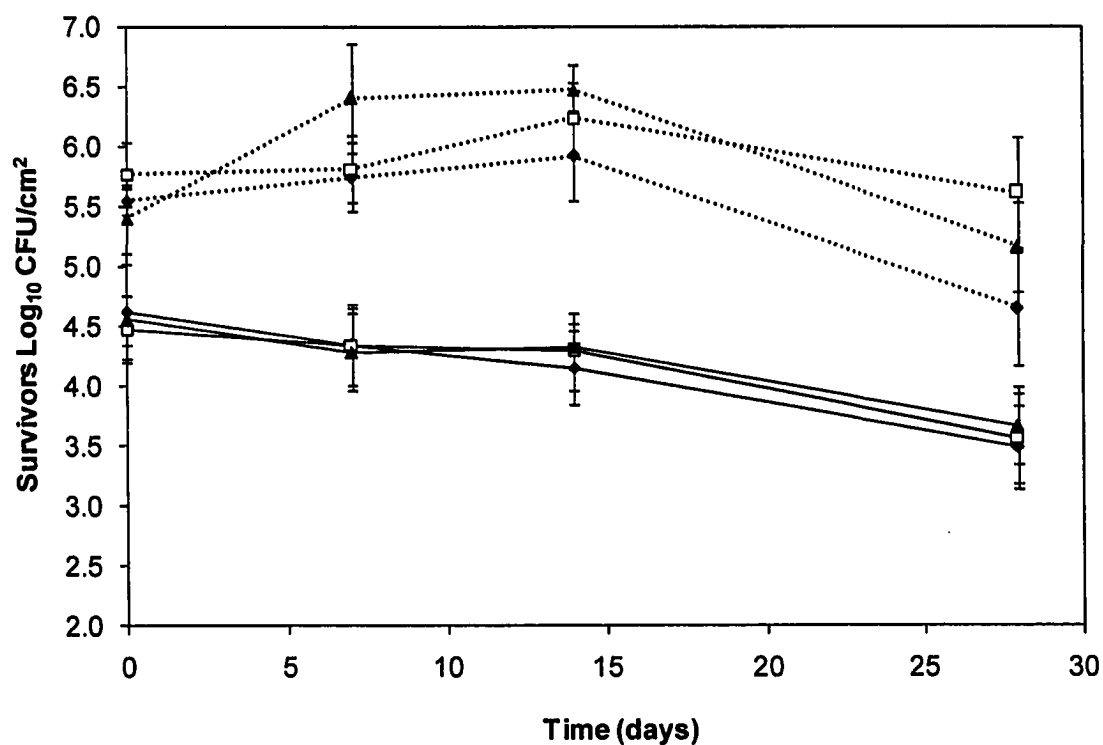
In conclusion, it was demonstrated for the first time that *Listeria* spp. can remain viable for relatively long periods on the surface of intact cabbage. All twenty four strains used in this investigation exhibited moderate growth at refrigeration temperatures when presented with damaged tissues. The subsequent mild thermal treatment of cabbage tissue significantly enhances the growth of *Listeria* spp. including the human pathogen *L. monocytogenes*. Since the goal of mild thermal processing is intended to extend the shelf life of shredded cabbage beyond 10 days, the results reported here would suggest that this practice may have serious risk implications if other effective deterrents are not implemented in the process. However, a more thorough investigation involving a broader range of variables (such as the influence of initial inoculum load) needs to be conducted to assess the true risk factor. Moreover, heat shock of *L. monocytogenes* may also induce enhanced resistance to other inimical barriers during downstream processing and this should be considered for future studies. The influence of mild heat on *L. monocytogenes* strain 568 is addressed in the following chapters.



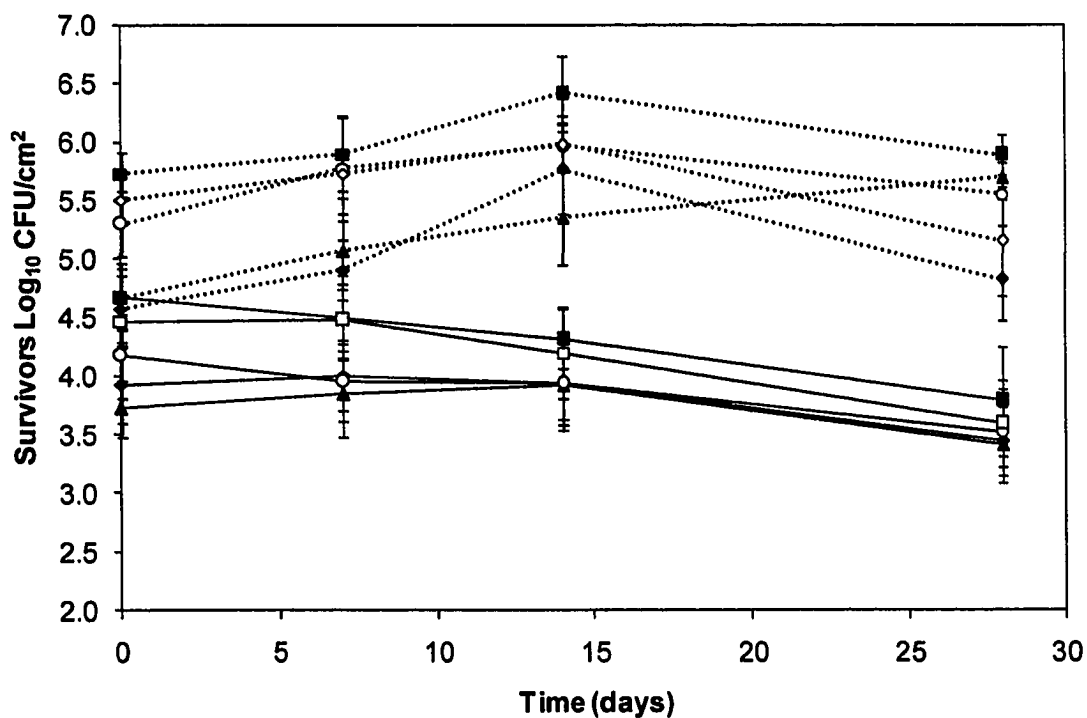
**Figure 5.1.** Changes in populations of *Listeria* spp. grouped by species or serotypes on A) intact or B) cut cabbage, stored at 5°C for 28 days. Bars for each group show the standard error of the means for the  $\text{log}_{10}$  change in  $\text{CFU/cm}^2$  relative to the initial population at day 0 for all strains in each group. Initial populations for individual strains in each group are given in Figures 5.2 to 5.6.



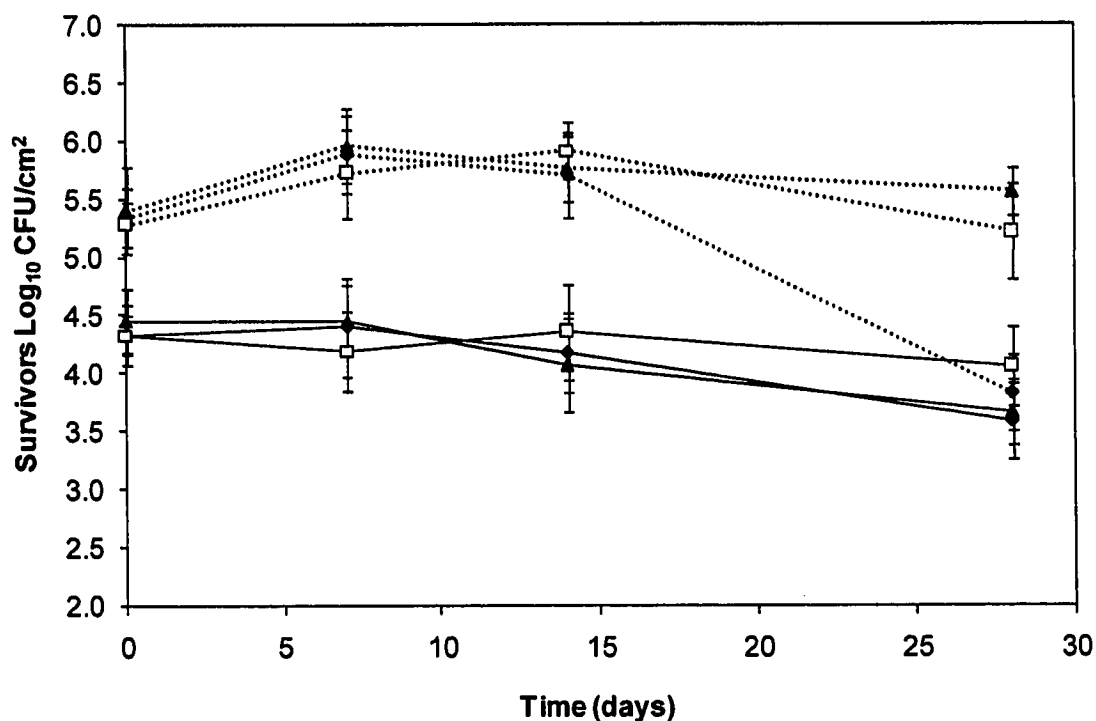
**Figure 5.2.** Survival and/or growth of stains of *Listeria monocytogenes* serotype 1/2a on intact (solid lines) and cut (dashed lines) cabbage coupons stored at 5°C for 28 days. The data represents the mean of eight coupons for each sample period (0, 7, 14 and 28 days) for each strain. The error bars represent the standard deviation for the populations on the eight coupons. The strains used were EGD-e (◆), Lm568 (■), Lmap1 (▲), Lmap2 (△), Lmap4 (□), and Lamp8 (○).



**Figure 5.3.** Survival and/or growth of stains of *Listeria monocytogenes* serotype 1/2c on intact (solid lines) and cut (dashed lines) cabbage coupons stored at 5°C for 28 days. The data represents the mean of eight coupons for each sample period (0, 7, 14 and 28 days) for each strain. The error bars represent the standard deviation for the mean populations on the eight coupons. The strains used were Lm7644 (◆), Lmap3 (□), and Lmap7 (▲).

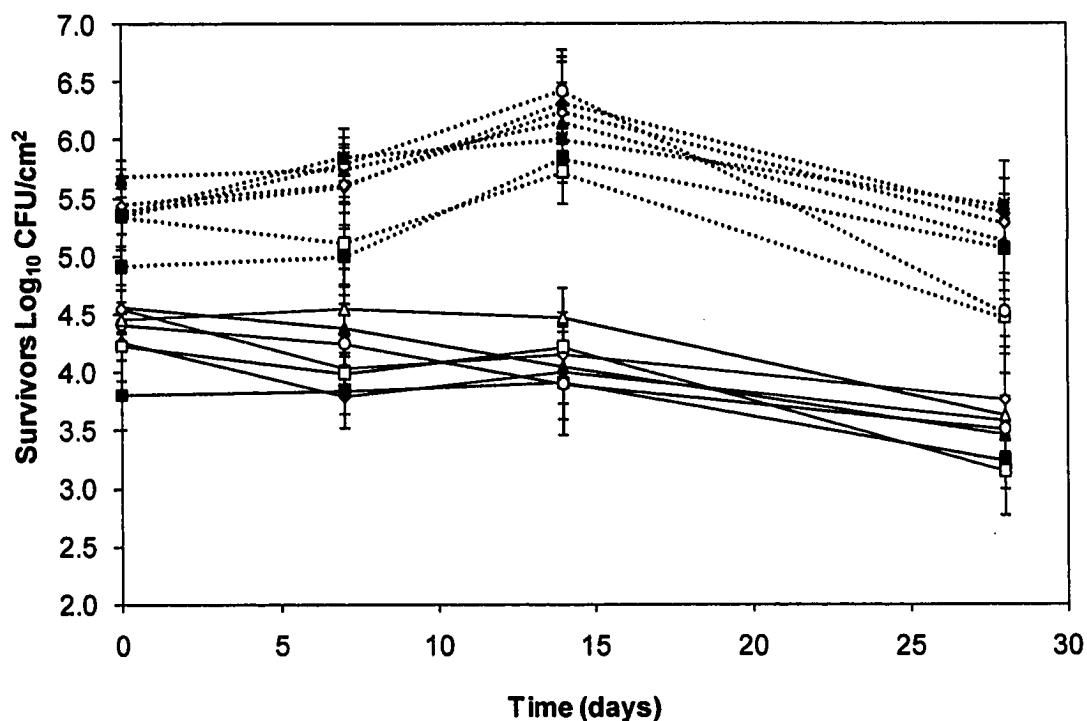


**Figure 5.4.** Survival and/or growth of strains of *Listeria monocytogenes* serotype 4b on intact (solid lines) and cut (dashed lines) cabbage coupons stored at 5°C for 28 days. The data represents the mean of eight coupons for each sample period (0, 7, 14 and 28 days) for each strain. The error bars represent the standard deviation for the populations on the eight coupons. The strains used were ScottA (◆), LmK (■), Lm1174 (▲), Lm19115 (○), and Lm4 (□).

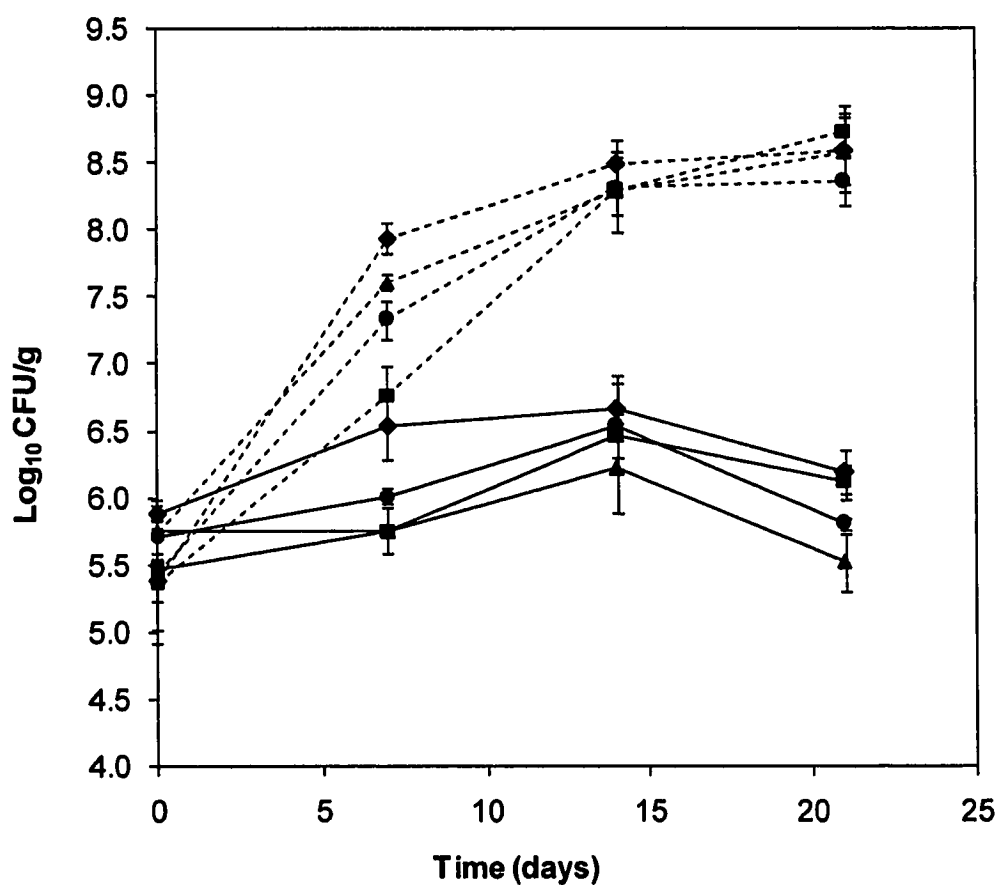


**Figure 5.5.** Survival and/or growth of strains of *Listeria innocua* on intact (solid lines) and cut (dashed lines) cabbage coupons stored at 5°C for 28 days. The data represents the mean of eight coupons for each sample period (0, 7, 14 and 28 days) for each strain. The error bars represent the standard deviation for the populations on the eight coupons. The strains used were Li228 (◆), Liap 5a (□), and Liap 9 (▲).

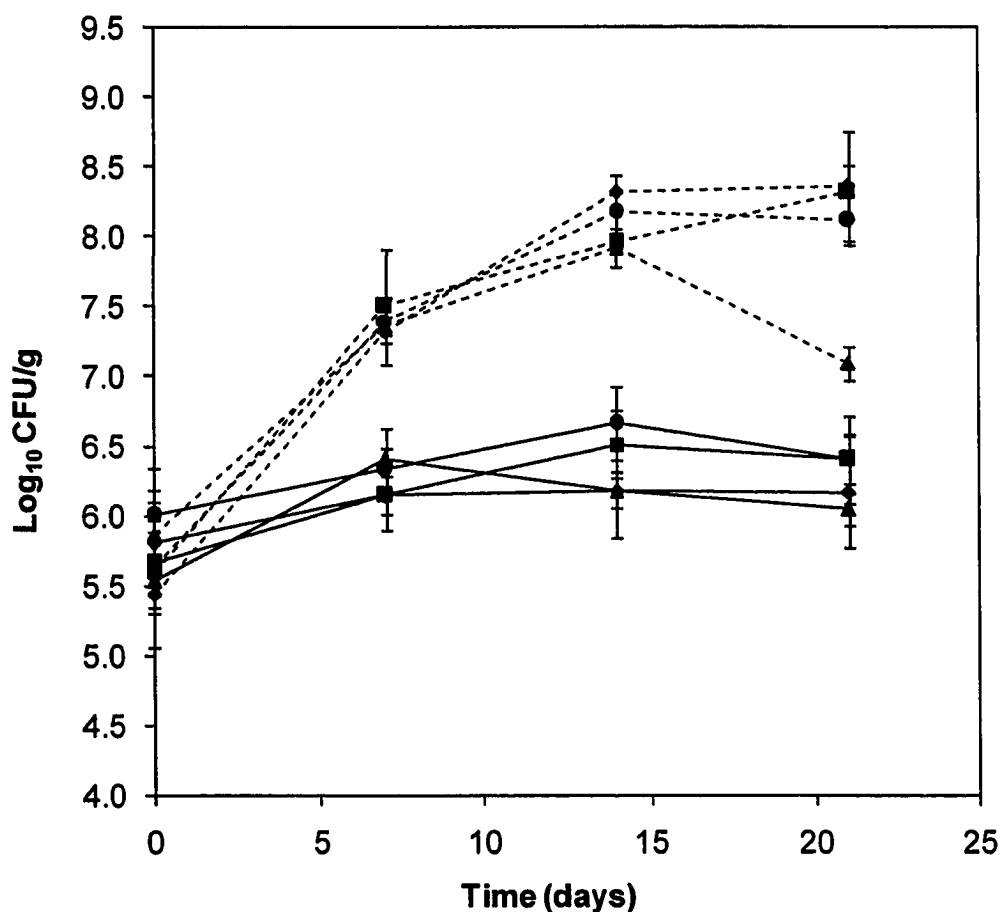




**Figure 5.6.** Survival and/or growth of strains of *Listeria welshimeri* on intact (solid lines) and cut (dashed lines) cabbage coupons stored at 5°C for 28 days. The data represents the mean of eight coupons for each sample period (0, 7, 14 and 28 days) for each strain. The error bars represent the standard deviation for the populations on the eight coupons. The strains used were Lwap6 (◆), Lwap7 (■), Lwap8 (▲), Lwap10 (◇), Lwap11 (□), Lamp12 (○), and Lwap13 (△).



**Figure 5.7.** Growth of *Listeria monocytogenes* (serotypes 1/2a and 4b) on shredded cabbage following a minimal thermal treatment. Strains used were ScottA (▲), EGD-e (■), Lm568 (◆), and Lm4 (●). Each strain was inoculated onto shredded cabbage that was either non-treated (solid lines) or treated (dashed lines) with mild heat (3 min at 50°C). Cabbage was stored in thin polypropylene packaging for 21 days at 5°C. Data points represent means of six samples (two samples from each time interval of three independent trials). Error bars represent the standard deviations for the populations for the six samples.



**Figure 5.8.** Growth of *Listeria* spp (*L. innocua* and *L. welshimeri*) on shredded cabbage following a minimal thermal treatment. Strains used were Li228 (▲), Liap5a (■), Lwap7 (◆), and Lwap13 (●). Each strain was inoculated onto shredded cabbage that was either non-treated (solid lines) or treated (dashed lines) with mild heat (3 min at 50°C). Cabbage was stored in thin polypropylene packaging for 21 days at 5°C. Data points represent means of six samples (two samples from each time interval of three independent trials). Error bars represent the standard deviations for the populations for the six samples.

## Chapter 6

### **Insertional mutagenesis of *Listeria monocytogenes* 568 reveals genes that may contribute to enhanced thermotolerance.**

#### **6.1 Introduction**

Thermal processing continues to be one of the most frequently used methods for the inactivation of microorganisms in food products. From a microbiological perspective, exposing a product to a specified time/temperature regime fulfills two primary objectives; 1) the elimination of pathogenic organisms from the food and 2) the reduction of spoilage organisms to extend product shelf life. Prior to the implementation of a heat process, much information needs to be considered to assure a safe and shelf-stable product. Hence, the design of any thermal process depends on factors such as the thermal resistance of the targeted microbial hazard, the thermal transfer properties of the food, the heat stability of the product (sensory considerations), and the intended end use and storage criteria after processing (shelf life) (Holdsworth, 2004).

*Listeria monocytogenes* is an important foodborne bacterial pathogen that has received a considerable amount of attention over the past 30 years. This attention is justified due to the high mortality rate for people contracting foodborne listeriosis and the fact that this Gram-positive, non-sporulating rod is widely distributed in the environment

(Vazquez-Boland, 2001). Therefore, it is not surprising that many food products may contain this bacterium. Moreover, the overall hardiness of *L. monocytogenes* is particularly problematic since it is capable of growing over a broad temperature range (0-46°C) (Doyle et al., 2002), at high osmolarities (up to 10% NaCl) (Sleator et al., 2003) as well as relatively low pH values (if acid adapted) (Sleator and Hill, 2003). *L. monocytogenes* is generally considered to have a relatively high thermotolerance compared to the vegetative cells of other foodborne bacterial pathogens (MacKey and Bratchell, 1989). This, combined with the organism's ability to grow at refrigeration temperatures, means that ineffective or inconsistent pasteurization processes resulting in low numbers of *L. monocytogenes* survivors may pose a serious hazard for the safety of minimally processed ready to eat foods. Consequently, the thermal resistance of *Listeriae* in laboratory media, and numerous food products, has been extensively studied (for an in-depth review see Doyle et al., 2001). Like other bacteria, the rate of thermal inactivation of *Listeria* spp. is dependent on several criteria. These parameters are related to the process, the heating medium, or the bacterium itself. With regard to process, temperature is of course the most important factor influencing inactivation rates. Secondly, the nature of the heating medium affects the heat transfer and the content of protective components may cause increases in bacterial resistance (Doyle et al., 2001). With respect to the target organism, the relative thermal tolerance depends on several factors including the growth phase of the cells, culture history and intrinsic properties of the strain itself (Pagán et al., 1997; Casadei et al., 1998; Doyle et al., 2001; Silk et al., 2002).

Numerous studies have reported broad variations in thermotolerance among strains of *L. monocytogenes* treated in a range of laboratory media or food items. The reported decimal reduction times (D-values) vary up to 4 times for cells treated under similar heat regimens. Mackey et al. (1990) reported  $D_{57^{\circ}\text{C}}$  values of 6.5 and 26 min in tryptone soy broth for the most sensitive and most tolerant *L. monocytogenes* strains, respectively. Similar differences have been found in food products, where Palumbo et al. (1996) reported that *L. monocytogenes* strain Scott A (clinical isolate) had a  $D_{64.4^{\circ}\text{C}}$  value 4.3 times greater than that of strain V-7 when heated in liquid egg yolk. Additionally, De Jesús and Whiting (2003) examined heat inactivation of twenty one *L. monocytogenes* strains from three genotypic lineages and found lineage III strains consistently had the lowest D-values. This observation also held true for both acid-adapted and non-adapted cells. Additionally, the strain to strain variation within each group was also significant regardless of the genotypic lineage.

It is because of this variance in thermal inactivation kinetics among strains that researchers have turned to the use of multi-strain cocktails of *L. monocytogenes* in order to evaluate the behaviour of the organism in various foods during thermal processing (Mazzotta, 2001a-c; Juneja, 2003; Nogueira et al., 2003). However, the reasons for these strain variations with respect to thermotolerance is unclear and research in this area is needed to provide a better understanding of molecular mechanisms involved in thermal resistance of this bacterium.

The objective of this study was to investigate some of the possible mechanisms which may contribute to the differences in thermotolerance between strains of *L.*

*monocytogenes*. Using the food isolate *L. monocytogenes* 568 (Lm568) as a model organism, it was hypothesized that mutants with increased thermotolerance could be obtained by random insertional mutagenesis in wild type Lm568 using the transposon Tn917. This approach would allow for the identification of important genes/mechanisms which contribute to the heat resistant phenotype and therefore enhance our understanding of how heat resistant strains of food pathogens evolve. This represents the first study whereby insertional mutagenesis is utilized to prospect for genes of potential importance for development of heat tolerant phenotypes in *L. monocytogenes* strain 568.

## 6.2 Materials and Methods

### 6.2.1 Bacteria strains, plasmids, media and culture conditions

The plasmids and bacterial strains used in this study are given in Table 6.1. Routine culturing of *L. monocytogenes* was carried out with either brain heart infusion (BHI) broth or agar (Oxoid Canada Inc., Nepean, ON, Canada). Luria-Bertani (LB, Oxoid) broth or agar was used to culture *Escherichia coli*. Stock cultures were stored at -80°C and reconstituted in BHI broth at 30°C. Where applicable, antibiotics (all from Sigma-Aldrich, Oakville, ON, Canada) were supplemented as follows: kanamycin (kan) 50 µg/ml; erythromycin (erm) 250 µg/ml for *E. coli*, and 0.03, 5 or 10 µg/ml for *L. monocytogenes*. Prior to heat treatments strains were pre-cultured for 24 h at 30°C before inoculation of test cultures. Recovery of *L. monocytogenes* survivors following heat treatments was carried out on Tryptic Soy Agar (Oxoid) supplemented with 6 g/L yeast

extract (Oxoid) and 10 g/L sodium pyruvate (Sigma-Aldrich) (TSA-YEP) and incubation at 35°C.

### *6.2.2 DNA extractions, purification and manipulations*

Large scale plasmid DNA extraction for the isolation and purification of the vector pTV1-OK was carried out using the Qiagen Maxi-Prep (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. Genomic DNA extractions for the Tn917 mutants were performed with an UltraClean™ Microbial DNA isolation kit as directed (MoBio Laboratories Inc., Carlsbad, CA, USA). All restriction endonucleases were provided by New England BioLabs (Pickering, ON, Canada).

### *6.2.3 Insertional mutagenesis of *L. monocytogenes* 568*

Insertional mutagenesis was carried out using the shuttle vector pTV1-OK which contains a temperature sensitive origin of replication and the Tn917 transposon (Gutierrez et al.1996). The vector was electroporated into competent *L. monocytogenes* 568 (Lm568) cells according to the method described by Alexander et al. (1990) with minor modifications. Briefly, the cells were grown overnight at 37°C in BHI. The culture was diluted 1/100 into 100 ml of pre-warmed BHI and incubated at 37°C on an orbital shaker at 200 rpm. When the culture reached an absorbance reading of 0.6 at 600 nm, it was placed on ice for 20 min. The cells were harvested by centrifugation at 6000 × g at 2°C for 15 min. Cell pellets were washed by resuspending in 100 ml of ice-cold 10% (v/v) glycerol (Sigma-Aldrich) followed by centrifugation as before. The wash was



repeated twice in 50 ml of 10% glycerol and the final pellet was resuspended in 0.5 ml of 10% glycerol. The suspension was divided into 40  $\mu$ l aliquots in pre-chilled 1.5 ml eppendorf tubes and used within 30 min of preparation. Electroporation of pTV1-OK was carried out in 0.1 cm cuvettes in a Micropulser™ (BioRad Laboratories Inc., Mississauga, ON, Canada) set to field strength of 1.0 kV. Following electroporation, the cells were resuspended in 1.0 ml of BHI and transferred to a disposable 10 ml culture tube. The cells were allowed to recover for 3 h at 28°C without shaking. Selection of transformed Lm568 harbouring the pTV1-OK plasmid was carried out by plating the suspensions on BHI containing kan (50  $\mu$ g/ml).

Transposon mutagenesis was performed following the protocol described by Gutierrez et al. (1996). Briefly, Lm568 transformed with pTV1-OK was grown overnight at 28°C in BHI broth containing 50  $\mu$ g/ml kan. This culture was used to inoculate fresh pre-warmed (44°C) BHI broth containing a sub-lethal concentration of erm (0.03  $\mu$ g/ml). Following overnight incubation at 44°C, the culture was diluted in peptone water and spiral plated (Spiral Systems, Bethesda, MD) onto several BHI agar plates supplemented with 5  $\mu$ g/ml of erm. To test for successful transposon insertion a Southern blot was performed on twelve isolated colonies, which were picked and grown overnight in BHI with 10  $\mu$ g/ml erm. The cultures were patched onto BHI plates with kan to confirm loss of the plasmid. Total DNA was then extracted from each culture using the UltraClean™ microbial DNA isolation kit (MoBio Laboratories Inc.). The DNA was cut with either *Eco*RI or *Hind*III restriction endonucleases, separated on a 1% TBE agarose gel (MoBio Laboratories Inc.) and transferred to a positively charged nylon membrane (Roche

Diagnostics, Laval, PQ, Canada) (Southern, 1975). Confirmation of the presence of Tn917 and randomized insertions was verified by probing the membrane with Digoxigenin (DIG) labelled (Roche Diagnostics) pTV1-OK. Subsequently, approximately 4300 mutants were picked from the original BHI plates and transferred to 96-well microtitre plates containing BHI with 10 µg/ml erm. Following overnight growth at 30°C, sterile glycerol was added to the cultures to a concentration of *ca.* 20% and the plates were then stored at -80°C.

#### *6.2.4 Selection of heat tolerant mutants*

Lm568 mutants were screened for increased tolerance to thermal stress. Frozen Tn917 library stocks were thawed and used to inoculate 100 µl of fresh BHI distributed in 96-well tissue culture plates (no antibiotics). The cultures were grown at 30°C overnight without agitation. For the thermotolerance screen, the overnight pre-cultures were used to inoculate 96-well thin-walled PCR plates containing 100 µl BHI in each well. The cultures were incubated for 16 h at 30°C. Following incubation, the PCR plates were placed in a Tgradient thermocycler (Whatman Biometra, Goettingen, Germany) heated at 52°C for 3 h and then cooled to 4°C. A multi-channel pipette was used to mix the cells in each well by pipetting the contents up and down several times. Five µl of cell suspension from each well was spotted onto BHI agar in square petri dishes (9.0 × 9.0 cm, Fisher Scientific, Ottawa, ON, Canada). The spots were allowed to dry on the agar surface in a bio-containment hood and the plates were incubated at 35°C for 72 h. Following incubation, the mutants were scored for growth. Most mutants did not grow

after the heat treatment. Thence, those displaying confluent or moderate growth were selected for a second round of screening.

The second screen involved the estimation of decimal reduction times (D-values) for each mutant at 52°C based on endpoint survivors after heat treatment for 1 h. The mutants and the Lm568 wild type strain were grown for 16 h at 30°C in BHI and diluted in 0.1% peptone water to achieve a cell density of *ca.*  $10^7$ - $10^8$  CFU/ml. For each mutant, 100 µl aliquots of the suspension were transferred to four thin-walled PCR tubes which were placed in the thermocycler programmed to maintain a constant temperature of 52°C. Once the thermocycler block reached 52°C, two duplicate tubes were removed and placed on ice for one min. Duplicate tubes were removed in the same manner after 60 min. Each pair of tubes, representing the initial and final exposure times (0 and 60 min), were diluted into 0.1% peptone water and spiral plated onto TSA-YEP with 1% sodium pyruvate. Survivors were enumerated after 72 h incubation at 35°C. The experiment was repeated three times for each mutant. The mean values for each trial were calculated and the  $D_{52^\circ\text{C}}$  -value for each mutant was calculated according to the formula:

$$D_{52^\circ\text{C}} = t/(\log_{10}N_0 - \log_{10}N_t) \quad (1)$$

where;  $N_0$  is the initial population density and  $N_t$  is the number of survivors after heating at 52°C for a specified time (i.e.,  $t = 60$  min). The mutants displaying a 15% increase in  $D_{52^\circ\text{C}}$  -value relative to the wild type strain in at least two of the three trials, were considered to have enhanced thermotolerance.

The final verification of thermotolerance was achieved by subjecting the mutants from the secondary screen to a 4D heat treatment (4 log reduction in survivors) relative to the wild type. A thermal death time curve for the Lm568 at 52°C was constructed. Lm568 was grown in BHI at 30°C for 16 h as before. One ml of culture was dispensed into a 1.5 ml microcentrifuge tube and then centrifuged at  $10,000 \times g$  for 1 min. In order to remove residual BHI, the pellet was washed twice and then re-suspended in 0.1% (w/v) peptone water. The suspension was diluted to a final cell concentration between  $10^7$  and  $10^8$  CFU/ml. One hundred microlitres of suspension was dispensed (in duplicate) into eight thin-walled PCR tubes and then heated in a thermocycler at 52°C. Two tubes were removed every 15 min (for 105 min) and cooled on ice for 1 min before diluting and spiral plating on TSA-YEP. Enumeration was conducted after a 72 h incubation period at 35°C. The entire experiment was repeated three times and the time required to obtain a *ca.* 4 log reduction in survivors was measured by plotting log survivors against treatment times. Once the 4D time was determined, suspensions of the transposon mutants selected from the second screening were prepared in the same manner as described above for the wild type. The cells were then subjected to the 4D heat treatment. Enumeration of survivors was carried out as previously described above. A subset of seven mutants displaying the highest thermal resistance at 52°C were chosen for further characterization at 56 and 60°C using the same protocol as described above.

### *6.2.5 Confirmation of single Tn917 insertions in selected thermotolerant mutants by Southern hybridization*

Total genomic DNA was isolated from each heat tolerant mutant using the UltraClean™ microbial DNA isolation kit according to the manufacturer's directions. In order to confirm a single event transposition of Tn917 into the chromosome of each mutant, the extracted DNA was digested with *Hind*III and fragments were separated by electrophoresis on a 1.0% Tris-Borate-EDTA (TBE) agarose gel. The DNA was transferred to a positively charged nylon membrane (Southern, 1975) and probed with DIG-labelled pTV1-OK.

### *6.2.6 Identification of disrupted genes containing Tn917 insertions*

Isolation of the flanking regions adjacent to the Tn917 transposon was carried out by inverse PCR. Briefly, each heat tolerant mutant was grown overnight in BHI at 37°C. Chromosomal DNA was extracted using the UltraClean™ microbial DNA isolation kit as described by the manufacturer. The DNA (*ca.* 200 ng) was digested with either *Hind*III or *Eco*RI restriction enzymes. The restricted DNA from either digest was serially diluted 1/10, 1/100 and 1/1000 and then self-ligated in a 100 µl reaction volume containing 1U of T4 ligase (Roche Diagnostics). Ligation mixes were then used as template for PCR amplification. The PCR reactions were performed using Klentaq LA (Sigma-Aldrich). Each 50 µl reaction volume contained: 5 µl 10× Klentaq buffer, 1 µl of dNTP mixture (10 µmol/l of each dATP, dCTP, dGTP and dTTP), 1 µl of each 10 mM stock primer pair, 5 µl of template DNA and 1 µl Klentaq LA. The primers for the

*Hind*III digested samples were as described by Begley et al. (2002); forward primer 4292 (5'-CAAAGCCTAGTAATGCGGTCATTCC-3') and reverse primer 4293 (5'-CTTGGA GAGTATAAATTTGACTTG-3'). The primers used for *Eco*RI digests were as described by Lévesque et al. (2004); Tn917-L (5'-TGTCACCGTCAAGTTAAATG-3') and Tn917-R (5'-GAACTATTTTTTGGGCGACG-3'). PCR cycle parameters consisted of a 5 min initial denaturation step at 95°C followed by 35 cycles of 94°C denaturation for 40 s, 55°C annealing for 40 s and 68°C elongation for 5 min. This was followed by a final extension step of 15 min at 68°C. PCR products were separated on a 1.0% TAE (Tris-acetate EDTA) agarose gel, excised and purified using GeneClean™ gel purification kit (Q-Biogene, CA, USA). TA cloning of the PCR products was carried out using the TOPO TA® cloning kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Sequencing of the cloned inserts was performed using the ABI BigDye™ Terminator Sequencing Reaction (Applied Biosystems Canada, Mississauga, ON, Canada). The sequences were aligned using BLASTn online software (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>) and comparisons were made using the GeneBank database for the complete sequence of *Listeria monocytogenes* strain EGD-e (Glaser *et al.*, 2001; GeneBank ID NC 003210).

#### 6.2.7 Statistical Analysis

Analysis of variance (ANOVA) was performed on the 4D reduction times for the mutant using SYSTAT® software. Comparisons of the means or individual treatments were carried out using the Tukey method at a 95% confidence interval. The students t-

test ( $\alpha = 0.05$ ) was used to compare the means of the 4D reduction time of the mutants to wild type Lm568.

## 6.3 Results

### *6.3.1 Screening Tn917 insertion library for heat tolerant mutants*

Approximately 4300 Tn917 insertion mutants were isolated and screened for increased thermotolerance. The spot plate method provided a satisfactory initial screen to identify potential mutants with increased resistance to an exposure to 52°C for 3 h. Since the method did not compensate for differences in initial cell numbers or growth phase, the authors acknowledge that some thermotolerant mutants may have been overlooked by this procedure. The 3 h treatment at 52°C, which was chosen based on preliminary trials (data not shown), resulted in few or no colonies on the agar plates for most mutants. A total of 132 mutants with either confluent growth or a large number of distinct colonies in the spotted area were selected from the initial screen (Figure 6.1). These mutants were further evaluated by the more robust second stage screening which entailed calibrating all cultures to equivalent initial cell densities and enumerating endpoint survivors. This allowed for a crude estimation of the  $D_{52^{\circ}\text{C}}$ -values for each mutant. Figure 6.2 is a graphical representation showing the number of survivors relative to initial population after 60 min at 52°C for six of the 132 tested mutants and wild type. Eighteen presumptive heat tolerant mutants displayed a *ca.* 15% increase in  $D_{52^{\circ}\text{C}}$ -values relative to Lm568 in at least 2 of 3 trials. These mutants were selected for the verification stage.

### 6.3.2 Comparison to 4D reduction process for Lm568

A thermal death curve was constructed for Lm568 at 52°C and the approximate time to achieve a four log reduction (4D) in survivors was estimated to be between 70 and 75 min. For convenience, thermal treatments of 75 min were used to evaluate the heat resistance of the transposon mutants. This time and temperature combination confirmed the increased heat tolerance of the mutants previously selected from the secondary screening protocol. All selected mutants were found to be significantly ( $P < 0.05$ ) more resistant than Lm568. This was achieved by using the student's t-test to compare the mean  $\log_{10}$  CFU/ml reduction for each mutant to the wild type. Table 6.2 lists the  $\log_{10}$  reduction in CFU/ml measured for the mutants after 75 min at 52°C. The  $\log_{10}$  numbers (CFU/ml) of survivors for wild type Lm568 were reduced by 4.30 while mutant 23D2 was highly resistant to this temperature treatment as the number of survivors ( $\log_{10}$ ) was only reduced by 1.87. The mutants are grouped according to the relative level of thermal resistance (Tukey pairwise comparison). Other notable mutants in order of resistance were 41D6, 32F11, 16H12, 7F6, 26A1, and 1B4 with  $\log_{10}$  decreases of only 2.26, 2.37, 2.70, 2.87, 2.99 and 3.01, respectively.  $\log_{10}$  reductions for the other remaining mutants ranged from 3.11 to 3.82.

The time required for 4D reductions for Lm568 at 56°C and 60°C were determined to be *ca.* 16 and 4 min, respectively, (Table 6.2) and the seven most thermotolerant mutants (groups A and B) at 52°C were chosen for further evaluation at these time/temperatures treatments. Interestingly, two of the mutants (7F6 and 16H12), that demonstrated higher thermal resistance relative to Lm568 at 52°C, were not more



heat resistant at elevated temperatures. However, the other five mutants continued to display significantly higher levels ( $P < 0.05$ ) of heat tolerance at both temperatures (Table 6.2). These heat regimes reduced the  $\log_{10}$  number of survivors for the wild type by 4.35 and 4.53 for 56 and 60°C, respectively. Mutant 23D2 continued to be the most resistant mutant at 56°C (2.31  $\log_{10}$  reduction after 16 min) but not at 60°C (3.81  $\log_{10}$  reduction after 4 min). The most resistant mutant to the 60°C regime was 41D6 with a  $\log_{10}$  reduction factor of only 2.81 which was significantly less than values obtained for all other mutants ( $P < 0.05$ ). Mutant 32F11, also demonstrated consistently high resistance to the higher temperatures as it displayed the second lowest  $\log_{10}$  reductions for both temperatures; 2.70 and 3.42 for 56°C and 60°C, respectively.

### 6.3.3 Identification of genes flanking transposon insertions

Southern hybridization of *Hind*III digested genomic DNA demonstrated a single Tn917 insert for each mutant (data not shown). The DIG-labelled pTV1-OK hybridized to three fragments for each mutant; two fragments of varying size containing flanking regions of mutant DNA, and a 1400 bp fragment common to all mutants which represents the internal *Hind*III fragment of Tn917. The gene regions flanking the Tn917 transposon insertions were isolated by inverse PCR and cloned into pCR® 2.1 (Invitrogen). The inserts were partially sequenced via the BigDye™ terminator reaction and the chromosomal DNA flanks for 15 of the 18 mutants were identified by referencing the complete genome sequence of *L. monocytogenes* strain EDG-e (serotype 1/2a) (Glaser et al., 2001; GenBank ID NC 003210). The site of the transposon insertion

could not be identified in three of the mutants (34F7, 39H2 and 41D6) since a PCR product could not be generated following self-ligation of the either *Hind*III or *Eco*RI restricted DNA. For the identifiable insertions, the affected genes included those involved in transport, metabolism, replication and repair, general stress, and structural properties. A list of the mutants with their respective positions of the transposon insertions and gene identities are given in Table 6.3.

There were only 2 pairs of duplicated insertions in the set of 18 mutants. Specifically, 1H1 and 16H11 were identified to have inserts in the same location of the proximal end of *lmo 1598*, a gene with high similarity to tyrosyl-tRNA synthetase (*tyrS*). The actual position was only 10 bases into the ORF. Downstream of this gene are ORFs encoding proteins known to be involved in catabolite control (CcpA), the formation of 3-deoxy-D-arabino-hept-2-ulosonate 7-phosphate from phosphoenolpyruvate and D-erythrose 4-phosphate (*aroA*), as well as a putative general stress protein (*lmo1601*). The other duplicated insert appeared in *lmo1413* for mutants 7F6 and 33E6. The phenotype for this insertion was an enhanced level of thermal resistance at 52°C, but not at the higher temperatures. This was identified in the EGD-e sequence to encode for a putative peptidoglycan bound protein containing a C-terminal LPXTG motif.

Genome maps were constructed for those mutants with the greatest level of thermotolerance (at 52°C) where the chromosome regions flanking the Tn917 had been identified by DNA sequencing. Figure 6.3 shows the relative position of transposon insertions and surrounding ORFs for the six most heat resistant mutants; 1B4, 7F6, 16H12, 23D2, 26A1 and 32F11. The transposon insertion for 1B4 was located in

*lmo1634* only 113 bp from the distal end of the ORF (Figure 6.3a). The predicted protein for this gene is highly similar to an alcohol-acetaldehyde dehydrogenase (*adh*). The fact that the transposon inserted so close to the distal end of the gene may indicate that a truncated form of the enzyme continued to be transcribed. Immediately downstream is an ORF that encodes a putative protein of unknown function. An ABC transporter (ATP-binding protein) and a putative membrane protein are also in close proximity. Upstream of the *adh* gene resides two operons involved in tryptophan biosynthesis.

Mutant 23D2 was found to possess the highest levels of thermal resistance at 52°C and 56°C (Table 6.2). The transposon was found to be inserted in ORF *lmo0219* (Figure 6.3b). The product of this gene is a predicted fusion protein of unknown function in *Listeria*. The N-terminal end of the protein is similar to the YacA protein found in *Bacillus subtilis* and the C-terminus is similar to hypoxanthine-guanine phosphoribosyltransferase. The adjacent gene downstream of this ORF encodes for the cell division protein FtsH (an ATP- and Zn<sup>2+</sup> protease). Immediately upstream are genes encoding for a putative protein with a polyribonucleotide nucleotidyltransferase domain (*lmo0218*), a protein similar to DviVC in *B. subtilis* (*lmo0217*), an operon containing a protein highly similar to *B. subtilis* YabO (*lmo0216*), a conserved membrane-spanning protein (*lmo0215*), and a transcription-repair coupling factor (*mfd*).

The point of insertion for mutant 26A1 was determined to be in a cluster of genes responsible for the transport of solutes (Figure 6.3c). Tn917 was inserted in the intergenic space between *lmo1423* and *lmo1424*. The later is a similar to the NRAMP (Natural resistance associated macrophage protein) magnesium transporters but the

function of *lmo1423* is currently unknown. The region contains the Bile operon (*lmo1412* and *lmo1422*). The 3.5 kb OpuC operon resides upstream of the putative manganese transport gene. Directly upstream of *lmo1421* on the opposite strand resides an operon thought to be involved in peptidoglycan biosynthesis.

An interesting mutant was 32F11 which demonstrated a substantial increase in thermal resistance at all three lethal temperature treatments used in this study. Tn917 was inserted at the distal end (150 bases before terminator) of an ORF encoding for alpha, alpha, phosphotrehalase (*lmo1254*); an enzyme responsible for the hydrolysis of trehalose-6-phosphate (Figure 6.3d). This gene is part of an operon that also includes a trehalose specific enzyme II<sub>BC</sub> of the phosphoenolpyruvate phosphotransferase system (PTS) and a regulatory gene with strong similarity to the GntR family of transcriptional regulators. The operon is essential for the utilization of trehalose as a carbon and energy source in related Gram-positive bacteria.

Transposon integration into the genome occurred at position 1370879/1370880 for mutant 16H12. This corresponds to protein 2 of the ComG operon (*comGB*) (Figure 6.3e). Expression products of this heptacistronic operon are essential for the uptake of exogenous DNA. Directly downstream of this operon, but in the opposite orientation is *lmo1340* which encodes for a protein with high similarity to *B. subtilis* YqgU (putative lipoprotein). Also in the opposite direction but upstream of the ComG operon, is another operon consisting of 3 genes; the first encoding for a putative aminomethyltransferase gene; the others encoding for subunits I and II, respectively, for a glycine dehydrogenase.

The remaining mutants displaying moderate increases in thermotolerance at 52°C had transposon insertions in a broad variety of genes (Table 6.3). The disrupted gene in mutant 7F5 was found to be located in the middle of *lmo0897*, which has high similarity to a sulfate transport gene. Interestingly, this gene is directly downstream of *rsbX*; an indirect negative control regulator of the alternative Sigma factor *sigB*. The Tn917 insertion for mutant 9E5 was located in an unusual position (1646472/1646473). This corresponds to the intergenic space between a hypothetical protein (*lmo1602*) in an operon also containing a putative general stress gene (*lmo1601*), and a putative aminopeptidase gene (*lmo1603*). Mutant 17A4 possessed a transposon insert in *lmo1412*, a gene encoding for FlaR, a DNA topology modulator which was previously described to affect transcription of *flaA* which encodes for flagellin (Sanchez-Campillo et al., 1995). The transposon insertion in mutant 35B4 was determined to be located in position 1010013/1010014 of the genome (*lmo0978*). The encoded protein is predicted to be similar to a branched chain amino acid aminotransferase. The role of these enzymes is in the biosynthesis and degradation of amino acids such as isoleucine, leucine and valine. Mutant 38A1, contained the Tn917 in an ORF with high similarity to a branched chain fatty acid kinase (*lmo1370*). The insertion was at the distal end of this gene which is the first in a polycistronic operon. Similar to mutant 9E5, the transposon insertion for 42F5 was also located in the intergenic space between two genes. Both flanking genes encode for proteins of unknown function. However, the upstream gene appears to belong to an operon involved in tRNA charging (*glyS*, glycyl tRNA synthetase, beta chain; *glyQ*,

glycyl tRNA synthetase, alpha chain). The downstream gene is the first in a bicistronic operon harbouring *dnaG* (DNA primase).

## 6.4 Discussion and Conclusions

Past research has demonstrated that strain variation influences the thermal resistance of *L. monocytogenes* in a variety of heating menstrea (Edelson-Mammel et al., 2005; Juneja, 2003; DeJesús and Whiting, 2003). The present study reveals for the first time that insertional mutagenesis with transposon Tn917 results in gene disruptions leading to the creation of mutants in a *L. monocytogenes* strain 568 background with enhanced heat tolerance. The structural and/or physiological changes that occur due to the altered genetic expression may provide insight into novel mechanisms leading to resistance to environmental stresses such as heat. Moreover, significantly different levels of thermal protection were obtained depending on the affected genes. For example, greater than a 100 fold increase in the number of survivors was observed for mutant 23D2 compared to the wild type at 52°C. For the other mutants, significant increases ( $P < 0.05$ ) in the numbers of survivors ranged from 0.5 to 2 log CFU/ml. Therefore, the progression to cellular death differs for each of these mutants, as the process depends on a complex array of molecular and physiological events prior to and during the exposure to the lethal temperature. The variance in observed phenotypes in this study appears to directly result from cellular changes incurred due to the disruption of the Tn917 harbouring genes, or result from cascading effects on other genes.

The loss of cell viability due to high temperatures cannot be attributed to a single event but instead, the progressive failure of multiple systems and cellular components including; the cell wall, outer membrane (Gram-negative bacteria), cytoplasmic membrane, ribosomes, proteins, and nucleic acids (Russell, 2003). Hitchener and Egan (1977) observed that 20% of the cellular lipopolysaccharide (LPS) was released from the outer membrane of *Escherichia coli* K-12 suspended in phosphate buffer when subjected to 48°C for 1 hour. Although, this LPS release contributed to cell death, it was not the sole mechanism, since increasing the leakage rate did not significantly alter the rate of inactivation. In comparison, the cell wall of Gram-positive bacteria is largely made up of highly cross-linked peptidoglycan and is far more resistant to thermal stress than the outer membrane of Gram-negative cells. Therefore, it is likely that lethal damage to other cellular components precedes damage to the cell wall. However, although dominated by peptidoglycans, the cell wall is highly complex displaying a host of surface structures (Navarre and Schneewind, 1999), which may affect the thermal death kinetics.

Several of the mutants generated in this study had transposon insertions within ORFs that could alter the surface topography of the cell. Mutant 7F6 contained a deletion in the *lmo1413* genes which encodes for a putative peptidoglycan bound protein with a LPXTG-like motif and unknown function. Interestingly, this protein has no ortholog in the non-virulent *L. innocua* which may suggest a role in pathogenesis (Calvo et al., 2005). LPXTG proteins are unique in that they are the only surface proteins shown to be covalently linked to the cell wall as the result of a membrane-anchored sortase (Desvaux et al. 2006). The actual linkage sites vary within the cell wall with Gram-positive genera,

and in *Listeria* the *meso*-diaminopimelic acid residue is the binding site in its peptidoglycan structure (Cossart and Jonquières, 2000). The LPXTG surface displayed proteins play a key role in the interaction with surfaces including host cells. Therefore, their role in surface adherence, cell to cell communication and virulence have intrigued researchers (Dahr et al., 2000; Cossart and Jonquières, 2000; Bierne et al., 2002; Cabanes et al., 2002). It is possible that the removal or augmentation of certain proteins on the cell surface alters cell flocculation or other cell-to-cell associations before and during the heat process. The clustering of cells during thermal inactivation studies has been identified as a contributing factor to the “shoulder and tailing” phenomenon (Russell, 2003). However, the microscopic examination of the mutants used here showed no evidence of cell “clumping” prior to, or after 30 min of heat exposure.

Another mutant (17A4) was found to have the Tn917 insertion in the ORF immediately upstream to *lmo1413*, but in the opposite orientation. The encoded protein is FlaR (Flagellum Regulator), a DNA topography modulator. The 18 kDa topoisomerase protein has specificity for sites along the DNA molecule therefore influencing its topography (Sanchez-Campillo et al., 1995). Moreover, FlaR is a regulator of *flaA*, the gene that encodes for flagellin. The disruption of *flaR* results in a non-motile phenotype devoid of flagella. Additionally, FlaR negatively regulates its own expression. The importance of flagella in motility, adhesion to surfaces and their thermally regulated production in *Listeria* has been discussed in detail (Peel et al., 1988; Gorski et al., 2003).

A common theme for a number of the disrupted genes in the heat tolerant mutants was transport and metabolism. These included mutants 1B4, 7F5, 26A1, 32F11 and



34F5. Mutant 1B4 was one of seven mutants demonstrating the greatest increase in thermal resistance. The point of Tn917 insertion was at the distal region of ORF *lmo1634*, which encodes for an alcohol acetaldehyde dehydrogenase (*adh*). Adh is a 104 kDa protein that was previously shown to be an important binding protein for the adherence of *L. monocytogenes* to mammalian cell lines and interestingly, the receptor for Adh was determined to be heat shock protein 60 (Hsp60) (Wampler et al., 2004; Kim et al., 2006). The *Listeria* homologue for Hsp60 is GroEL, which belongs to a group of molecular chaperonins produced at elevated levels during exposure to stressful environmental conditions (Gahan et al., 2001). The interaction of these chaperonins with other heat labile proteins within the cell during heat stress prevents misfolding and denaturation (Ben-Zvi and Goloubinoff, 2001). During normal growth conditions, basal amounts of the protein continue to be produced which is suggestive of a general physiological “housekeeping” role. The influence of a deletion to the *adh* gene on GroEL within *L. monocytogenes* is unknown. However, based on its specificity for Hsp60 from several mammalian cell lines, the heat tolerant phenotype selected here may suggest an interaction or regulatory function for Adh on heat shock proteins within *L. monocytogenes* itself. The role of this gene on thermal resistance as well as other stress conditions is currently under investigation.

The synthesis or uptake of compatible solutes is a common mechanism to combat stress conditions and *Listeria* possesses at least three mechanisms for the acquisition of compatible solutes, including two glycine betaine transport systems and a third system specific for L-carnitine. The latter system is a substrate binding protein-dependent ABC

transporter encoded by the tetracistronic OpuC operon (Fraser et al., 2000). It was reported that *L. monocytogenes* mutants lacking the OpuC continued to accumulate L-carnitine suggesting the presence of a secondary transport system (Fraser and O'Byrne, 2002). The authors speculated that, because of their close proximity (2.1 kb) and resemblance in DNA sequences to the OpuC operon, the ORFs *lmo1421* and *lmo1422* (formerly OpuB operon) could act as a low affinity transporter for L-carnitine. However, it was later demonstrated that although up-regulated during cold stress, this operon was not responsible for enhanced cold tolerance (Wemekamp-Kamphuis et al., 2004). Most recently, *lmo1421* and *lmo1422* have been designated the Bile operon (bile exclusion system), *bileA* and *bileB*, respectively. The expression of these genes is necessary for survival in the presence of bile (Sleator et al., 2005). Mutant 26A1, demonstrating increased thermotolerance at 52, 56 and 60°C, contained a Tn917 insertion in the intergenic space between *lmo1423* and *lmo1424*. The function of ORF *lmo1423* is not known and the predicted product of *lmo1424* resembles a NRAMP-like manganese transport protein. The positioning of *lmo1423* between the Bile and the OpuC operons is intriguing and it is easy to speculate whether or not its transcription product has an effect on either operon. Additionally, the close proximity of these genes to the Tn917 insertion in the 7F6 mutant raises more interesting questions about this region of the *Listeria* genome. The roles of both of these genes in heat stress tolerance and their relationship to the already characterized adjacent operons need to be investigated.

The role of the non-reducing disaccharide trehalose in the stabilization of both eukaryotic and prokaryotic cells under various inhospitable conditions has been

established (Argüelles, 2000; Elbein et al., 2003). Many microorganisms are able to synthesize *de novo* trehalose in the presence of various stressors (Eleutherio et al., 1993; Soto et al., 1999; Bearoudj et al., 2001; Kandrór et al., 2002). One of the most heat resistant mutants was 32F11 with a Tn917 insertion in a gene that encodes for the enzyme,  $\alpha$ ,  $\alpha$  phosphotrehalase (*treA*). This gene is part of a three gene operon that is organized similarly to the well characterized operon in *B. subtilis* (Helfert et al., 1995; Schöck and Dahl, 1996). In addition to *treA*, the operon also includes a trehalose specific enzyme II<sub>BC</sub> of the phosphoenolpyruvate transport system (PTS) (*treB*) and a regulatory gene similar to the *GntR* family of transcriptional regulators (*treR*). Trehalose is imported into the cell across the cytoplasmic membrane via the PTS and as a result, it is phosphorylated giving rise to trehalose-6-phosphate (T6P). Phosphotrehalase is specific for T6P, cleaving the molecule into two subunits; glucose and glucose-6-phosphate, which are immediately available for glycolysis. It can be speculated that the abolishment of phosphotrehalase activity in mutant 32F11 results in the accumulation of T6P in the cytosol. The phosphorylated sugar is not thought to serve as a compatible solute and it has been reported that the accumulation of T6P in yeasts is toxic to cells and impairs growth (Van Dijck et al., 2001). To our knowledge, T6P does not have similar glycolytic regulatory functions in Gram-positive bacteria since impaired growth does not occur in phosphotrehalase negative mutants, unless trehalose is the sole source of carbon (Duong et al. 2006). It may be plausible that this mutant may be benefiting from increased levels of the trehalose which is a well known osmolyte (Argüelles, 2000). Since the transposon insertion in 32F11 is located at the extreme distal end of the ORF, it is also possible that

a truncated protein continues to be transcribed for this gene. As a result, the product may have reduced enzymatic activity and as such, helps the cells to maintain a balance between toxicity and stability.

The cytoplasmic membrane is an important point of attack during a thermal process and damage results in leakage of intracellular constituents depending on the intensity and duration of the treatment (Russell, 2003). Microbial membrane fatty acid composition is carefully adjusted to match the growth temperature in order to maintain the proper liquid crystalline state and thereby membrane integrity (Suutari and Laakso, 1994). More than 90% of the fatty acid content of *L. monocytogenes* can be attributed to branched-chain fatty acids and the changes that occur to the fatty acid composition in response to, for example, decreasing temperatures, include the shortening of fatty acid chain length and switching from iso to anteiso branched forms (Annous et al., 1997). The transposon insertions for mutants 35B4 and 38A1 were determined to be in ORFs that could impact fatty acid synthesis and as a consequence, membrane structure. DNA flanking Tn917 in mutant 35B4 was sequenced to *lmo0978*, which encodes a protein with similarity to branched-chain amino acid aminotransferases. The production of branched chain fatty acids can be achieved through the metabolism of amino acids and  $\alpha$ -keto acids (Massey et al. 1976) and transcription of genes encoding these enzymes is up-regulated during stress conditions (Chia et al., 2001). Mutant 38A1 in this study was identified to have a disruption in a putative branched-chain fatty acid kinase (*lmo1370*). The primary role of these enzymes is to convert amino acids into branched chain fatty acids via substrate level phosphorylation. This ORF is part of a cluster of six genes

(*lmo1369* to *lmo1374*) that comprise the *bkd* (branched-chain  $\alpha$ -keto acid dehydrogenase) region of the *Listeria* genome (Zhu et al., 2005a). Northern blot analysis of this region suggests that all six genes are co-transcribed as a single 7 kb transcript. Transposon mutagenesis in ORFs *lmo1371* and *lmo1374* in *L. monocytogenes* 10403S were shown to increase cold sensitivity (Zhu et al., 2005b). Therefore, since the Tn917 insertion rests between these co-transcribed ORFs it is likely that normal synthesis of branch-chain fatty acids is affected which may give rise to the variant thermo-response in 38A1.

Fatty acid composition is not the only determinant that could affect the resilience of the cytoplasmic membrane to withstand environmental stresses. The membrane contains a complex array of proteins that act as enzymes, regulators, sensors, transporters, as well as having structural roles. Therefore, changes to the protein profile could alter the membrane's sensitivity to heat. Mutant 16H12 in this study was determined to have a transposon insert in the ComG operon; specifically in the second gene, *comGB*. In *B. subtilis*, ComGB is thought to be a membrane integrated protein (Albano et al., 1989; Chung et al., 1998) that closely resembles PilC in *Pseudomonas aeruginosa* which is essential for Type IV pili assembly (Dubnau, 1999). The coordinated synthesis of products from this operon is necessary for the development of competency for the uptake of exogenous DNA in *P. aeruginosa* (Albano et al., 1989). Although, competency in *Listeria* spp. has not been demonstrated, it is clear that these genes are transcribed hence impacting membrane and cell wall composition (Borezee et al., 2000). One may speculate that the inhibition of the formation of certain transmembrane structures involving ComGB may make the membrane less leaky during

exposure to elevated temperatures. Enhanced thermotolerance was only observed at 52°C and not the higher temperatures tested here. This may suggest that membrane plays less of a role in delaying cell death at high temperatures.

Mutant 7F5 was determined to have a transposon in *lmo0897*. The encoded protein is a putative sulphate transporter that likely spans the cytoplasmic membrane. Interestingly, the position of this ORF in the *Listeria* genome is immediately downstream of the *sigB* operon.  $\sigma^B$  is an alternative sigma factor that is responsible for the transcription of a subset of genes upon entry into stationary phase or during exposure to various stress conditions. These encoded products under the control of  $\sigma^B$  may act against the stressors directly or they may act as regulatory proteins for other genes. The close proximity of this transposon event to the *sigB* operon brings into question whether the insertion interferes with the regulation of  $\sigma^B$  expression in this thermotolerant mutant.

It is interesting that two of the thermotolerant mutants contained Tn917 insertions in non-coding regions of the genome. However, reports of altered phenotypes for osmotic stress, cold stress, and antibiotic resistance have previously been encountered due to intergenic transposon insertions (de Jesus Ferreira et al., 2001; Hu and Coates, 2005). In some cases, the intergenic inserts resulted in a disruption where adjacent genes are normally co-transcribed (de Jesus Ferreira et al., 2001). However, the insert for 9E5 is between two open reading frames oriented in opposite directions, hence co-transcription is highly unlikely. As for mutant 42F5, the Tn917 insert resides between two putative operons; *dnaG* and *glyS/Q*; the former encoding for a DNA primase and the latter encoding for glycyl-tRNA synthetase. It was also observed that another mutant (16H11)

with moderate thermotolerance at 52°C containing an insertion in a tyrosyl tRNA synthetase. Therefore, the mutation in 42F5 may be due to its close proximity to the *glyS* gene.

One of the most thermal resistant mutants encountered in this study was 23D2, which demonstrated increased thermotolerance at all three temperatures. The Tn917 insertion was mapped to a fusion protein of unknown function in *Listeria*. However, the encoded protein has similarity at the N- and C- termini to the YacA protein of *B. subtilis* and a hypoxanthine-guanine phosphoribosyl transferase (HGPT), respectively. The latter is a purine salvage protein responsible for the conversion of purines to nucleotides. Recently, it was found that a *Staphylococcus aureus* transposon mutant with the insert in a homologue of HGPT exhibited limited growth under high salt and limiting amino-acid conditions (Lithgow et al., 2004). Moreover, the organization of this area of the *S. aureus* genome is similar to that of *Listeria* since the ORF directly downstream of the HGPT homologue gene is similar to the *ftsH* gene in *B. subtilis*, an ATP- and  $\text{Zn}^{2+}$  protease. Mutation in *ftsH* results in restrictive growth when cells were exposed to numerous stress conditions (Lithgow et al., 2004). The reasons for the heat tolerant phenotype for this mutant are unknown; however, continued studies are ongoing.

In this study, it was demonstrated that various gene disruptions via transposon mutagenesis led to increased thermal resistance in *L. monocytogenes* strain 568. The extent of this enhancement varied depending on the affected genes. Therefore, the thermal inactivation of bacteria must be a “multi-hit” process whereby a cascade of events can lead to cell death. In order to develop safe food processes designed to

inactivate pathogenic bacteria, it is important to have a clear understanding of what makes certain strains more resistant over others when subjected to various stress conditions. The information presented here is useful in enhancing our understanding of the inactivation of bacteria by heat and other preservation processes important to the food industry. Further work will focus on the characterization of a number of these heat tolerant mutants to elucidate the mechanisms responsible for the observed phenotype.

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Table 6.1 List of bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	Reference or Source
<b>Strains</b>		
<i>Listeria monocytogenes</i>		
Lm568	Wild type; serotype 1/2a; shrimp processing plant	Hefford et al. (2005)
1B4	Tn917 mutant	This study
7F5	Tn917 mutant	This study
7F6	Tn917 mutant	This study
9E5	Tn917 mutant	This study
16H11	Tn917 mutant	This study
16H12	Tn917 mutant	This study
17A4	Tn917 mutant	This study
23D2	Tn917 mutant	This study
26A1	Tn917 mutant	This study
32F11	Tn917 mutant	This study
34F7	Tn917 mutant	This study
35B4	Tn917 mutant	This study
38A1	Tn917 mutant	This study
39H2	Tn917 mutant	This study
41D6	Tn917 mutant	This study
42F5	Tn917 mutant	This study
<i>Escherichia coli</i>		
RR1	HB101 recA <sup>+</sup>	
TOP10	F- mcrA $\Delta$ (mrr-hsdRMS-mcrBC) $\phi$ 80lacZ $\Delta$ M15 $\Delta$ lacX74 deoR nupG recA1 araD139 $\Delta$ (ara-leu)7697 galU galK rpsL(str <sup>R</sup> ) endA1 $\lambda$ -	Invitrogen
<b>Plasmids</b>		
pTV1-OK	repA(Ts)-pWV01Ts aphA3 Tn917(erm)	Gutierrez et al. (1996)
pCR $\otimes$ 2.1 TOPO	ColE1, amp <sup>R</sup> , Kan <sup>R</sup> , lacZ	Invitrogen

Table 6.2. Heat resistance of *Listeria monocytogenes* strain 568 and transposon mutants displaying increased thermal resistance expressed as log<sub>10</sub> reductions in survivor numbers (CFU/mL). Cells were suspended in 0.1% peptone water and treated at three temperatures (52, 56 and 60°C) for times previously determined to give approximate four log reductions (4D) for the wild type strain.

Mutant#	LOG <sub>10</sub> Reduction		
	75 min at 52°C	16 min at 56°C	4 min at 60°C
Lm568 (WT)	4.30 ± 0.23	4.35 ± 0.133	4.53 ± 0.33
1B4	3.01 ± 0.02 B	3.04 ± 0.23 A	3.60 ± 0.21 B
7F5	3.30 ± 0.41 C		
7F6	2.87 ± 0.34 B	4.40 ± 0.26	4.62 ± 0.23
9E5	3.52 ± 0.16 D		
16H11	3.35 ± 0.26 C		
16H12	2.70 ± 0.092 B	4.26 ± 0.20	4.65 ± 0.28
17A4	3.21 ± 0.27 C		
23D2	1.87 ± 0.20 A	2.31 ± 0.50 A	3.81 ± 0.05 B
26A1	2.99 ± 0.33 B	3.58 ± 0.26 B	3.82 ± 0.10 B
32F11	2.37 ± 0.27 A	2.70 ± 0.39 A	3.42 ± 0.12 B
34F7	3.73 ± 0.19 D		
35B4	3.58 ± 0.18 D		
38A1	3.82 ± 0.15 D		
39H2	3.11 ± 0.50 C		
41D6	2.26 ± 0.17 A	2.98 ± 0.52 A	2.81 ± 0.10 A
42F5	3.30 ± 0.42 D		

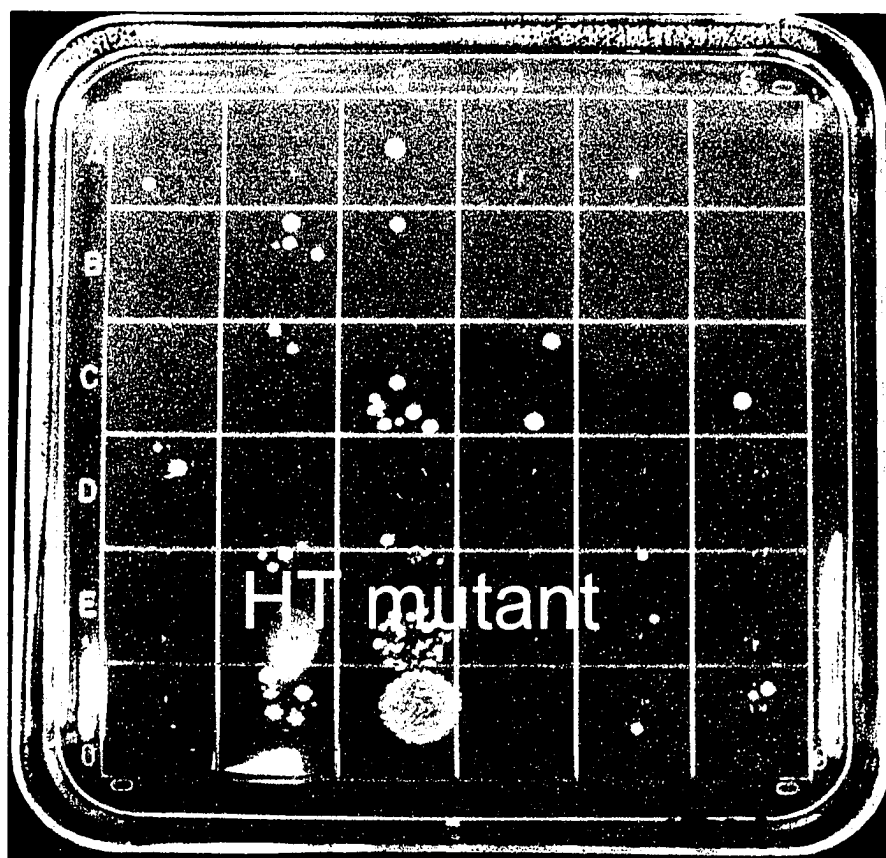
<sup>a</sup>Results represent the means for six samples (two replicates for each of three independent trials) ± standard deviation.

<sup>b</sup>Means followed by a letter were significantly different ( $P < 0.05$ ) than the wild type strain as determined by the students t-test.

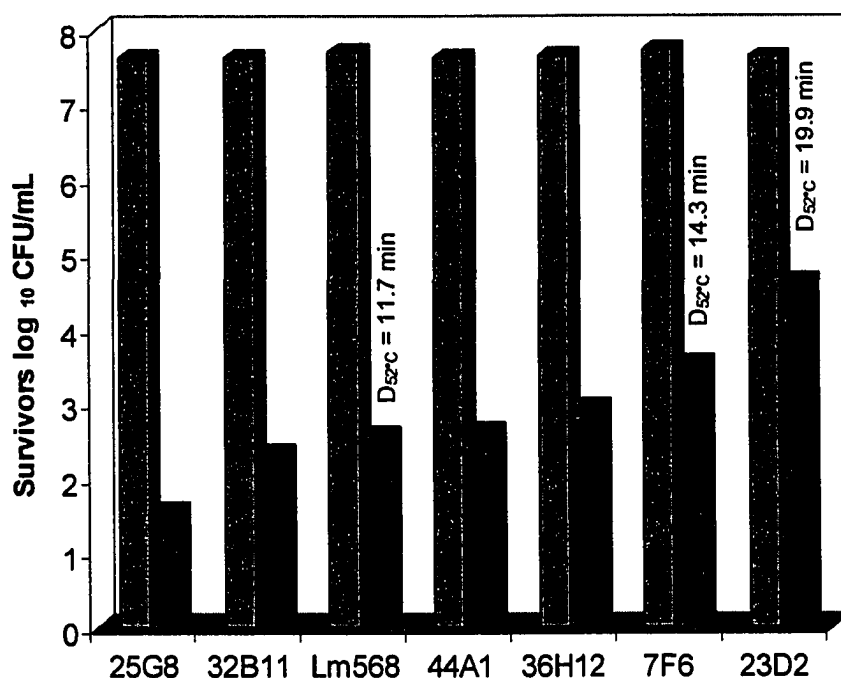
<sup>c</sup>Means followed by the same letter were similar in their relative thermal resistance as determined by pairwise comparisons (Tukey test).

Table 6.3. *Listeria monocytogenes* strain 568 transposon mutants displaying increased thermotolerance. The position of the Tn917 insertions are listed relative to the published genome sequence of *Listeria monocytogenes* EGD-e strain (GeneBank ID NC 003210).

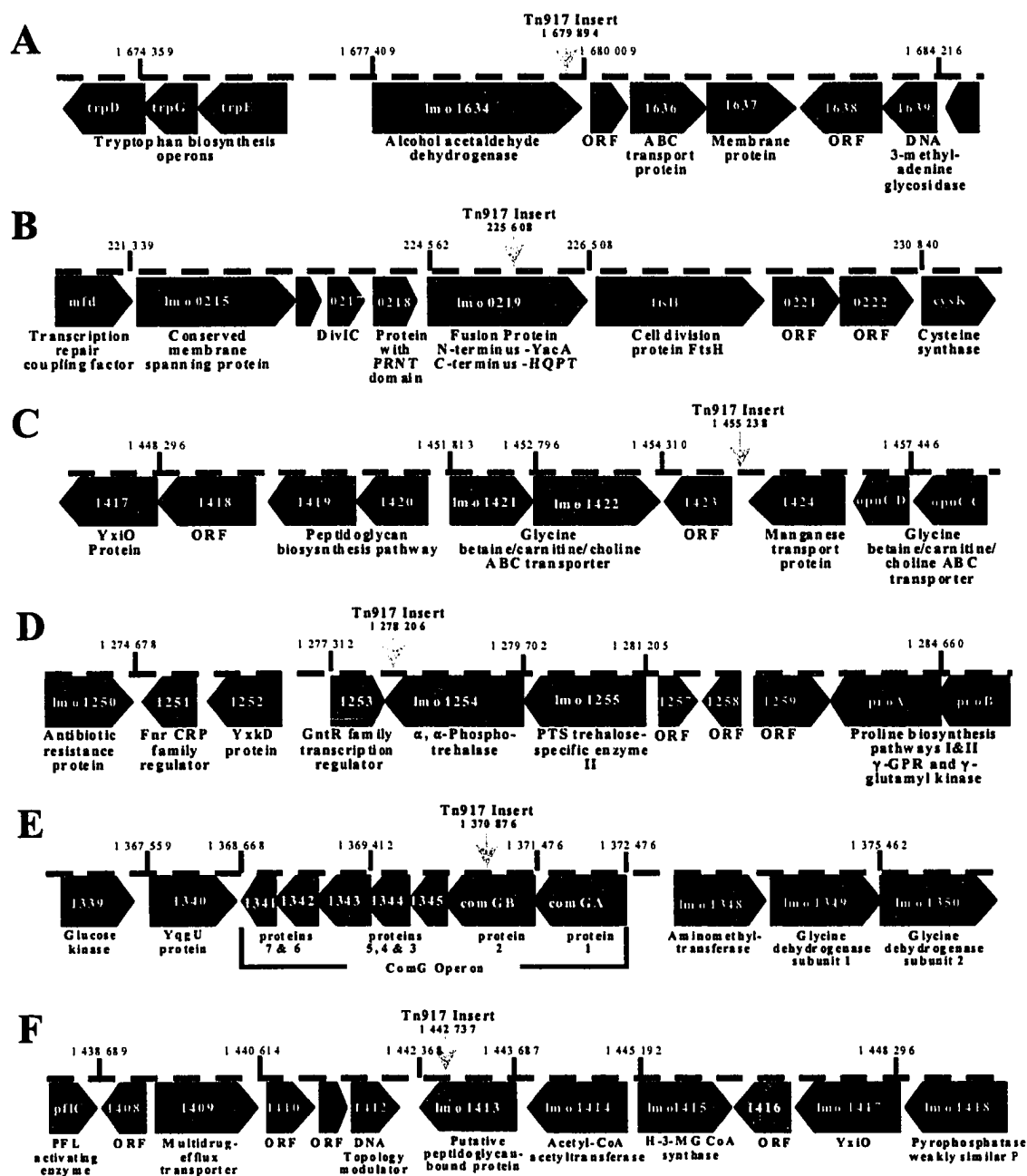
Mutant #	Similar Gene in GeneBank Entry for <i>L. monocytogenes</i> strain EGD-e (GeneBank ID NC 003210)	Position of Tn917
1B4	lmo 1634 - alcohol acetaldehyde dehydrogenase	1679894/1679893
7F5	lmo 0897 - putative sulphate transporter protein	0932833/0932834
7F6	lmo 1413 - putative peptidoglycan bound protein	1442737/1442738
9E5	Intergenic space between lmo1602 and lmo1603	1646472/1646473
16H11	lmo 1598 - tyrosyl tRNA synthetase	1641294/1641295
16H12	lmo 1344 - similar to comG operon protein 2	1370879/1370880
17A4	lmo 1412 - DNA topology modulation FlaR	1442003/1442004
23D2	lmo 0219 - fusion protein; N-terminus similar to <i>B. subtilis</i> YacA protein and C-terminus similar to hypoxanthine-guanine phosphoribosyl transferase.	225608/225609
26A1	Intergenic space between 1423 and 1424	1455238/1455239
32F11	lmo 1254 - alpha, alpha, phosphotrehalase	1278206/1278207
34F7	NA- unable to obtain PCR amplicon	
35B4	lmo 0978 - similar to branched-chain amino acid aminotransferase	1010013/1010014
38A1	lmo 1370 - branched-chain fatty acid kinase	1394515/1394516
39H2	NA- unable to obtain PCR amplicon	
41D6	NA- unable to obtain PCR amplicon	
42F5	Intergenic space between lmo1456 and lmo1457	1490145/1490146



**Figure 6.1.** Example of a spot-plate used for the primary screen for heat resistant mutants. Mutants were heat treated in a thermocycler at 52°C for 3 hours and 5  $\mu$ L of each was spotted onto TSA-YE amended with 1% sodium pyruvate. The plates were incubated for 72 hours at 35°C. Spots showing confluent growth or a large number of individual colonies were regarded “presumptive” heat tolerant mutants.



**Figure 6.2.** Secondary screening protocol used to determine prospective heat tolerant transposon mutants. Approximations of decimal reduction times (D-values) were calculated based on the initial population (light grey bars) and endpoint survivors (dark grey bars) after 60 min at 52°C. The graph demonstrates the variable death rate for 6 of the 132 mutants relative to wild type strain Lm568. Mutants displaying 15% increase in D<sub>52°C</sub> were considered to have enhanced “heat resistance” (cut-off was 13.5 min). The D<sub>52°C</sub> -value of the wild type was 11.7 min. For the mutants shown above, only 7F6 (D<sub>52°C</sub> = 14.3 min) and 23D2 (19.9 min) were deemed “thermotolerant”.



**Figure 6.3.** Map of genome regions showing the position of Tn917 insertions for the most heat tolerant mutants; A) 1B4, B) 23D2, C) 26A1, D) 32F11, E) 16H12 and F) 7F6 (mutant 41D6 was not mapped). Each ORF is indicated as a horizontal arrow indicating the abbreviated name or number (i.e. *Imo*). ORFs with the same colour in each locus are considered to be part of an operon. Nucleotide base numbers indicated are in reference to the sequencing origin from the *Listeria* genome project (Glaser et al., 2001). The position of Tn917 in each scheme is indicated by a downward facing arrow.

## CHAPTER 7

**Inactivation of *treA*, the gene encoding a phospho-(1,1) glucosidase, causes increased thermal and osmotic resistance in *Listeria monocytogenes* 568 when grown in the presence of trehalose.**

### 7.1 Introduction

The disaccharide trehalose ( $\alpha$ -D-glucopyranosyl-1,1- $\alpha$ -D-glucopyranoside) has a wide distribution in nature and is found in many biological samples including shellfish, insects, plants, mammals, bacteria and fungi. It is a non-reducing sugar with unique physicochemical properties that result in a stabilization effect when interacting with other molecules. It is thought that trehalose affords this stabilization in two ways; 1) acting in a similar manner as molecular chaperones interacting with macromolecules (i.e., proteins) to prevent their misfolding (Welch and Brown, 1996), and 2) stabilizing membranes and lipid assemblies through hydrogen bonding with phospholipids (Rudolph et al., 1986). Therefore, the presence of trehalose plays an important role in the long term preservation of biological materials under desiccation and freezing conditions. Moreover, the survival of many organisms during exposure to adverse conditions such as temperature extremes, desiccation, high osmolarity and oxidative stress has been documented (reviewed by Elbein et al., 2003).

Many different microorganisms have the ability to synthesize trehalose for a multitude of roles, including as energy source, structural component of cell walls and stress protectant (Elbein et al., 2003). As an energy source, it has been shown that trehalose and glycogen accumulate in the stationary cells of the yeast *Saccharomyces cerevisiae* during periods of starvation (Lillie and Pringle, 1980). This is interesting in consideration of the fact that exogenous trehalose cannot be utilized as a carbon source by most yeasts (Argüelles, 2000). However, it has been demonstrated that the rise in intracellular trehalose concentrations also serves to protect cells during heat stress (Eleutherio et al., 1993; Hottiger et al., 1994), high osmolarity (Sharma, 1997) and oxidative stress (Benaroudj et al., 2001). Structurally, esterified derivatives of the carbohydrate (i.e., trehalose monocorynomycolate and trehalose dicorynomycolate) form an integral component of the unique hydrophobic outer lipid bi-layer of the cell wall complex (cord factor) of *Mycobacteria* and *Corynebacteria*. Moreover, these related mycolic-acid-containing bacteria, possess all three known bacterial biosynthetic pathways for trehalose biosynthesis; an apparent necessity for the synthesis and incorporation of trehalose-containing cell wall components (De Smet et. al., 2000; Tzvetkov et al., 2003). Although, several reports regarding trehalose metabolism in various Gram-negative bacteria such as *Enterobacter sakazakii* (Breeuwer et al., 2003), *Pseudomonas fluorescens* (Matthijs et al., 2000), *Rhizobium japonicum* (Streeter, 1985), and *Salmonella enterica* serovar Typhimurium (Cánovas et al., 2001) have been documented, *Escherichia coli* is the only member of this group that has received a thorough analysis for trehalose metabolism (Rimmele and Boos, 1994; Klein et al., 1995;



Horlacher and Boos, 1997). Large amounts of trehalose can be accumulated via *de novo* synthesis when *E. coli* is subjected to high osmolarity. In this case, the molecule acts as a compatible solute to stabilize the internal cellular environment against an osmotic gradient created by the high osmolarity outside the cell. Perhaps the most interesting phenomenon surrounding this accumulation is the simultaneous high osmolarity-dependent degradation of the disaccharide (Horlacher and Boos, 1997). Spatial separation of these two metabolic processes allows for their functional co-existence; hydrolysis of trehalose is carried out in the periplasm whereas the synthesis of the molecule is localized in the cytoplasm (Horlacher and Boos, 1997). Moreover, when the osmolarity of the external environment is low, *E. coli* does not accumulate trehalose but instead uses exogenous trehalose as a carbon and energy source. This involves a separate pathway where trehalose is imported via a trehalose-specific phosphotransferase system followed by cleavage of the phosphorylated disaccharide by trehalose-6-phosphate hydrolase (Rimmele and Boos, 1994). Similarly, PTS-dependent uptake systems for trehalose catabolism are found in Gram-positive bacteria such as *Bacillus subtilis* which do not appear to accumulate the sugar for starvation or stress relief. Instead trehalose is used solely as a carbon/energy source after its translocation across the cytoplasmic membrane via the enzyme IIBC trehalose specific phosphotransferase system (PTS) (Schöck and Dahl, 1996).

*Listeria monocytogenes* is an important Gram-positive foodborne pathogen that has received much attention due to the serious nature of its pathogenesis for susceptible individuals and its ability to survive and/or grow in many foods and the food processing

environments. In consideration of the vast amount of literature pertaining to the physiology of this organism, it is somewhat surprising that information regarding trehalose metabolism in *Listeria* spp. is restricted to inference based on the published genomic sequence of the putative trehalose operon (Glaser et al., 2001; Andersson et al., 2005) and drawing comparisons to other related Gram-positive bacteria for which data has been compiled through experimentation (Bhumiratana et al., 1974; Helfert et al., 1995; Schöck and Dahl, 1996a & 1996b; Duong et al., 2006). However, general growth studies examining the use of several carbon sources including trehalose have been carried out in the past (Premaratne et al., 1991). In general, *Listeria* spp. are able to utilize the disaccharide as their sole source of carbon and energy (Bergey, 1986) and like many other simple carbohydrates, the utilization of trehalose is repressed by the presence of glucose in the growth medium (Evans Gilbreth et al., 2004).

In the previous chapter, a transposon insertion mutant created in *Listeria monocytogenes* strain 568 (Lm568) was isolated that displayed enhanced thermotolerance. The location of the Tn917 insertion was determined to be at the distal end of the putative *treA* gene (*lmo 1453*), the gene encoding for phosphotrehalase, a phospho-(1,1) glucosidase. The objective of the current study was to elucidate the reason for the observed thermal resistant phenotype. Therefore, a deletion mutant for the *treA* gene in Lm568 was created to assess its impact on the biochemistry and physiology of the organism. Furthermore, the cross protective capacity of this heat tolerant phenotype was elucidated by subjecting the mutant to a multitude of other environmental stressors.

Currently, this is the first report involving experimental analysis of any of the three genes of the putative trehalose operon in *Listeria monocytogenes*.

## 7.2 Materials and Methods

### 7.2.1 Bacterial strains and culture conditions.

The bacterial strains and plasmids used in this study are given in Table 7.1. For long term storage, all strains and transformants were kept at -80°C in either brain heart infusion (BHI) broth (Oxoid Canada Inc, Mississauga, ON) for *Listeria monocytogenes* or Luria-Bertani (LB) broth (Oxoid) for *Escherichia coli*, each medium supplemented with 15% glycerol. Reconstitution of frozen stock cultures was done overnight in the aforementioned media without glycerol at 37°C. Recovery of survivors following stress treatments was carried out on Tryptic Soy Agar (Oxoid) supplemented with 0.6% yeast extract (TSA-YE) and 1% sodium pyruvate (Sigma-Aldrich Chemical Company, Oakville, ON). Survival studies for oxidative stress, acid stress, tolerance to ethanol, and osmotic stress were performed in BHI broth augmented according to the methods listed below. Trehalose utilization experiments were carried out using modified Welshimer's broth (MWB) according to the recipe of Premaratne et al. (1991). All ingredients were purchased from Sigma-Aldrich. Antibiotics used in selective media were at the following concentrations; cloning *treA* into *E. coli* using TOPO pCR®2.1, ampicillin (amp), 100 µg/ml; selection of *E. coli* carrying pAUL-A with the *treA* deletion insert (pAULA:ΔT6P), erythromycin (erm), 250 µg/ml; selection of pAULA:ΔT6P transformed *L.*

*monocytogenes* 568, erm, 5 µg/ml; complementation experiments with *E. coli* carrying pAM401, chloramphenicol (crm), 50 µg/ml and selection of pAM401:TREOP transformed *L. monocytogenes*, crm at 10 µg/ml. All antibiotics were purchased from Sigma-Aldrich Chemical Company.

### 7.2.2 DNA extractions, purification and manipulations.

Large scale plasmid DNA extractions for the isolation and purification of the vectors pAUL-A and pAM401 were carried out using the Qiagen Maxi-Prep kit (Qiagen Inc, Mississauga, ON) according to the manufacturer's instructions. Small scale genomic DNA extractions were performed using the UltraClean™ Microbial DNA isolation kit as specified by the manufacturer (MoBio Laboratories Inc, Carlsbad, CA). DNA modifying enzymes which included, *xbaI* restriction endonuclease, T4 ligase, and calf intestinal alkaline phosphatase (CIAP), were provided by New England BioLabs (Pickering, ON). DNA TOPO cloning kits were supplied by Invitrogen (Burlington, ON). KlenTaq and Taq DNA polymerases were supplied by Sigma-Aldrich.

### 7.2.3 Construction of a chromosomal $\Delta T6P$ deletion mutant.

A 462 bp deletion in the central region of the *treA* gene encoding for phosphotrehalase in *L. monocytogenes* 568 (Lm568) was carried out by allelic replacement with a plasmid construct generated through PCR by gene splicing and overlap extension (SOE) (Horton et al., 1991). Briefly, genomic DNA from an overnight culture of Lm568 was extracted using the UltraClean™ Microbial DNA isolation kit.

The DNA was used as the template for two separate PCR reactions. In the first reaction, the primers SOE-T6P-A (5'-ACGGTCTAGAACTGATTCTCTGGCGCTCAT-3') and SOE-T6P-B (5'-GGATTGATAAA GGCGTGGAAGGCGCAACGATTCATTTTAT-3') were used to generate a 591 bp amplicon at the 5'-end of the *treA* gene using KlenTaq polymerase (Sigma-Aldrich). The second reaction was used to produce a 491 bp amplicon at the 3'-end of the gene. This employed the primers SOE-T6P-C (5'-TTCCACGCCTTTATCAATCC-3'), which was complementary to the 5'-tail of SOE-T6P-B, and SOE-T6P-D (5'-CTGCTCTAGAGGGGATATCCCGGTA TCAC-3'). Sequences for the restriction endonuclease *XbaI* (underlined regions) were introduced into primers SOE-T6P-A and SOE-T6P-D to facilitate cloning. The PCR reactions were cleaned with the EZ-10 Spin Column PCR purification kit (Bio Basic, Inc, Markham, ON). Each of the two amplicons from the above PCR reactions was diluted 1000-fold and equal portions were mixed together. One  $\mu$ l of the mixture was used as template for a PCR using the primers SOE-T6P-A and SOE-T6P-D. PCR products were separated on a 1% Tris-acetate EDTA (TAE) agarose gel and the 1061 bp amplicon was excised and purified using the GeneClean™ gel purification kit (Q-Biogene, CA). The purified fragment was TA cloned into *E. coli* TOP10 using the TOPO TA<sup>®</sup> cloning kit (Invitrogen) and the presence of insert was confirmed by digesting the extracted plasmid with *XbaI* and fractionation on a TAE agarose gel. The gel fragment was excised and the  $\Delta treA$  fragment purified as before and then ligated into the dephosphorylated temperature-sensitive shuttle vector pAUL-A with T4 DNA ligase (New England BioLabs). The resulting plasmid, designated pAULA: $\Delta$ T6P, was electroporated into *E.*

*coli* DH5 $\alpha$  (Ausubel et al., 1987; Miller and Nickoloff, 1995). Transformants were selected on LB agar containing 250  $\mu$ g/ml erm.

*7.2.4 Electroporation into L. monocytogenes 568 and selection of isogenic treA deletion mutant.* A maxi-prep of the pAULA: $\Delta$ T6P plasmid was prepared using a large-scale plasmid DNA extraction and purification kit according to the manufacturer's instructions (Qiagen Inc.) and the DNA was electroporated into Lm568. *Listeria* cells were made competent by the method of Gorski et al. (2004) with minor modifications. In brief, the cells were pre-cultured overnight at 37°C in BHI containing 0.5 M sucrose. The culture was diluted 1/100 into 100 ml of fresh BHI/sucrose medium and incubated at 37°C on an orbital shaker at 150 rpm. Once the culture reached an absorbance of 0.2 at 600 nm, penicillin G was added to a final concentration of 10  $\mu$ g/ml and the culture was incubated for another 2 h. The cells were then harvested by centrifugation at 6000  $\times$  g at 0°C. The pellets were washed by resuspending in 100 ml of electroporation buffer (1mM Hepes pH 7.0; 0.5 M sucrose) followed by centrifugation as before. The wash was repeated twice in 50 ml of electroporation buffer. The pellet was then resuspended in 10 ml of electroporation buffer and lysozyme was added to a final concentration 100  $\mu$ g/ml. The suspension was incubated at 37°C for 20 min without agitation. The cells were collected by centrifugation for 15 min at 4000  $\times$  g at 0°C. The pellet was gently washed twice in 10 ml of electroporation buffer and then resuspended in 0.5 ml of the same buffer. The cells were transferred in 40  $\mu$ l aliquots to pre-chilled 1.5 ml eppendorf tubes and used for electroporation within 30 min of preparation. Electroporation of

pAULA:ΔT6P was carried out using 0.1 cm cuvettes in a Micropulser™ (BioRad) set to a field strength of 1.0 kV using varying amounts of DNA. Pulse times ranged from 3.8 to 5.1 ms. Following electroporation, the cells were resuspended in 1 ml of BHI/sucrose broth and transferred to a disposable 10 ml culture tube. The cells were allowed to recover for 3 h at 28°C without shaking. Selection of transformed *Listeria* harbouring the pAULA:ΔT6P plasmid was carried out by plating the suspensions on BHI agar containing erm (5 µg/ml) and incubating at 28°C for 3-4 days. Confirmation of presence of the pAULA:ΔT6P plasmid was conducted by miniprep plasmid extraction followed by restriction digestion of the DNA with *Xba*I. For the integration of pAULA:ΔT6P into the chromosome of Lm568, a single colony of a confirmed plasmid-carrying transformant was patched onto BHI agar supplemented with 5 µg/ml erm which was subsequently incubated at 42°C for 2 days. The colony was transferred to a fresh plate and incubated under the same conditions and time period. A total of three transfers/incubations were performed. Merodiploid intermediates were confirmed by the presence of two amplicons (wild-type allele, 1523 bp; deleted allele, 1061 bp) by PCR using primers SOE-T6P-A and SOE-T6P-D. Spontaneous excision of the integrated plasmid via intramolecular homologous recombination of the deleted allele was achieved by 3 subsequent overnight incubations in BHI at 28°C in the absence of erm followed by a final overnight incubation at 42°C in BHI. The culture was diluted in 0.1% peptone water and spiral plated onto BHI agar. The plates were incubated at 35°C for 48 h. One hundred colonies were patched onto BHI and then replica plated onto minimal medium (modified Welshimer's agar) containing 0.5% trehalose as the sole source of carbon. The plates

were incubated at 37°C for 48 h and the patched colonies that grew poorly on the MWB/trehalose medium were subjected to PCR using primers SOE-T6P-A and SOE-T6P-D. Those recombinants displaying a single amplicon of 1061 bp were selected. The in-frame deletion was confirmed by DNA sequencing and the deletion mutant was named 568:ΔT6P.

### 7.2.5 Complementation of 568: ΔT6P

Phosphotrehalase activity was restored in 568:ΔT6P by transforming the mutant with the shuttle vector pAM401 carrying an intact copy of the *treA* gene. Briefly, a full length amplicon for the *treA* and *treB* genes which included the promoter region upstream of *treB* was amplified from Lm568 using the following primers: TreOp-F (5'-CCTTCTAGAGGTCACCGGTTGGAT AAA TTCC-3') and TreOp-R (5'-AATCTAGAACCGGCAATGTCCAATAATTT-3'). The underlined bases denote *Xba*I restriction sites to facilitate cloning. The amplicon was TA cloned into TOPO pCR®2.1 according to the methods described by the manufacturer (Invitrogen) and transformants were selected on LB with ampicillin (100 µg/ml). The plasmid carrying the insert was isolated and purified using the MoBio plasmid DNA miniprep kit and then digested with *Xba*I. The insert was gel purified and ligated to pAM401 linearized with *Xba*I and dephosphorylated with CIAP. The resulting construct pAM:TREOP was electroporated into the competent deletion mutant 568:ΔT6P as described above. Simultaneously, pAM401 containing no insert was also electroporated into the wild-type Lm568. This was done to offset any potential differences in later experiments when comparing the



mutant to wild-type cultures due to the presence of the plasmid in the complemented mutant. The transformants were selected on BHI containing crm (10 µg/ml). The presence of the construct was verified by PCR using the TreOp-F and TreOp-R primers. The restored activity of *treA* was confirmed by both culturing with MWB with 0.5% trehalose as the sole source of carbon, and measuring the enzymic activity in crude cell extracts (see below).

#### *7.2.6 Preparation of crude cell extracts for enzyme assays*

Extracts for the mutants and wild-type Lm568 were prepared according to the method of Helfert et al. (1995) with some modifications. The cultures were grown in 50 ml of BHI supplemented with 1% trehalose for 16 h at 30°C and 200 rpm in an orbital shaker. The cells were harvested by centrifugation at  $6000 \times g$  for 15 min at 0°C. The pellets were washed twice by re-suspending in 40 ml of sonication buffer (50 mM Tris-Bis, pH 7.0; 10 mM KCl; 1 mM MgCl<sub>2</sub>; 1 mM CaCl<sub>2</sub>; 2 mM dithiotreitol and; 1 mM phenylmethylsulphonylfluoride (PMSF) and repeating the centrifugation. The pellet was then re-suspended in 5 ml of sonication buffer and cells were disrupted with a Virsonic 600 ultrasonic cell disrupter (Virtis, Gardiner, NY). Sonication was performed at 60% power for 20 s with a 30 s rest between pulses. Total pulse time was 5 min and the tubes were kept on ice for the entire procedure. Cellular debris was removed by centrifuging the samples at  $10,000 \times g$  for 30 min at 0°C. The quantity of total protein in the supernatant was determined using the Ettan™ 2D-Quant Kit (Amersham Biosciences, Inc, Baie d'Urfé, PQ).

### 7.2.7 Assay for $\alpha, \alpha, (1-1)$ phosphotrehalase activity

The crude cell extracts were used to confirm the loss of  $\alpha, \alpha, (1,1)$  phosphotrehalase activity in the Tn917 transposon 32F11, (see Chapter 5) and the deletion mutant (568: $\Delta$ T6P). This was performed by measuring the amount of glucose liberated from the hydrolysis of trehalose-6-phosphate (T6P) using a commercial glucose hexokinase (HK) kit according to the manufacturer's instructions (Sigma-Aldrich). A 500  $\mu$ l aliquot of crude cell extract was incubated with 10 mM trehalose-6-phosphate (T6P) for 2 h at 37°C. Since crude extracts were used to assess enzyme activity, controls were included containing the same amount of crude extract but no added T6P. This was done to assure that other constituents in the extract did not produce glucose. Following the prescribed incubation period, 200  $\mu$ l of each sample was combined with 1.0 ml of glucose assay reagent and mixed by vortexing. At the same time, 200  $\mu$ l of the sample extract was combined with 1.0 ml sterile ddH<sub>2</sub>O which was used as a sample blank for the glucose HK procedure. The absorbance of each sample was read at 340 nm and the amount of glucose was calculated according to the manufacturer's instructions using a glucose standard curve.

### 7.2.8 The effect of glucose on the expression of *treA*

In order to assay the repressive nature of glucose on *treA* activity, Lm568 was grown overnight at 37°C in MWB containing 10 mM trehalose. The overnight culture (10  $\mu$ l) was used to seed 50 ml of fresh MWB +10 mM trehalose and 4 different levels of glucose (0, 1, 5 and 10 mM) and the flasks were incubated at 37°C on an orbital shaker at

200 rpm. The cultures were grown to 20 h and then harvested by centrifugation. Crude cell extracts were prepared in sonication buffer and the activity of *treA* was estimated using the glucose HK assay as described above.

#### *7.2.9 Determination of T6P levels in cell extracts*

Accumulated T6P in Lm568 and the *treA* deletion mutant were estimated based on the method of Van Vaeck et al. (2001). The inducible expression vector pCCV1 containing the intact *treA* gene from *Bacillus subtilis*, was a gift from Drs. Van Vaeck and Thevelein (Katholieke Universiteit, Leuven, Flanders, Belgium) and used to produce the phosphotrehalase enzyme which subsequently was used in the assay to determine the T6P levels in cells.

*7.2.9.1 Production of the phosphotrehalase enzyme:* The pCCV1 vector was electroporated into competent *E. coli* XL10-Gold (Stratagene, LaJolla, CA) and the transformants were recovered on LB containing 100 µg/ml ampicillin. Isolation and purification of the over-expressed enzyme was carried out following the methods of Gotsche and Dahl (1995) and Van Vaeck et al. (2001) with some modifications. In brief, *E. coli* carrying pCCV1 was pre-cultured overnight at 37°C in LB containing amp. Ten ml of this culture was used to seed 1 L of LB broth containing 0.5 mM IPTG (isopropyl β-D-thiogalactoside) and amp (100 µg/ml) in a 2 L flask and the culture was grown for 5 h at 37°C and 200 rpm. The cells were centrifuged at 6000 × *g* for 10 min at 4°C. Washing and lysis of the pellets was carried out in Bis-Tris buffer (50 mM Bis-Tris, pH

7.0; 10 mM KCl; 1mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>). Sonication parameters were as described above for the crude cell extracts. Cellular debris was removed by ultracentrifugation at 40,000 × *g* for 30 min at 4°C in Beckman SW40 rotor. The supernatant was treated with a saturated solution of ammonium sulfate added stepwise to achieve 80% saturation. The solution was allowed to stir overnight at 4°C and the precipitate was collected by centrifugation at 15,000 × *g* at 4°C for 20 min. The pellet was dissolved in 5 ml of extraction buffer and then dialyzed against several changes of Bis-Tris buffer. Proteins were first fractionated by passage through a DEAE Sephadex A-50 anion exchange column equilibrated with Bis-Tris buffer. Proteins were eluted in 5 mL fractions with a NaCl gradient. Confirmation of fractions harbouring the 64 KDa phosphotrehalase enzyme was determined on a 12% SDS-PAGE gel by the method of Laemmli (1970). The fractions were pooled and precipitated with ammonium sulfate as before. The sample was centrifuged and the resulting pellet was dissolved in Bis-Tris buffer. For size fractionation, the sample was loaded on a Superdex 75 gel filtration column equilibrated with Bis-Tris buffer. Elution was carried out with the same buffer with the collection of 2 ml fractions. The fractions were analyzed on a 12% SDS-PAGE gel and those containing a prominent 64 kDa band were pooled. The pooled fractions were analyzed for enzyme specificity using pure trehalose-6-phosphate as a substrate and the glucose HK reaction to measure liberated glucose (see above). In addition, the activity of the purified enzyme was quantified using PNPG (p-nitrophenyl-α-D-glucopyranoside) as a substrate, where 1 unit of phosphotrehalase will hydrolyze 1 nmol

of PNPG /min at 37°C. The specific activity was calculated based on units/mg of protein as determined by the Ettan™ 2D Quant protein kit.

7.2.9.2. Extraction of intracellular listerial trehalose-6-phosphate: Lm568 and the mutant were grown in 50 ml cultures using 5 different media; TSB, TSB-YE, TSB with 1% trehalose, BHI and MWB with 1% glucose to determine the intracellular accumulation of T6P. Briefly, cultures were grown for 18 h at 30°C and the cells were harvested by centrifugation at  $6,000 \times g$  for 10 min at 0°C. Cells were re-suspended in 1 ml of PBS pH 7.0 and sonicated on ice as before. Following sonication, an equal volume of ice cold 2 M  $\text{HClO}_4$  was added to the tubes along with glass beads and tubes were shaken for 10 min. The cellular debris was removed by centrifugation at  $20,000 \times g$  for 30 min at 0°C and the samples were then neutralized with 3 M  $\text{K}_2\text{CO}_3$ . The supernatant was passed through LC- $\text{NH}_2$  solid phase ion exchange columns (Supelco, Bellefonte, PA). Elution was carried out with 2 ml of 2.5 M ammonium hydroxide (pH 12.5). The ammonia was removed from the sample by freeze drying and the sample was re-dissolved in 50 mM Bis-Tris buffer (pH 7.0).

7.2.9.3 Trehalose-6-phosphate (T6P) assay using purified phosphotrehalase: The extracts from the cells grown in the 5 different media or 4 different vegetable juice (see below) extracts were assayed for T6P. Varying quantities of extracts were mixed with 2.0 U of purified phosphotrehalase and the total reaction volume was brought to 200  $\mu\text{l}$ . The mixture was incubated at 37°C for 2 h. The reactions were run in duplicate. The amount

of glucose liberated in the reaction was measured using the glucose HK kit (Sigma-Aldrich).

#### *7.2.10 Extraction and assay of trehalose from bacterial cultures*

Overnight TSB cultures were grown at 30°C. The cultures were used to inoculate fresh 100 ml cultures in TSB, BHI and TSB supplemented with 1% trehalose. The cultures were grown for 16 h at 30°C at 200 rpm. The cells were harvested by centrifugation at  $6000 \times g$  for 15 min at 2°C. The pellets were washed twice by resuspending in PBS (pH 7.0) followed by centrifugation as before. The cells were resuspended in 4 ml of sterile ultrapure water tempered to 2°C and then sonicated on ice using a Virsonic 600 ultrasonic cell disrupter (Virtis, Gardiner, NY). Sonication parameters consisted of 30 s pulses at 18 W followed by 30 s cooling intervals. The total cumulative pulse time was 5 min. Cellular debris was removed by centrifugation at  $10,000 \times g$  for 20 min and saved for protein analysis. Clarification of the supernatant was achieved by the addition of acetic acid to reduce the pH to 2.0 to precipitate proteins. The acidified supernatant was allowed to stand on ice for 1 h followed by centrifugation as before. The pH of each extract was adjusted to 6.0 by the addition of 1 M KOH and the extracts were stored at -80°C until assayed. The pellet from the acid precipitation was combined with the original pellet and solubilized in Laemmli buffer (Laemmli, 1970). The amount of extracted protein in the pellet was determined using the Ettan™ 2D-Quant kit according to the manufacturer's instructions (Amersham Biosciences).

Since trehalose consists of two glucose molecules, the glucose HK assay (Sigma-Aldrich) was used to measure the amount of glucose liberated following the hydrolysis of trehalose with purified porcine kidney trehalase (Sigma-Aldrich). Two hundred microlitres of clarified cell extracts were incubated with 0.05 U of trehalase at 37°C for 8 h. In order to compensate for glucose in the samples that did not originate from trehalose, a separate 200 µl aliquot omitting trehalase was incubated along with each trehalase treated sample. Standards using pure trehalose were also treated with trehalase simultaneously. Following incubation, the samples were assayed for glucose by the glucose hexokinase reaction. Since two glucose units are formed from each molecule of trehalose calculations were adjusted by a factor of two.

#### *7.2.11 Preparation of vegetable and mushroom juice*

All food items were obtained from a local supermarket. Included were; whole white cabbage heads, shrink wrapped whole *Agaricus* mushrooms and bagged frozen beans and peas. The cabbage and mushrooms were hand diced and then homogenized in a commercial juicer. The beans and peas were thawed prior to juicing. For all food items, the resultant pulp was squeezed through four layers of cheese-cloth for maximum recovery of juice. The pulp was discarded and the juice was clarified by centrifugation at  $15,000 \times g$  for 30 min at 4°C. The juice then underwent a sequential filtration regime to achieve sterility. This involved first passing the juice through coarse Whatman filter paper and then a series of micropore filters ranging from 10 µm down to 0.45 µm

(Sartorius AG, Goettingen, Germany). The final sterile juices were stored frozen until use.

#### *7.2.12 Bacterial growth in juice extracts*

Lm568 and 568:ΔT6P were pre-cultured overnight in 5 ml of each vegetable juice at 30°C at 200 rpm. Ten µl of pre-culture was used to inoculate 50 ml of the respective vegetable extracts in a 250 ml culture flask. The flasks were incubated at 30°C at 200 rpm. Cell numbers were periodically determined by diluting cultures in 0.1% peptone water and then spiral plating on TSB-YE agar. When cultures reached stationary phase, the pellets were harvested by centrifugation and used for either T6P determinations (see above) or thermal death time assays (see below).

#### *7.2.13 Sugar analysis by high performance liquid chromatography (HPLC)*

HPLC was used to quantify the amount of trehalose in the bacterial growth media and the vegetable extracts (see above). The samples were prepared by first precipitating the protein by adding 5% TCA to the extracts followed by 15 min on ice. The samples were then centrifuged at  $12,000 \times g$  for 30 minutes at 2°C and the supernatant was neutralized with 1 M KOH. The supernatants were then passed through Sep-Pak C18 (Waters) reversed-phased columns conditioned according to the manufacturer's specifications. Particulates in the flow through liquid were removed by filtration through a 0.22 µm filter. Sugars were separated on a Beckman Coulter System Gold 126 HPLC system (Beckman Coulter) equipped with an Aminex HPX-87P column (300 × 7.8 mm,



BioRad, Hercules, CA, USA) and a Waters 2414 refractive index detector (Frequency 10.0006 Hz; Detector Mode RIU; Measurement Range 250 $\mu$  RUI-FS). Experimental conditions were: Column temperature 85°C; mobile phase was nanopure water; flow rate, 0.6 ml/ min; run time, 30 min; Identification and external quantification were done on the basis of relative retention times of authentic standards (Sigma-Aldrich). Retention time for trehalose was 10.59 min.

#### *7.2.14 Assessment of stress tolerance*

The *treA* deletion mutant and wild-type were subjected to several stress conditions to determine the mutant's ability to survive relative to Lm568. For heat treatments, overnight pre-cultures in BHI, TSB, TSB with 0.6% yeast extract (TSB-YE) and TSB with 1% trehalose (TSB-Tre), were grown at 30°C. A 10  $\mu$ L aliquot from each overnight culture was used to inoculate 5 mL of the same media which was subsequently incubated for 16 h at 30°C. For cold temperature growth curves, the cells were pre-cultured in 5 mL of BHI for 5 days at 4°C. Ten microlitres of this culture was used to inoculate BHI cultures tempered to 4°C. For the desiccation experiments and all other stress studies, the cells were pre-cultured in BHI and then grown in 5 mL of BHI + 1% trehalose for 16 h at 30°C. The cultures were then harvested by centrifugation at 6000  $\times$  g for 10 min at room temperature and pellets were washed once with 0.1% peptone water and then re-suspended in the appropriate test medium (see below).

**7.2.14.1 Heat Treatments:** The pellets from the various media or vegetable extracts were washed twice in 0.1% peptone water and then diluted in the same medium to obtain initial concentrations of  $10^7$ - $10^8$  CFU/mL. Each suspension was distributed as 100  $\mu$ L aliquots into duplicate sets of 8 thin-walled 0.2 ml PCR tubes. The tubes were placed in a Biometra Tgradient thermocycler (Whatman Biometra, Göttingen, Germany) and heat treated at 52°C for 105 min. Duplicate samples were removed at 15 min intervals and cooled in an ice-water bath for 1 min. The samples were diluted in sterile 0.1% peptone water and spiral plated onto TSA-YE containing 1% sodium pyruvate. Colony forming units were enumerated following 72 h incubation at 35°C and the log values were plotted against time to achieve thermal death time curves. The entire experiment was repeated 3 times.

**7.2.14.2 Cold Growth:** Duplicate tubes containing 5 ml of BHI were tempered to 4°C and then inoculated with 10  $\mu$ L of the 5 day pre-culture. The tubes were incubated at 4°C without shaking and aliquots were removed periodically for absorbance measurements and colony enumerations. Absorbance measurements were taken at 600 nm and cell enumerations were performed by diluting cultures in 0.1% peptone water and spiral plating on TSA-YE. The plates were incubated for 48 h at 35 °C. The experiment was repeated twice.

**7.2.14.3 Resistance to freeze-thaw cycles:** The washed cell suspensions from above were washed once more in sterile phosphate-buffered saline (PBS), pH 7.0, and then re-

suspended in PBS to obtain cell densities of  $10^7$  to  $10^8$  CFU/ml in 10 ml of PBS in 50 ml polypropylene centrifuge tubes. The initial cell numbers were determined by spiral plating on TSA-YE as before. The tubes were frozen to  $-80^{\circ}\text{C}$  in an ultra low temperature freezer. After 2 hours, the suspensions were thawed at  $37^{\circ}\text{C}$  in a water bath and an aliquot was diluted and spiral plated. The tubes were placed in the freezer again and viable CFU's were determined after 24 h. The freeze/thaw cycles were continued for 10 days with samples being taken every 24 h. Colony enumerations were performed after 72 h at  $35^{\circ}\text{C}$ . The experiment was repeated 3 times.

7.2.14.4 Resistance to desiccation: The washed pellets from each growth medium were re-suspended in 0.1% peptone water to achieve a cell density *ca.*  $10^8$  CFU/ml. Ten microlitres of each suspension (i.e.,  $10^6$  CFU) were aseptically transferred to 3 wells of a 12-well flat bottomed tissue culture plate. The plates were dried in a biosafety cabinet (Baker Co., Sanford, ME) with their lids removed for 2 h. The lids were replaced and the plates were stored in a vacuum jar containing 10-20 mesh Drierite desiccant (W A Hammond Drierite Co., Xenia, OH) at ambient temperature for 14 days. Following the incubation period, 5 ml of BHI was added to the 12 wells of each culture plate and the plates were placed in an incubator at  $35^{\circ}\text{C}$ . The wells were monitored for growth by measuring the absorbance at 600 nm. Absorbance readings were taken until the cells reached stationary phase. Three replicates of this experiment were carried out.

**7.2.14.5 *Survival in acid, ethanol, hydrogen peroxide, and sodium chloride*:** The pellets were re-suspended to  $10^7$ - $10^8$  CFU/ml in BHI adjusted to achieve one of the following stress conditions; pH 3.5, adjusted with HCl; 18% (v/v) ethanol; 0.1% H<sub>2</sub>O<sub>2</sub> (v/v) and 20% NaCl (w/v). For each sample treatment, a 100 µl aliquot was removed immediately following mixing, diluted in peptone water and spiral plated on TSA-YE. Samples were removed and spiral plated at specified time intervals for each sample treatment. All plates were incubated at 35°C for 72 h before colony enumerations. These experiments were replicated either 2 or three times.

#### **7.2.15 *Statistical analysis***

Stress test data was subjected to Analysis of variance (ANOVA ) using SYSTAT® software and pair wise comparisons were assessed by the Tukey method at the 95% confidence level. Critical parameters for analysis of thermal death curves were obtained using the Pruitt and Kamau (1993) model (equations 1 and 2). Where appropriate, the students t-test ( $\alpha = 0.05$ ) was used for direct comparison of the means obtained for trehalose or T6P accumulation in the mutant versus the wild type.

### **7.3 Results**

#### **7.3.1 *Growth kinetics of the deletion mutant***

The mutant, 568:ΔT6P, was constructed such that a 462 bp portion was deleted from the central region of the putative *treA* gene which encodes for  $\alpha, \alpha$ , (1,1)

phosphotrehalase. DNA sequencing demonstrated that the recombined gene containing the deletion was indeed in frame. The deletion mutant had similar colony morphology as the wild-type strain when grown on TSA-YE or BHI agar. The growth kinetics in BHI broth, TSB and MWB with glucose, were also similar with respect to lag time, rate of growth in exponential phase and time to stationary phase (data not shown). Surprisingly, replacing the 1.0 % glucose in MWB with 1.0% trehalose as the sole carbon source did not prevent growth of the mutant. The mutant was able to grow but with a slower growth rate reaching a lower maximum cell density (Figure 7.1). In order to confirm that the mutant was indeed using trehalose at a much reduced rate and not another constituent in the medium, both the wild-type and the mutant were inoculated into MWB with no added carbon source. Neither culture grew after 48 h at 37°C. Also investigated, was the possibility that the apparent utilization of trehalose by the mutant may be due to natural degradation of the disaccharide after prolonged incubation times at 37°C; with the observed growth therefore being due to the released glucose and not the trehalose. However, no glucose could be detected in the uninoculated MWB + 1% trehalose medium following 48 h at 37°C using neither the HK assay nor HPLC analysis (data not shown).

### 7.3.2 Enzymic activity of crude cell extracts

According to the published genome map of *L. monocytogenes* EGD-e (Genebank accession number; AL591824) there does not appear to be another putative transport mechanism other than the *treA* for the utilization of trehalose (Glaser et al., 2001). In

order to verify this fact, the glucose hexokinase was used to assay the amount of glucose liberated from pure trehalose during incubation for 4 h at 37°C with crude cell proteins extracted from the mutant and wild-type. In neither case were an increase in glucose detected in comparison to the control extracts containing no added trehalose. Therefore, the possibility was investigated that this limited capacity to grow on trehalose may be due to the transcription and subsequent translation of a truncated version of the enzyme with reduced activity. RT-PCR demonstrated that a truncated transcript was indeed produced (Figure 7.2). However, free glucose could not be detected by the hexokinase assay when pure trehalose-6-phosphate was exposed to crude cell extracts from the mutant for 1, 4 and 8 h at 37°C. Conversely, extracts from the wild-type strain had high levels of phosphotrehalase activity since 10 µl of cell extract liberated large quantities of glucose after only 1 h at 37°C (Figure 7.3).

### 7.3.3 Repression of *treA* by glucose

We examined the effect of the presence of glucose on the *treA* activity in early stationary cells of Lm568. Crude cell extracts from cells grown on different concentrations of glucose demonstrated that the transcription of *treA* was repressed by the presence of glucose (Figure 7.3). Using trehalose-6-phosphate (T6P) as a substrate and the glucose hexokinase reaction, cells grown in the presence of 10 mM trehalose as the sole carbon source exhibited the highest level of phosphotrehalase activity with more than 80 µg of glucose/mg cell protein being liberated from trehalose-6-phosphate after 1 hour at 37°C. The addition of glucose concentrations of 5 and 10 mM to MWB cultures

resulted in only 28.9 and 11.6  $\mu\text{g}$  of glucose/mg protein being released from T6P, respectively. At 1 mM glucose, catabolite repression was not as strict since crude cell extracts from these cultures continued to show enzyme activity with the liberation of 71.2  $\mu\text{g}$  of glucose/mg cell protein. This result clearly indicated that the presence of glucose in the medium repressed the utilization of trehalose hence the regulation of these pathways is closely linked.

#### 7.3.4 Accumulation of intracellular trehalose-6-phosphate (T6P)

Lm568 and 568: $\Delta$ T6P were assessed for intracellular levels of T6P after growth in different substrates. The growth medium affected the concentration of T6P in both the wild-type and the mutant (Figure 7.4). Neither strain grown in MWB with glucose as the carbon and energy source displayed detectable levels of T6P. However, the phosphorylated sugar was present in low amounts after growth in TSB; 2.4 and 3.2  $\mu\text{g}/\text{mg}$  cell protein for Lm568 and 568: $\Delta$ T6P, respectively. This was interesting since trehalose could not be detected in this medium using HPLC (data not shown).

Supplementing TSB with yeast extract (0.6% w/v) resulted in a significant increase ( $P < 0.001$ ) in the intracellular pool of T6P for the mutant (8.2  $\mu\text{g}/\text{mg}$  cell protein) but not the wild-type strain (2.5  $\mu\text{g}/\text{mg}$  cell protein), in comparison to the unmodified medium. The addition of 1% trehalose to TSB did, however, result in more than twice as much T6P in the wild-type (6.3  $\mu\text{g}/\text{mg}$  cell protein) compared to cells grown in TSB without trehalose. However, growing Lm568 in BHI did not significantly raise T6P levels (2.7  $\mu\text{g}/\text{mg}$  cell protein) for the wild-type strain ( $P > 0.05$ ), although, HPLC analysis detected the

disaccharide in this medium. In contrast to Lm568, mutant 568:ΔT6P showed a gradient of increasing quantities of T6P depending on the type of medium. Although T6P levels for cells grown in TSB were not significantly greater than that of the wild-type ( $P > 0.05$ ), the addition of yeast extract resulted in an increase in T6P from 3.2 to 8.2 μg/mg cell protein. Not surprising, the presence of 1% trehalose in TSB medium resulted in the greatest amount of accumulated T6P (22.8 μg/mg cell protein). Moreover, the free trehalose in BHI, as demonstrated by HPLC (data not shown), was also effectively transported into the 568:ΔT6P mutant since cells grown in this medium accumulated the second highest levels of T6P (10.7 μg/mg cell protein).

#### *7.3.5 Quantification of trehalose in cells extracts and phosphatase activity*

Since it has been suggested that T6P does not act as a compatible solute (O'Byrne and Booth, 2002), the possibility that free trehalose was being accumulated in the cytosol of 568:ΔT6P was investigated. Sugars were extracted from the cell pellets of Lm568 and 568:ΔT6P after growth in 3 different media. The extracts were then passed through an LC-NH<sub>2</sub> ion exchange column to remove phosphorylated species and the flow-through was then subjected to enzymatic hydrolysis for 2 hours using pure porcine kidney trehalase and then assayed for free glucose content. Liberated glucose was detected in all cell extracts regardless of treatment or strain thus indicating intracellular presence of trehalose (Figure 7.5). Trehalose levels for the wild-type did not vary significantly ( $P > 0.05$ ) with growth medium as concentrations ranged from 0.95 to 1.3 μg/mg cell protein. However, trehalose levels in 568:ΔT6P extracts from treatments were consistently



significantly greater ( $P < 0.05$ ) than for Lm568. Mutant cells grown in TSB had a nearly 2-fold greater trehalose concentration than Lm568 cells from the same medium.

Concentrations increased significantly ( $P < 0.05$ ) to approximately 6.6  $\mu\text{g}/\text{mg}$  protein when BHI was the growth medium. Even higher amounts (11.6  $\mu\text{g}/\text{mg}$  protein) accumulated in mutant cells grown in TSB with the addition of 1% trehalose.

Conventional wisdom suggests that the only pathway for the uptake of trehalose in *Listeria* is via a sugar-specific PTS system. No endogenous enzymatic activity was found in the crude cell extracts for the direct hydrolysis of the sugar (i.e., levels of glucose in cell extracts not treated with trehalase remained unchanged). Therefore, the formation of trehalose as the result of the dephosphorylation of T6P was investigated. The mutant 568: $\Delta\text{T6P}$  was grown in MWB + 1% glucose and the proteins were extracted as before. The crude extracts were incubated in the presence of 10 mM pure trehalose-6-phosphate for 4 h at 37°C and then passed through an anion exchange column. Following a 2 hour digest with pure trehalase, free glucose was indeed detected following the treatments ( $0.89 \pm 0.21$   $\mu\text{g}$  glucose/ mg protein), suggesting that the original crude extracts contain phosphatase activity capable of cleaving the phosphate group from T6P.

### *7.3.6 Heat tolerance as a function of growth medium*

Lm568 and 568: $\Delta\text{T6P}$  were grown to early stationary phase in four different broth media. The washed cells were re-suspended in 0.1 % peptone water and heated to 52°C. The thermal death curves are presented in Figure 7.6a. All curves displayed a lag period prior to a log linear region of maximum inactivation. Therefore, to compare the thermal

resistance of each treatment, the curves were modelled based on a modification of the Fermi distribution model of Whiting (1993) for curves not exhibiting a tailing phenomenon:

$$\text{Log } N/N_0 = \text{Log} [(1+e^{-\beta * t_{cm}}) / (1+e^{-\beta (t-t_{cm})})] \quad (1)$$

Where,  $N$  is the number of survivors at any time ( $t$ );  $N_0$  is the initial population at  $t = 0$ ;  $\beta$  is the maximum specific death rate and  $t_{cm}$  is the lag period. The results for the model parameters are given in Table 7.2. For comparison of the overall thermal resistance for the wild type and mutant under the conditions used in this experiment, a logistic decimal reduction time ( $D_L$ ) was calculated as previously described by Pruitt and Kamau (1993). This allowed for a simple comparison of the time required to achieve the initial log reduction in population taking into account both the lag period and the maximum inactivation rate.

$$D_L = t_{cm} + [\ln(9+10e^{-\beta})]/\beta \quad (2)$$

When the wild-type strain was grown in BHI, the  $D_L$  (*ca.* 30 min) was significantly longer ( $P < 0.05$ ) than  $D_L$ -values obtained for cells grown in TSB or TSB-YE (22.6 and 22.1 min, respectively). However, there was no significant difference ( $P > 0.05$ ) between Lm568 cells grown in BHI or TSB supplemented with trehalose. This indicated that BHI in general induces a higher level of heat resistance in Lm568 than the other media used in this study. The mutant grown in TSB was not noticeably more heat tolerant than the wild-

type grown in the same medium. However, supplementation of TSB with either 0.6% yeast extract or 1% trehalose resulted in a significant increase ( $P < 0.05$ ) in heat resistance relative to the TSB cultures (Figure 7.6). The trehalose supplemented cultures showed the highest level of thermotolerance ( $D_L = 33.3$  min) for all cells grown in TSB-based media (Table 7.2). This progression in thermal resistance appeared to correlate with the level of T6P accumulated from the uptake of trehalose (Figure 7.4).

Interestingly, 568: $\Delta$ T6P grown in BHI were by far the most thermal resistant cells observed in this study ( $D_L = 44.1$  min) despite the fact that the amount of T6P found in these cells was only about half the levels in detected in cells grown in TSB + 1% trehalose. This indicates that other growth factors contributed by BHI, in concert with the uptake of trehalose, increased the listerial thermotolerance. The enhanced thermal resistance of the mutant appears to be mainly due to an enhancement of the lag period prior to maximum inactivation (Table 7.2).

### *7.3.7 Tolerance to other environmental stresses*

In addition to heat stress, several other stress factors were examined to evaluate the phosphotrehalase mutant's capacity for cross-protection. These included: growth at 4°C; exposure to, 0.1% H<sub>2</sub>O<sub>2</sub>, 18% ethanol, high acidity (pH 3.5), and high osmolarity (20% NaCl); and resistance to freeze/thaw cycles as well as desiccation. In addition to heat, mutant 568: $\Delta$ T6P demonstrated an increased capacity to survive other stress factors. After 6 hours in BHI at ambient temperature in the presence of 20% NaCl, survivors for the phosphotrehalase mutant were significantly greater ( $P < 0.05$ ) than for Lm568 at all

subsequent sample times. After 48 h there were 1.5 log more survivors for 568:ΔT6P than exhibited by the wild-type (Figure 7.7a). Additionally, it was observed that 568:ΔT6P demonstrated an enhanced ability to survive desiccation in comparison to Lm568. The test regime was designed to examine the cell's ability to recover following an extended period of desiccation. The lag period for the initial load of cells not subjected to desiccation was only about 4 hours for both wild type and mutant following addition of BHI. For desiccated cells, 568:ΔT6P had a substantially shorter lag time than Lm568 prior to entry into the exponential growth phase (Figure 7.7b). The mean entry time into exponential phase was *ca.* 17 and 22 h for the mutant and the wild-type, respectively. However, there was no significant difference ( $P > 0.05$ ) between the two strains with respect to specific growth rate or maximum cell densities. The protection offered by the intracellular presence of trehalose during lowering of the intracellular water activity was also extended to the results observed for the survival following repeated freeze/thaw cycles. A significant media effect ( $P < 0.05$ ) was observed for the mutant as survivor numbers were *ca.* 1 log greater than for the wild-type (7.7c) after 10 freeze/thaw cycles spread over 240 h. Interestingly, there were no significant differences ( $P > 0.05$ ) between the wild-type and 568:ΔT6P when subjected to the other stress factors (low temperatures, low pH, ethanol, H<sub>2</sub>O<sub>2</sub>) examined in this study (data not shown).

#### 7.3.8 Complementation of 568:ΔT6P

In order to confirm that the deletion in the *treA* gene was responsible for the observed phenotype with enhanced tolerance to heat stress, 568:ΔT6P was back-

complemented with a vector containing the intact *treA* gene. The *treA* gene does not contain its own promoter site. Instead, the upstream *treB* gene encoding the trehalose-specific PTS enzyme IIBC contains the promoter for the operon. Therefore, the entire *treA/treB* region was cloned into the *xbal* site of the Gram-positive shuttle-vector, pAM401 to create pAM:TREOP. The construct was electroporated into mutant 568:ΔT6P and the resultant complemented strain named ΔT6P pAM:TREOP. The restoration of phosphotrehalase activity in ΔT6P pAM:TREOP was clearly exhibited as crude cell extracts were able to liberate nearly 50 μg of glucose/mg cell protein from 10 mM trehalose-6-phosphate after 1 h at 37°C. This indicated an overexpression of *treA* since extracts from Lm568 liberated less than half this amount (22.5 μg/mg cell protein) of glucose under the same conditions (Figure 7.8). Moreover, the thermal death profile for the complemented mutant cells demonstrated a marked reduction in their enhanced thermotolerance relative to mutant 568:ΔT6P (Figure 7.9). The calculated logistic D-values for Lm568 and ΔT6P pAM:TREOP were 30.5 and 23.1 min, respectively. However, the D<sub>L</sub>-value of 45.9 min for mutant 568:ΔT6P was significantly greater ( $P < 0.05$ ) (Table 7.3). It should be noted that the resulting phenotype was the result of the activation of the *treA* gene and not due to the presence of the pAM401 vector, since the profile of Lm 568:pAM401 did not vary substantially from that of wild type Lm568. The D<sub>L</sub>-value for Lm 568:pAM401 was 28.5 min.

### 7.3.9 Heat resistance following growth in natural food substrates

The mutant 568:ΔT6P was examined for its ability to import extracellular trehalose from natural food sources using the PTS system (intact treB gene product). Its thermotolerance was then measured following growth in juices made from cabbage, beans, peas and mushrooms. HPLC analysis of the filter sterilized juices revealed the presence of varying levels of trehalose, glucose and mannose in all the juices (data not shown). Unidentified sugar peaks with long retention times were also present. Cabbage juice contained only trace amounts of trehalose while the greatest quantities were found in the beans and peas. The mushrooms contained intermediate levels of trehalose. Following growth of Lm568 and 568:ΔT6P in the juices, the sugars were extracted from washed cell pellets and examined for the presence of intracellular T6P (Figure 7.10). The accumulated T6P levels paralleled the available trehalose quantities in the juices with the highest levels of accumulated T6P found in 568:ΔT6P grown in pea juice (*ca.* 7.7 μg/mg cell protein). However, these amounts were not significantly greater than those found in beans (*ca.* 7.0 μg/mg cell protein). Trehalose-6-phosphate levels for the mutant grown in mushroom and cabbage juice were *ca.* 4.4 and 3.0 μg/mg cell protein, respectively. In all cases, significantly higher T6P levels ( $P < 0.05$ ) were found in the mutant as compared to Lm568 cells for all substrates tested (Figure 7.10). Moreover, the mutant grown in the mushroom, bean and pea juices, where T6P reached the highest levels, appeared to be more thermotolerant than the wild-type (Figure 7.11). However, there was no difference between the mutant and wild-type when grown in cabbage juice. The mutant cells grown in pea juice displayed the greatest increase in relative thermal resistance compared to

Lm568 (Figure 7.11c). The logistic D-values for cells grown in pea juice were 69.5 and 58.2 min for 568:ΔT6P and Lm568, respectively (Table 7.3). It is noteworthy that the thermal resistance of both Lm568 and 568:ΔT6P was significantly greater ( $P < 0.05$ ) when grown in mushroom and pea juice (Figure 7.11b & d) compared to cabbage and bean extracts (Figure 7.11a & c), indicating the presence of other factors that contribute to the overall heat tolerance of the organism.

#### 7.4 Discussion and Conclusions

In this study, it was demonstrated that a 462 bp deletion in the *treA* gene (*lmo1453* for strain EGD-e) of *Listeria monocytogenes* strain 568 results in a thermo- and osmotolerant phenotype when the cells are grown in complex media in the presence of trehalose. This represents the first published experimental work investigating any of the genes in the putative trehalose operon of *Listeria*. However, the corresponding gene and its operon in the closely related Gram-positive bacterium, *Bacillus subtilis*, have been examined in detail (Gotsche and Dahl, 1995; Schöck and Dahl, 1996a & 1996b; Dahl, 1997). The trehalose operon in the Gram-negative rod *Pseudomonas fluorescens* (ATCC strain 17400) was also shown to be highly similar to that of *Bacillus subtilis* in terms of gene function and regulation (Matthijs et. al., 2000). The conserved nature of this operon may be suggestive of its importance and evolutionary success. Most recently, the predicted trehalose operon in *Listeria* was shown to be consistent in organization with the catabolic operons of other closely related bacteria such as *Lactococcus lactis* and

*Lactobacillus plantarum* (Andersson et al., 2005). In general, the operon consists of 3 genes; 1) *treB*, encoding for a trehalose-specific enzyme IIBC transport protein complex required for the phosphorylation of the disaccharide via the phosphoenol-pyruvate transport system (PEP:PTS) 2) *treA*, a phosphotrehalase which cleaves trehalose-6-phosphate to glucose and glucose-6-phosphate, and 3) a regulator with high similarity to the GntR-like family of transcriptional regulators. Additionally, a *cis*-acting catabolite responsive element (CRE) has been identified in the promoter region of the operon just upstream of the OFR for *treB* (Miwa et al., 2000).

Based on DNA sequence, the predicted protein in *L. monocytogenes* EGD-e encoded by *treA*,  $\alpha$ ,  $\alpha$ , (1,1) phosphotrehalase, is a glucosidase that is highly similar to the characterized *B. subtilis* enzyme as well as trehalose-6-phosphate hydrolase, the product of the *treC* gene of *E. coli* (Rimmele and Boos, 1994). In *B. subtilis*, the phosphotrehalase hydrolyses trehalose-6-phosphate, the sugar derivative that forms during the translocation of trehalose across the cytoplasmic membrane via the PEP:PTS. In the present study, it was clearly shown that the product of *lmo 1453* (from EGD-e sequence) is indeed required for the cleavage of trehalose-6-phosphate in *L. monocytogenes* 568. Crude cell lysates from the wild-type strain efficiently hydrolyzed pure trehalose-6-phosphate as determined by assaying for liberated glucose by the glucose hexokinase method. On the other hand, glucose could not be detected when using crude cell lysates from the *treA* mutant. According to the genomic map of *L. monocytogenes* strain EGD-e (Genebank accession number, AL591824), there does not appear to be another obvious mechanism for the passage of trehalose to the cytoplasm nor is the disaccharide split into two glucose



subunits by exogenous trehalases since no glucose was detected in the medium following growth of Lm568 in MWB supplemented with trehalose as the sole carbon source. However, growth of the mutant in MWB-trehalose was not completely abolished. Therefore, there may be a secondary, less efficient permease for the transport of trehalose in *L. monocytogenes*. The pooling of cell components in crude cell extracts may inhibit enzymatic activity of another possible trehalase, hence the absence of activity in our assays especially when activities are naturally low. Nevertheless, it appears that the primary mechanism requires that trehalose must first pass through a trehalose-specific or semi-specific channel in the cytoplasmic membrane and must then be phosphorylated by a trehalose-specific enzyme IIBC in order to enter into the cytosol (Postma et al., 1993). The phosphorylated sugar is then cleaved by the action of the phosphotrehalase and the end products, glucose and glucose-6-phosphate are ready for entry into the glycolytic pathway.

Our *treA* mutant accumulated high levels of trehalose-6-phosphate which appeared to be dependent on the trehalose concentrations in the growth media and vegetable juice extracts. For *B. subtilis*, it has been shown that trehalose is the external molecular inducer of the operon, whereby, in its absence, the TreR repressor turns down the transcription of *treA* (Schöck and Dahl, 1996). Moreover, trehalose-6-phosphate acts as the internal inducer for *treA*, where the phosphorylated sugar interferes with the repressor/*tre*-operator interaction, thus allowing for the transcription of the *treA* gene to proceed. Also, media constituents other than extracellular trehalose will affect the transcription of the gene. Catabolites such as glucose, fructose or mannitol have been

found to be highly repressive for the uptake of trehalose (Helfert et al., 1995; Dahl, 1997; Evans Gilbreth et al., 2004). In this study, glucose was shown to act as a repressor in a concentration-dependent manner on the transcription of *treA* as shown with lower enzymatic activities found in extracts from Lm568 cells cultured in the presence of glucose.

Studies conducted with yeasts have indicated that a build up of trehalose-6-phosphate is toxic to the cells thence resulting in increased sensitivity to environmental stresses (De Virgilio et al., 1993; Franco et al., 2000; Van Vaeck et al., 2001; Van Dijck et al., 2002). Duong et al. (2006) also found that a *Lactobacillus acidophilus treB* (phosphotrehalase) deletion mutant was intolerant to stresses that were normally endured by the wild-type strain. Their study showed that in the presence of trehalose, repeated freeze/thaw cycles (-70°C, 5 min; 20°C, 10 min) had a more detrimental effect on the mutant (< 10% viability), than it did on the wild-type strain (>80% viability). Additionally, a phosphotrehalase mutant of *Bacillus popillae*, found to be unable to grow in a basal medium containing 0.2% trehalose as the only carbon source, continued to be inhibited even following the subsequent addition of glucose to the same culture (Bhumiratana et al., 1974). By monitoring [<sup>14</sup>C]-trehalose uptake, it was demonstrated that high levels of [<sup>14</sup>C]-trehalose-6-phosphate accumulated in the cells. The authors speculated that the accumulation of the phosphorylated derivative was responsible for the inhibition. However, increased concentrations of intracellular trehalose-6-phosphate in the deletion mutant did not negatively affect its ability to withstand environmental stresses. In fact, a significant increase in the thermo- and osmotolerance compared to the

wild-type Lm568 was observed. However, this observation may be merely coincidental since it has been demonstrated that the phosphorylated version of the disaccharide did not provide the same protective capacity as trehalose when the activity of a number of purified enzymes were tested *in vitro* following heat stress (Hottiger et al., 1994). Moreover, a mutation in the TPS2 locus of a *Saccharomyces cerevisiae* (encoding for trehalose-6-phosphate phosphatase) resulted in a heat sensitive phenotype that could no longer synthesize trehalose and accumulated trehalose-6-phosphate (Elliot et al., 1996). With regard to the *treA* mutant, a concomitant build up of trehalose was detected in the cell extracts along with trehalose-6-phosphate, although the latter was always present in *ca.* 2-fold greater concentrations than trehalose. Since no detectable trehalase activity was observed in crude cell extracts, which would allow for the direct cleavage of trehalose into two glucose molecules, the transport of the unmodified disaccharide into the cell may serve no purpose in terms of catabolism. Moreover, assaying for the presence of possible phosphatases with an affinity for trehalose-6-phosphate did demonstrate low level activity in repeated experiments. Therefore, it may be concluded that the increasing concentrations of trehalose were likely due to the dephosphorylation of trehalose-6-phosphate by non-specific phosphatases previously described by Elbein et al. (2003). This theory also helps to explain the dilemma of high levels of trehalose-6-phosphate not causing stress sensitivity as seen in other organisms, since these levels are being simultaneously reduced and replaced by the compatible solute, trehalose.

When exposed to certain environmental stresses, the accumulation of trehalose has been clearly shown to provide protection to cells of both bacteria and yeast. In fact, in

*Saccharomyces cerevisiae* the biosynthesis of the disaccharide is initiated by the same stimuli that induce a heat shock response (Neves and François, 1992; Hottiger et al., 1994; Elliot et al., 1996). Furthermore, Hengge-Aronis et al. (1991) showed that the putative stationary-phase sigma factor  $\sigma^S$  controls the expression of *otsA* and *otsB*, the genes encoding for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, in *Escherichia coli*. However, this was only for stationary phase associated thermotolerance, whereas the ability to synthesize trehalose is dispensable during the development of adaptive thermotolerance in logarithmic phase cells (Hengge-Aronis et al., 1991). Similarly, it was also found that the synthesis and subsequent accumulation of trehalose in *Salmonella enterica* Serovar Typhimurium is thermo-regulated (Cánovas et al., 2001). The observed increase in thermotolerance at 52°C for the mutant was manifested through the extension of the shoulder region of the thermal death curve and also an increased D-value in the log linear region of the curve. Moreover, the pAM:TREOP complement was slightly more thermal sensitive than the wild-type Lm568. This may be explained by the fact that the complemented mutant over-expresses phosphotrehalase activity. Under normal circumstances, the *treA* gene is induced by the presence of T6P which presumably must reach a threshold level to activate the gene. Therefore, in the presence of extracellular trehalose, a basal T6P level must exist within the cell. However, if *treA* is constitutively expressed, there would be no opportunity for T6P to accumulate. Hence it follows that no dephosphorylation of T6P could occur and trehalose would be absent in the cytoplasm of the complemented *treA* mutant. This would support other findings where the overproduction of trehalose

provided elevated tolerance to high osmolarity in *E. coli* (Purvis et al., 2005). The mechanism by which trehalose confers thermotolerance is thought to be due to its interaction with cellular proteins, preventing their denaturation and aggregation. Therefore, trehalose may act as a molecular chaperone working synergistically with heat shock proteins at elevated temperatures (Welch and Brown, 1996; Kandrór et al., 2002).

In addition to elevated heat resistance, the *treA* mutant was also more resistant than the wild-type strain to high osmolarity. A 2-log increase in survivors was observed for the mutant over Lm568 after 48 h in the presence of 20% NaCl. Since desiccation can be viewed as an extreme case of osmotic stress (Potts, 1994) further evidence for increased resistance to osmotic stress and membrane protection was also provided by the finding that shorter recovery times for dried *treA* mutant cells reconstituted in BHI was required than for the wild-type. The observation of shortened times to reach exponential growth was presumably due to the presence of more surviving and/or undamaged cells. The ability for *Listeria* to survive under such osmotic stress is generally achieved by the accumulation of the compatible solutes glycine betaine, carnitine and proline (Sleator et al., 2003). However, other bacteria display an osmotically induced accumulation of trehalose. For example, *E. coli* will accumulate trehalose under high osmolarity if the preferred osmolyte, glycine betaine, is not available (Ström and Kaasen, 1993). This preference for glycine betaine (if present) over trehalose under desiccation is interesting since the accumulation of the former osmolyte by *E. coli* was shown to provide no survival advantage, yet endogenous accumulated trehalose provided significant protection for these cells (Welsh and Herbert, 1999). Additionally, Breeuwer and co-workers (2003)

determined that stationary phase cells of *Enterobacter sakazakii* were able to withstand desiccation far more effectively than logarithmic phase cells. They attributed this resilience in part to the significant increase in trehalose present in the former cells (0.040 versus 0.003  $\mu\text{mol/mg}$  cell protein).

The importance of intracellular trehalose accumulation in providing microorganisms protection against high alcohol concentrations, oxidative stress, and cold temperature survival has also been demonstrated (Sharma, 1997; Benaroudj et al., 2001; Kandror et al., 2002). However, a significant enhancement was not observed for the survival of the *treA* mutant in comparison to the wild-type for any of these stressors. In fact, the wild-type strain appeared to have relatively high natural resistance to 18% ethanol and 0.1%  $\text{H}_2\text{O}_2$ , relative to other published data regarding *Listeria monocytogenes* (Lin and Chou, 2004). Therefore, the margin of protective capacity may be too narrow to expect a significant enhancement in survivability for the mutant. Pertaining to cold growth experiments, *Listeria* are psychrotrophs, thence the organism is well adapted not only to cold survival, but to growth at refrigerated temperatures which is somewhat unusual for a foodborne human pathogen (Bayles et al., 1996). During cold adaptation in BHI it has previously been shown that *L. monocytogenes* takes up large amounts of carnitine and glycine betaine via transporters encoded by OpuC and GbuABC, respectively (Wemekamp-Kamphuis et al., 2004). Additionally, *Listeria* adjusts their membrane fatty acid profiles to shorter chain fatty acids with a higher degree of unsaturation to maintain the proper membrane fluidity at low temperatures (Tasara and Stephan, 2006). Therefore, since the ability to proliferate at very low temperatures is a

hallmark of *Listeria* it is not surprising that the accumulation of trehalose by the mutant, did not affect its growth profile in BHI broth since its action would not be expected to influence growth rates. Furthermore, the presence of trehalose-6-phosphate does not appear to have a negative effect on its ability to grow at 4°C. Conversely, the accumulation of trehalose for cold survival by the mesophilic enteric bacterium, *E. coli*, is highly advantageous for its survival at 4°C. It has been shown that the transcription of the *otsA/B* genes is initiated early in the temperature downshift in order to prepare the cells for impending lower temperatures (Kandror et al., 2002).

To conclude, this is the first study where the effect of a deletion in any of the genes of the putative trehalose operon in *Listeria monocytogenes* has been reported. It has been clearly demonstrated that the ORF *lmo1453* in *Lm568* encodes for a phospho- $\alpha$ -(1,1)-glucosidase required for the hydrolysis of trehalose-6-phosphate. Moreover, disruption of this gene did not completely abolish growth when trehalose was the sole carbon source. It is possible that a secondary but less efficient transport system exists for the utilization of trehalose and experimentation is on going to further elucidate this hypothesis. Furthermore, growth in the presence of trehalose, results in the accumulation of trehalose-6-phosphate and the evidence obtained here suggests that it is subsequently dephosphorylated resulting in a build up of the compatible solute trehalose. Although, there is no mechanism for listerial synthesis of trehalose, as in other microorganisms, this study has shown that the disruption of *treA* and subsequent accumulation of intracellular trehalose leads to a hypertolerant phenotype against heat, high osmolarity, desiccation and freeze/thaw cycling. Therefore, the *treA* mutant, artificially induced for the

accumulation of trehalose, provides a model system to help understand the protective capacity of trehalose as a compatible solute in other Gram-positive foodborne pathogens.



Table 7.1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	Reference or Source
<b>Strains</b>		
<i>L. monocytogenes</i>		
Lm568	Wild type; serotype 1/2a; shrimp processing plant.	Hefford et al. (2005)
32F11	Tn917 mutant of Lm568; erm <sup>R</sup>	Ells and Truelstrup Hansen (2006)
568:ΔT6P	Lm568 deletion mutant, TreA <sup>-</sup>	This study
ΔT6P pAM:TREOP	pAM401:T6P complement of Lm568ΔT6P; crm <sup>R</sup> , tet <sup>S</sup> , TreA <sup>+</sup>	This study
TCELm:ΔT6P	Lm568 carrying PAUL-ΔT6P with treA deletion insert	This Study
<i>E. coli</i>		
TCTOP:ΔT6P	TOP10 carrying pCR®2.1 TOPO with Lm568 treA insert with deletion	This study
TCTOP:T6P	TOP10 carrying pCR®2.1 TOPO with intact Lm568 treA	This study
DH5α	Cloning host; F- endA1 glnV44 thi-1 recA1 gyrA96 deoR nupG Φ80LacZ ΔM15 Δ(lacZYA-argF)U169, hsdR17(r <sub>K</sub> -m <sub>K</sub> <sup>+</sup> ), λ-	Hanahan, (1985)
XL10-GOLD KanR	Cloning host; endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tetR F'[proAB lacIqZ ΔM15 Tn10(TetR Amy Tn5(KanR))]	Stratagene
XLCVV1	XL10-GOLD carrying pCVV1 expression vector	This study
TCE-PAUL:T6P	DH5α host carrying pAUL-A with treA deletion insert	This study
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) ψ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 λ-	Invitrogen
<b>Plasmids</b>		
pAUL-A	Gram positive and Gram negative shuttle vector, ori <sup>TS</sup> , LacZα', erm <sup>R</sup>	Chakraborty et al. (1992)
pAM401	Gram positive and Gram negative shuttle vector; crm <sup>R</sup> , tet <sup>R</sup>	Wirth et al. (1986)
PCR:ΔT6P	pCR®2.1 TOPO with Lm568 treA insert with deletion	This study
pAULA:ΔT6P	pAUL-A with truncated Lm568 treA gene, erm <sup>R</sup>	This study
pAM401:TREOP	pAM401 with intact Lm568 treA gene, crm <sup>R</sup> , tet <sup>S</sup> , treA <sup>+</sup>	This study
pCVV1	pCYB1 derived expression vector carrying <i>B. Subtilis</i> treA. Tac promoter, amp <sup>R</sup>	Van Vaecck et al. (2001)
pCR®2.1 TOPO	ColE1, amp <sup>R</sup> , Kan <sup>R</sup> , lacZα	Invitrogen

Table 7.2. Thermal inactivation parameters for *Listeria monocytogenes* strain 568 and *Lm568:ΔT6P* at 52°C following growth on different media. Inactivation kinetics was modeled using a modification of the Fermi distribution function proposed by Pruitt and Kamau (1993).

Strain and Growth medium	$\beta$	$t_{cm}$	A.S.E. ( $\beta$ )	A.S.E. ( $t_{cm}$ )	RSS	Logistic D-value (min)
<i>Lm568</i>						
TSB	0.235	12.86	0.009	1.70	0.489	22.57
TSB-YE	0.228	12.09	0.010	1.63	0.113	22.11
TSB-Tre	0.202	15.62	0.019	3.26	0.332	26.35
BHI	0.196	18.29	0.013	2.27	0.694	29.95
<i>Lm568:ΔT6P</i>						
TSB	0.203	11.80	0.010	2.27	0.576	23.05
TSB-YE	0.176*	14.31	0.012	3.13	0.765	27.14
TSB-Tre	0.161*	19.21*	0.005	1.43	0.208	33.31*
BHI	0.152*	29.03*	0.004	1.10	0.102	44.09*

N.B. The symbol  $\beta$  indicates inactivation rate parameter for the log linear region of the death curve;  $T_{cm}$  – lag parameter prior to the region of maximum inactivation rate;  $D_L$  – is the time for the initial log decline when a lag phase exists (Eq. 2)

ASE is the asymptotic standard error

\* denotes the parameter is significantly greater than that of the wild type *Lm568* for the same treatment ( $P < 0.05$ ).

Table 7.3. Thermal inactivation parameters at 52°C for *Listeria monocytogenes* 568, *Lm568:ΔT6*, complemented mutant *ΔT6 pAM:TREOP* and *Lm568:pAM401*. Inactivation kinetics were modeled using a modification of the Fermi distribution function proposed by Pruitt and Kamau (1993).

Strain	$\beta$	<i>tcm</i>	A.S.E. ( $\beta$ )	A.S.E. ( <i>tcm</i> )	RSS	Logistic D-value (min)
<i>Lm568</i>	0.175	17.47	0.006	1.76	0.387	30.54
<i>Lm568:ΔT6</i>	0.141*	29.64*	0.005	1.79	0.338	45.88*
<i>ΔT6 pAM:TREOP</i>	0.186	10.79*	0.008	2.12	0.371	23.08
<i>Lm568:pAM401</i>	0.174	15.35	0.012	3.66	1.248	28.49

N.B. The symbol  $\beta$  indicates inactivation rate parameter for the log linear region of the death curve; *Tcm* – lag parameter prior to the region of maximum inactivation rate; *D<sub>L</sub>* – is the time for the initial log decline when a lag phase exists (Eq. 2)

ASE is the asymptotic standard error

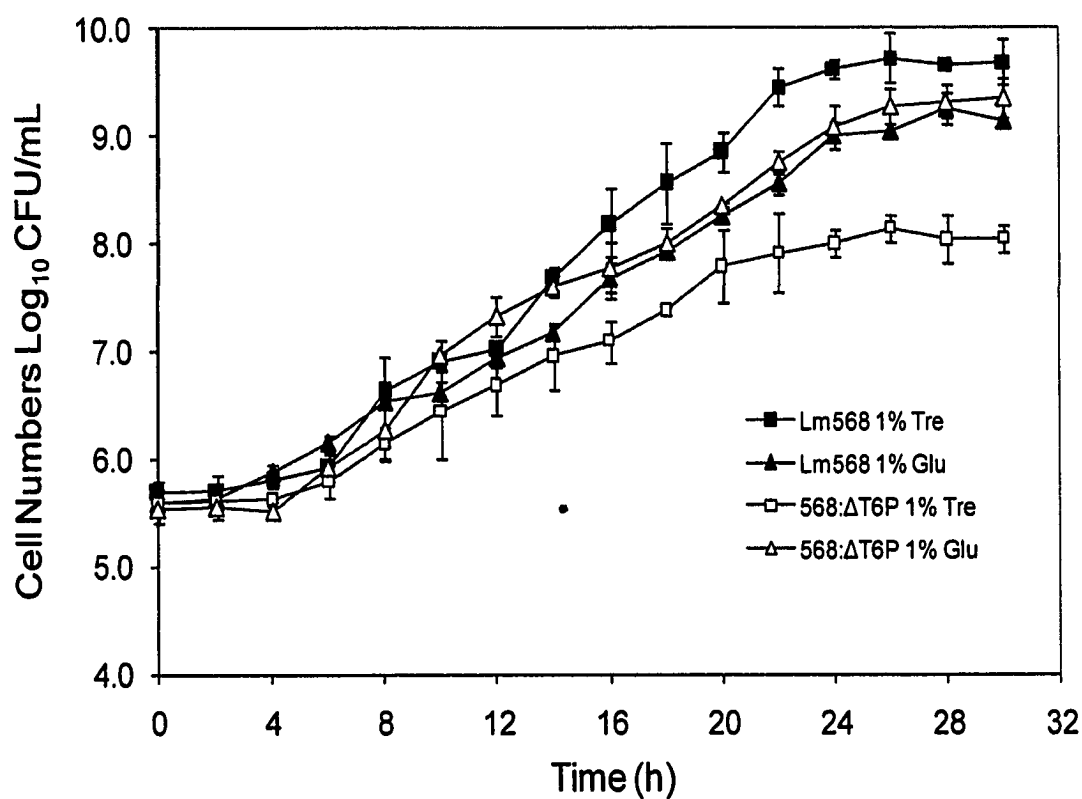
\* denotes the parameter is significantly different than *Lm 568* ( $P < 0.05$ ).

Table 7.4. Thermal inactivation parameters for *Listeria monocytogenes* strain 568 and *Lm568:ΔT6P* at 52°C following growth in vegetable and mushroom juices. Inactivation kinetics were modeled using a modification of the Fermi distribution function proposed by Pruitt and Kamau (1993).

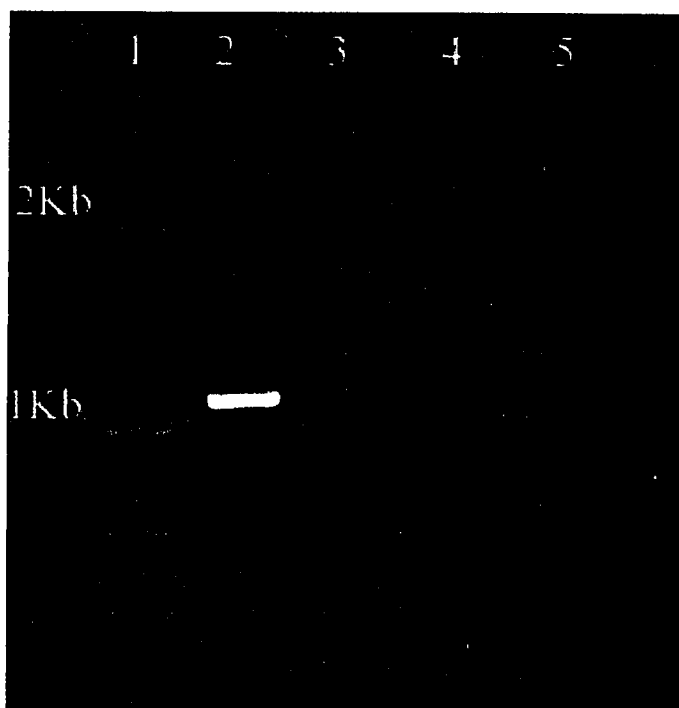
Strain and Growth medium	$\beta$	<i>tcm</i>	A.S.E. ( $\beta$ )	A.S.E. ( <i>tcm</i> )	RSS	Logistic D-value (min)
<i>Lm568</i>						
mushroom	0.116	40.02	0.007	2.59	0.469	59.83
cabbage	0.177	42.45	0.015	3.29	1.399	55.37
bean	0.153	29.87	0.017	5.41	3.467	44.82
pea	0.138	41.62	0.012	3.82	1.272	58.21
<i>Lm568:ΔT6P</i>						
mushroom	0.089	43.64	0.010	4.85	0.704	69.42
cabbage	0.211	50.74	0.015	2.54	0.311	61.56
bean	0.148	41.77	0.014	3.93	1.576	57.23
pea	0.116	49.77	0.013	4.23	0.940	69.52

N.B. The symbol  $\beta$  indicates inactivation rate parameter for the log linear region of the death curve; *Tcm* – lag parameter prior to the region of maximum inactivation rate; *D<sub>L</sub>* – the time for the initial log decline when a lag phase exists (Eq. 2)

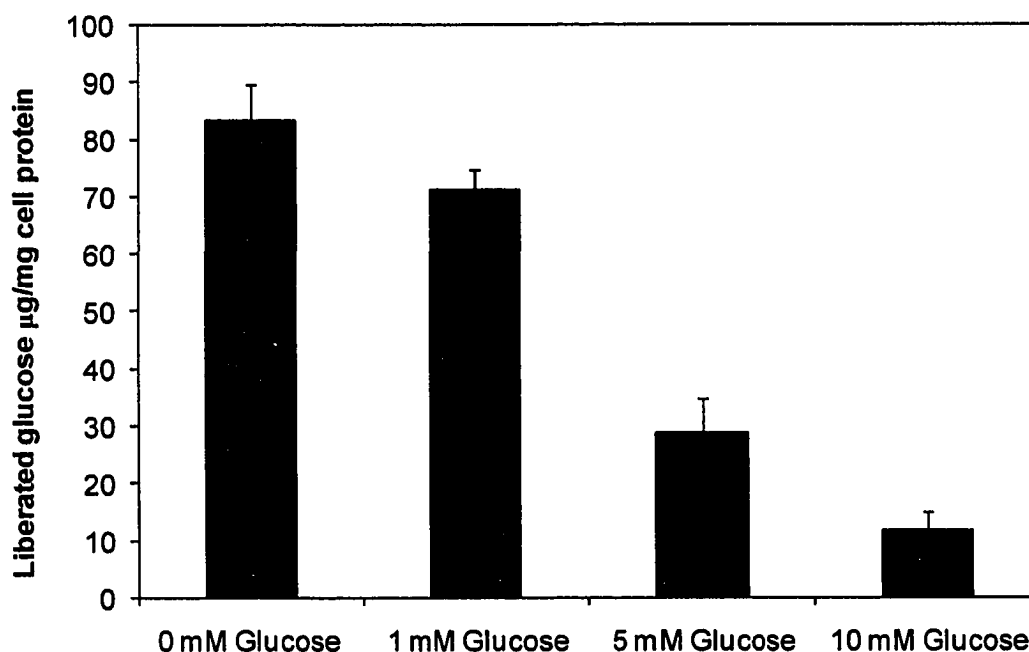
ASE is the asymptotic standard error



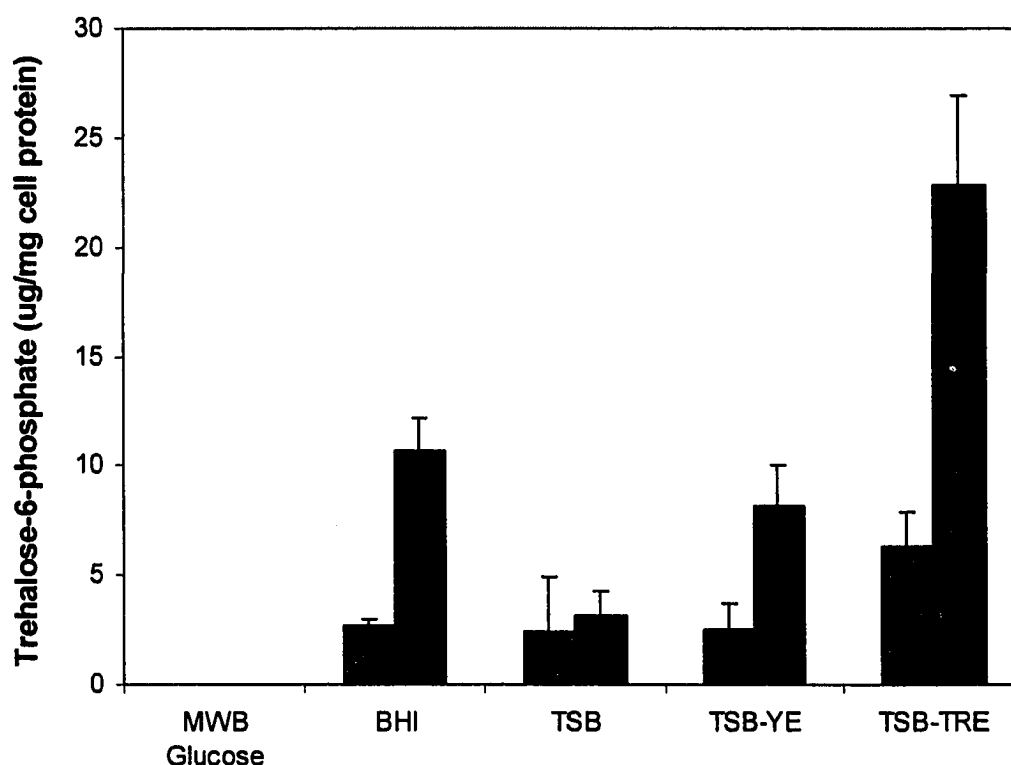
**Figure 7.1.** Growth curves for Lm568 and *treA* mutant 568:ΔT6P in Modified Welshimer's Broth (Premaratne *et al.*, 1991) at 37°C and 150 rpm. The values displayed for each data point are the mean of four samples (two replicates from two independent experiments). The error bars represent the standard deviations for four samples.



**Figure 7.2.** Reverse transcriptase PCR of *treA* gene in Lm568 and 568: $\Delta$ T6P. Lane 1 DNA ladder; Lane 2, Lm568: $\Delta$ T6P amplicon from cDNA; Lane 3, control for Lm568: $\Delta$ T6P with no RT added to the reaction mixture; Lane 4, Lm568 amplicon from cDNA; Lane 5, control for Lm568: $\Delta$ T6P with no RT added to the reaction mix.

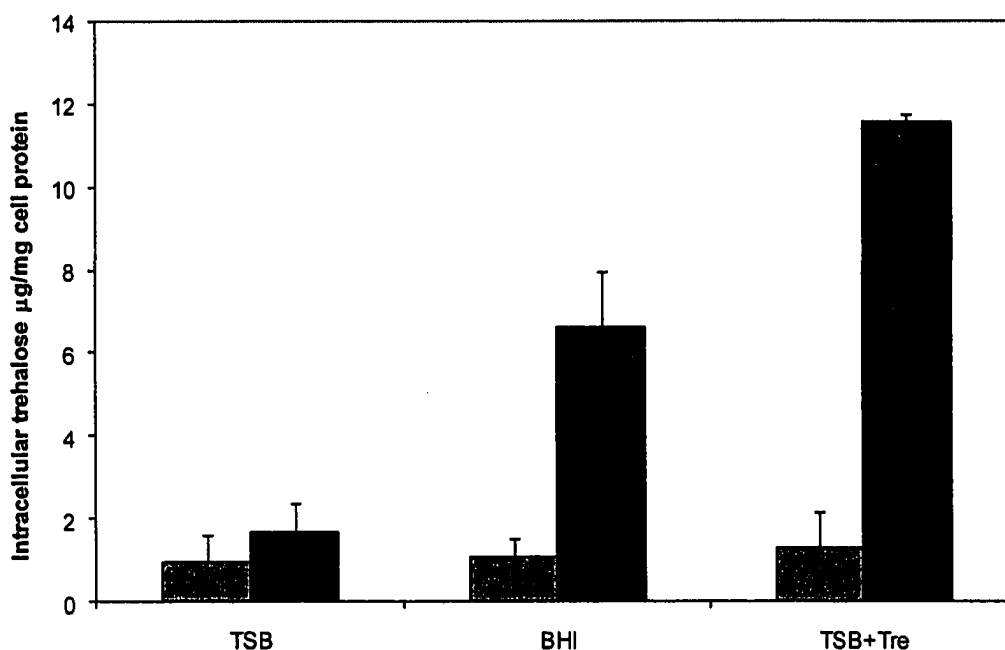


**Figure 7.3.** The repression of phosphotrehalase activity by the presence of glucose in minimal growth media. Crude cell extracts were prepared from *Listeria monocytogenes* strain 568 grown in modified Welshimer's broth (MWB) supplemented with 10 mM trehalose and varying concentrations of glucose. Pure trehalose-6-phosphate was added to the cell extracts at a concentration of 10 mM and the samples were incubated at 37°C for 1 hour. Hydrolysis of trehalose-6-phosphate was quantified by measuring the amount of free glucose liberated by the glucose hexokinase reaction. The results show the means of four samples (two replicates from two independent trials). Error bars represent the standard deviations of the four samples.

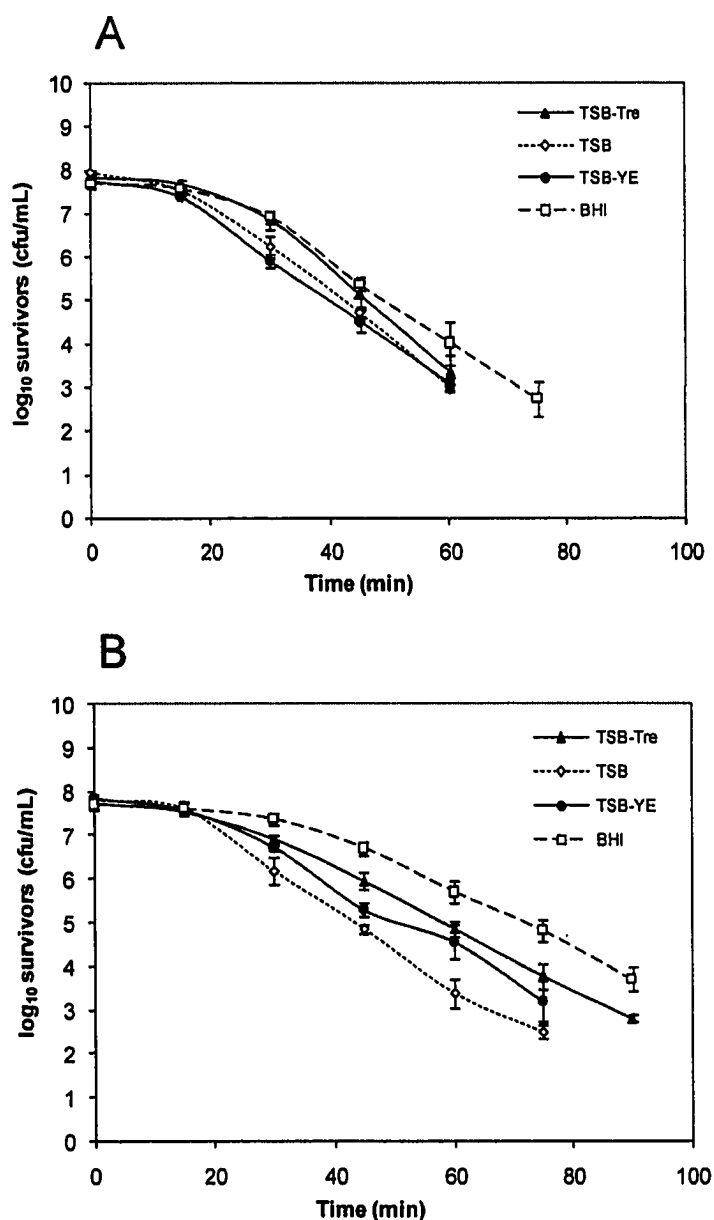


**Figure 7.4.** Intracellular accumulation of trehalose-6-phosphate by Lm568 (grey bars) and 568:ΔT6P (black bars). The wild-type and *treA* mutant were grown on five different media (MWB+Glu, TSB, TSB-YE, TSB-Tre and BHI) and sugars were extracted from sonicated cell pellets. The amount of trehalose-6-phosphate was determined according to the enzyme assay developed by Van Vaeck et al. (2001) using purified phosphotrehalase extracted from a recombinant *Escherichia coli* carrying the *treA* gene from *Bacillus subtilis* on a pCYB1-derived expression vector. The results show the means of four samples (two replicates from two independent trials). Error bars represent the standard deviations of the four samples.

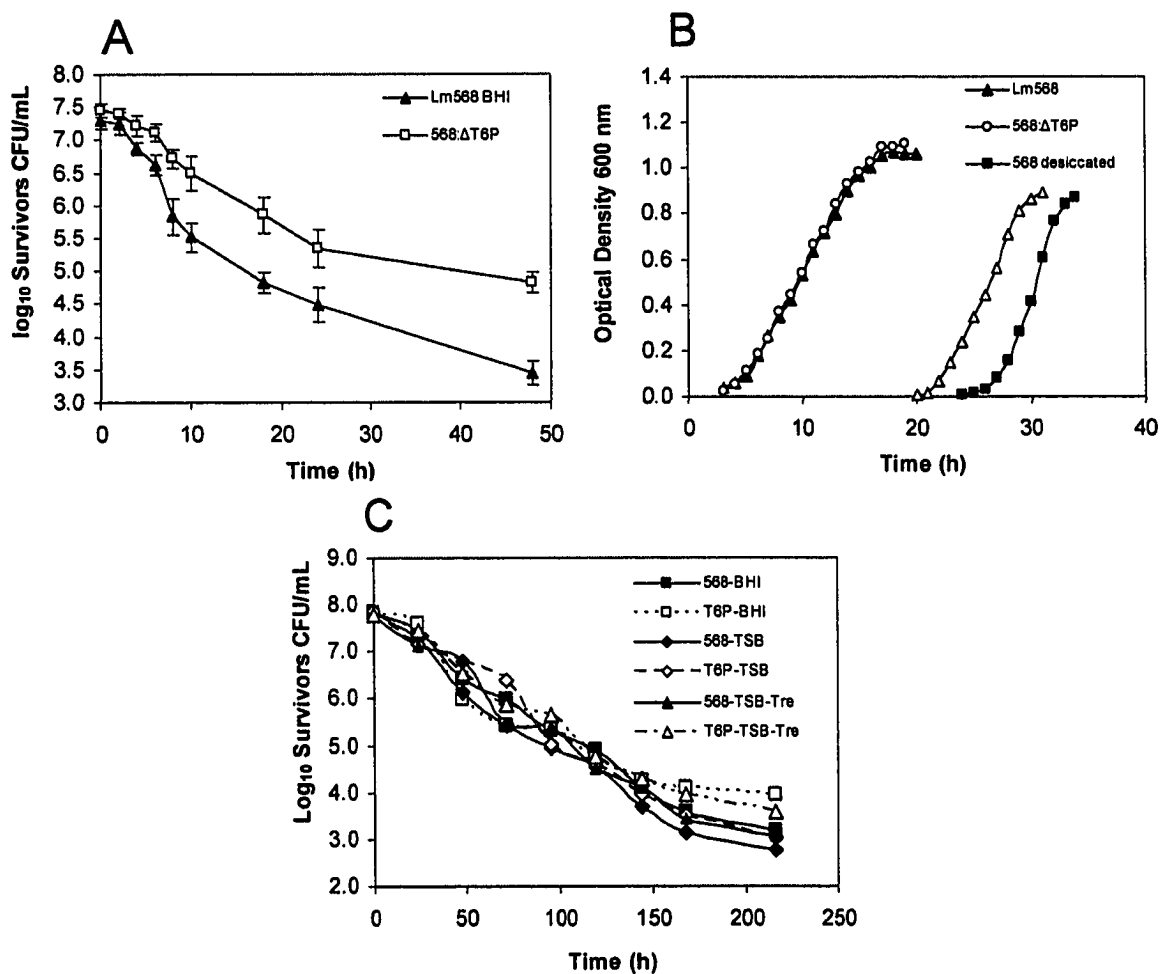




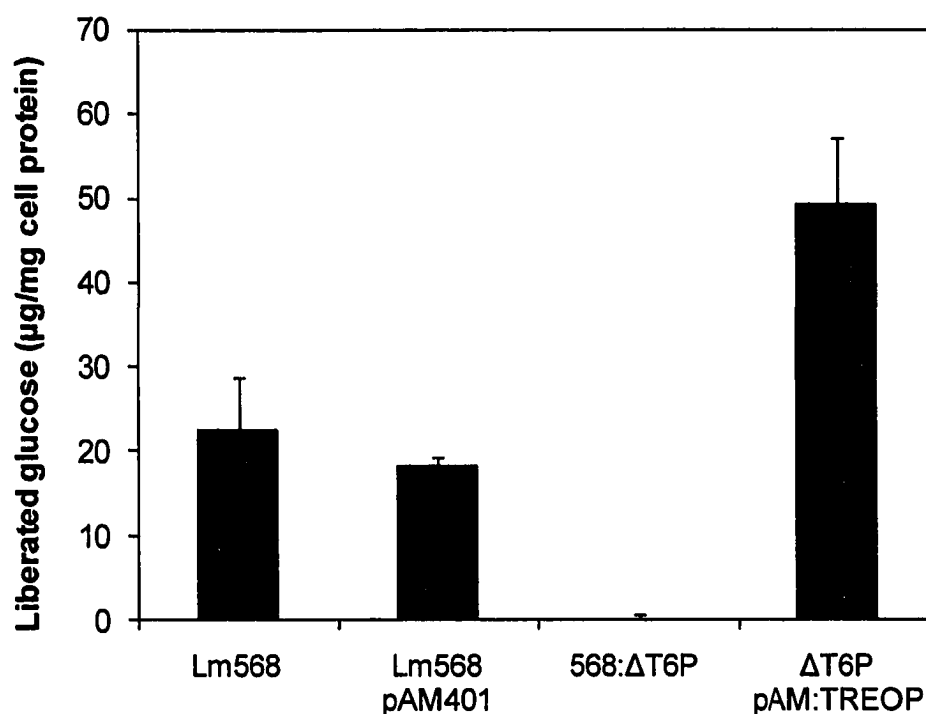
**Figure 7.5.** Intracellular trehalose measurements from Lm568 (grey bars) and 568:ΔT6P (black bars). Cultures were grown for 16 h at 37°C in TSB, TSB+1% trehalose or BHI. Sugars were extracted from washed cell pellets. The extracts were incubated in the presence of 0.05 mM porcine kidney trehalase for 2 h at 37°C and the trehalose concentrations were measured by indirectly by determining the amount of liberated glucose by the glucose hexokinase reaction. The results show the means of four samples (two replicates from two independent trials). Error bars represent the standard deviations of the four samples.



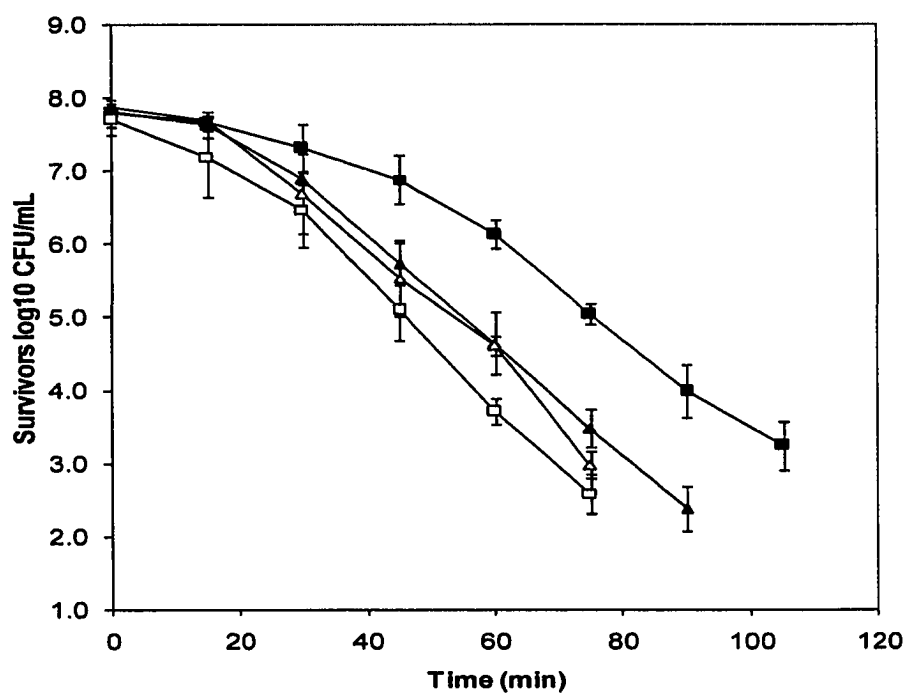
**Figure 7.6.** Survival curves for heat treated *L. monocytogenes* strain A) 568 (Lm568) and B) its *treA* deletion mutant, 568:ΔT6P, as influenced by growth media. The bacteria were cultured in one of 4 different media [tryptic soya broth (TSB), TSB + 0.6% yeast extract (TSB-YE), TSB + 1% trehalose (TSB-Tre), and brain heart infusion broth (BHI)], washed and resuspended in 0.1% peptone water. The cell suspensions were dispensed in thin-walled PCR tubes and then heat treated at 52°C in a thermocycler. Results show the means of three independent experiments with corresponding standard error bars.



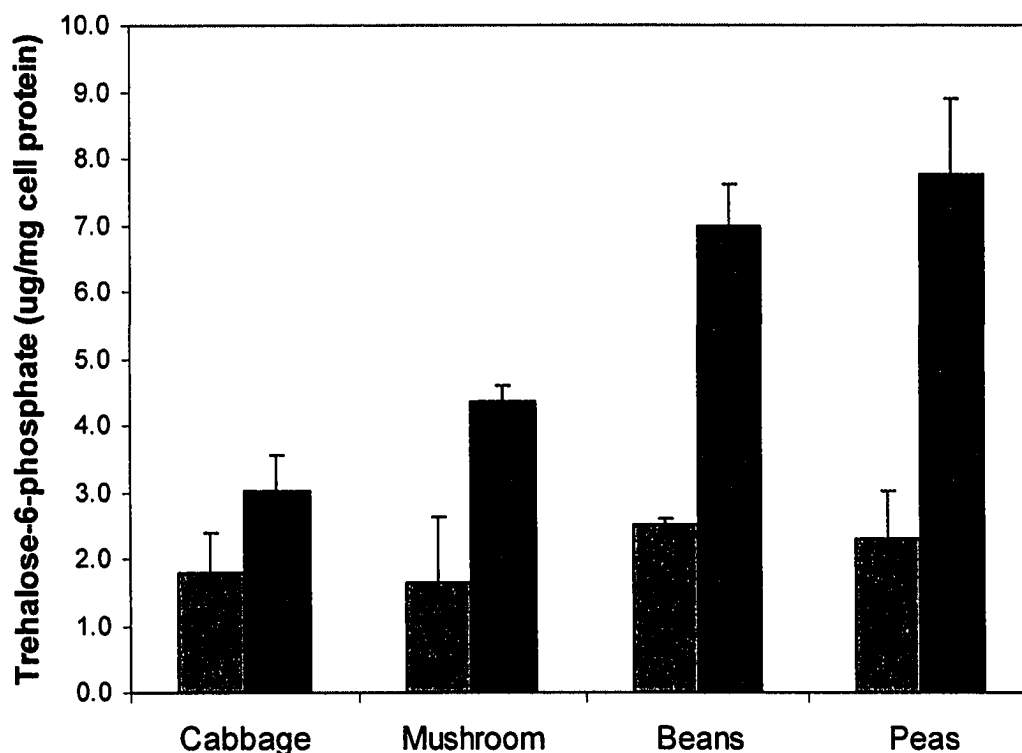
**Figure 7.7.** The effect of A) high osmolarity, B) desiccation, and C) repeated freeze/thaw cycling on the survival of *L. monocytogenes* strain 568 (Lm568) and its *treA* deletion mutant, 568:ΔT6P. For high osmotic stress and desiccation experiments, early stationary phase cells were harvested from BHI cultures. Cells were either resuspended in BHI+20% NaCl, or desiccated and stored at ambient temperature for 14 days. For the freeze/thaw experiments, the cells were grown to stationary phase in three different media (BHI, TSB and TSB supplemented with 1% trehalose), then resuspended in phosphate buffered saline (pH 7.0) and subjected to repeated freeze/thaw cycles ( $-80^{\circ}\text{C}/37^{\circ}\text{C}$ ) every 24 hours. Tolerance to high osmolarity and freeze/thaw cycling was estimated by enumerating survivor numbers by plating onto TSA-YE. Resistance to desiccation was measured by estimating time to recovery by absorbance readings at 600 nm. The results for the survival in high salt (A) show the means of three independent experiments and their corresponding standard errors. The displayed results for desiccation recovery (B) show one of three trials. The results shown for the freeze/thaw experiment (C) are the mean populations for three independent experiments with corresponding standard errors.



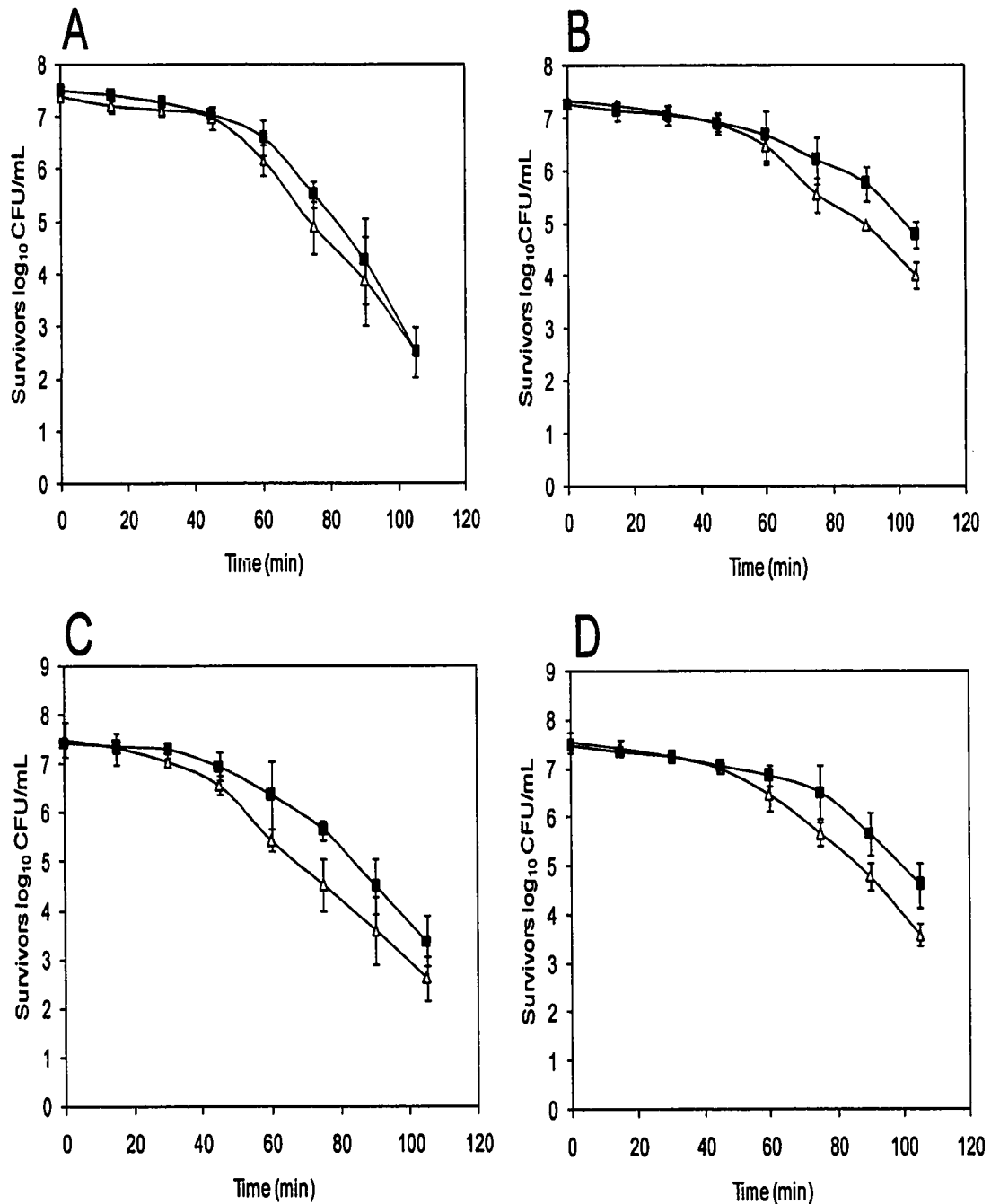
**Figure 7.8.** Complementation of 568:ΔT6P with intact *treA*. An amplicon encompassing the *treA* and *treB* genes from Lm568 was cloned into the shuttle vector pAM401. The resulting construct pAM401:TREOP was transformed into the *treA* mutant 568:ΔT6P to create ΔT6P pAM:TREOP and phosphotrehalase activity in crude cell extracts was measure using the glucose hexokinase assay. The results show the means of four samples (two replicates from two independent trials). Error bars show the standard deviations of the four samples.



**Figure 7.9.** Heat resistance of the complemented *treA* mutant. Wild-type strain Lm568 (-▲-), Lm568 pAM401 (-Δ-), deletion mutant 568:ΔT6P (-■-), and pAM401:TREOP transformed *treA* mutant, ΔT6P pAM:TREOP (-□-), were grown for 16 h at 37°C in brain heart infusion broth. The cultures were washed and re-suspended in 0.1% peptone water, and then subjected to a heat treatment of 52°C. The plot represents the mean survivors from three independent heat trials and corresponding standard errors.



**Figure 7.10.** The accumulation of trehalose-6-phosphate by cells grown in vegetable juice extracts. The wild-type Lm568 (gray bars) and *treA* deletion mutant 568:ΔT6P (black bars) were grown for 20 h at 30°C in sterile juice extracts prepared from cabbage, mushrooms, beans and pea. Sugars were extracted from sonicated cells and the trehalose-6-phosphate concentrations was determined by enzymatic cleavage with purified phosphotrehalase and subsequently assaying for liberated glucose using the glucose hexokinase method. The results show the means of four samples (two replicates from two independent trials). Error bars represent the standard deviations of the four samples.



**Figure 7.11.** Thermal death curves for wild-type strain Lm568 (-Δ-) and its *treA* mutant 568:ΔT6P (-■-) following growth in various vegetable extracts. Cells were grown in A) cabbage, B) mushroom C) bean, and D) pea juices for 20 h at 30 °C. Cells were harvested by centrifugation, washed and resuspended in 0.1% peptone water. Thermal death times were carried out at 52°C. Results show the mean populations for three separate experiments with corresponding standard error bars.

## 8. CONCLUSIONS

The increased consumption of raw or minimally processed fruit and vegetables has led to food safety concerns. Numerous recent outbreaks and spontaneous occurrences of foodborne disease associated with produce are evidence that more research is required to better our understanding of how human pathogens survive and/or grow on foods of plant origin, and what impact minimal processing has on the pathogens. Although, the 1981 outbreak of *L. monocytogenes* associated with coleslaw demonstrated for the first time that this pathogen could be transmitted via contaminated food, there have been few incidents involving *L. monocytogenes* and produce since that time. However, *L. monocytogenes* is one of the leading causes of death among foodborne pathogens in North America and its hardy nature requires vigilance, especially for foods previously shown to support the growth of the organism. To fully understand the potential risks associated with a particular pathogen and process, one must dissect the food process and examine important features of the organism such that appropriate interventions may be implemented.

In this study several important genetic and environmental factors associated with *L. monocytogenes* and mild thermal processing of shredded cabbage were examined. These factors impact the ability of *L. monocytogenes* to successfully survive and proliferate on minimally processed cabbage. The approach was sequential in that the ability of this pathogen to attach to cabbage tissues was first examined. Once attached, growth experiments of *L. monocytogenes* on different cabbage tissues were conducted and the effect of mild thermal processing on the capacity of the treated cabbage to support the



growth of *L. monocytogenes* was elucidated. Lastly, since the application of mild heat on produce has become an acceptable practice for enhanced sensory quality and shelf life stability, the genetic aspects of thermal resistance of *L. monocytogenes* was evaluated. In particular, the focus was on the molecular alterations that resulted in greater thermal resistance.

Preceding all other considerations for the success of *L. monocytogenes* in the food processing continuum is its ability to effectively attach to surfaces. Due to its ubiquitous nature, *L. monocytogenes* may come into contact with produce prior to harvest or at any point during post harvest handling, processing, transport or retail. The results from this study demonstrates that *Listeria* attach to cabbage surfaces in a strain specific manner. Although some strains were shown to attach significantly ( $P < 0.05$ ) better than others, it was evident that all strains were capable of binding rapidly to cabbage tissues and their removal through washing was difficult. Moreover, damaged tissues act as better receptor sites for the congregation of listerial cells. Therefore, abrasions occurring in the field or during handling may provide more attractive surfaces for the attachment of *Listeria* spp. It was also demonstrated that *Listeria* spp, including *L. monocytogenes* can remain viable on intact cabbage for a substantially long period of time (four weeks at 5°C). Since *Listeria* spp. is so widespread and attaches very effectively to plant surfaces, there is a strong probability that produce entering the fresh market or processing facilities may contain this pathogen. In respect to cabbage, these cells would be found on the outer leaf layers and hence it is important that these leaves are carefully removed and the striped cabbage heads do not touch surfaces previously in contact with the contaminated

cabbage. Moreover, based on the ability of *Listeria* spp. to exist in biofilms, contact areas in packing sheds and processing facilities must be properly sanitized to reduce the risk of persistent contamination problems.

Processors of fruits and vegetables are continuously seeking ways to improve the quality of retail products. Processed cabbage is normally shredded and packaged for use in salad mixes and in particular, coleslaw. The shelf-life of shredded cabbage is usually 10 days at 4°C before the development of off-colours and flavours become unacceptable. The implementation of mild heat treatments have shown promise to delay this deterioration, thereby extending the shelf life of the product to between 14 and 21 days. All *Listeria* strains used in this study showed a moderate ability to grow (1.0 log cycle) on cut cabbage tissues stored at 5°C. However, subjecting shredded cabbage to mild heat (three min at 50°C), substantially increased the ability of *Listeria* spp. to grow on the cabbage. Increases of more than 3.0 log CFU/g for two strains of *L. monocytogenes* were observed. Therefore, it is conceivable that if this pathogen was present in low numbers on shredded cabbage having received a mild thermal treatment, dangerous levels could be achieved during the extended shelf-life of the product. The reasons for this increased growth potential in heat treated cabbage are currently not known; however, it is possible that the mild heat deactivates natural inhibitory compounds within the cabbage. Further research is required to elucidate the mechanisms responsible for these observations.

Exposure to mild heat alters the cabbage substrate to support the growth of *Listeria* spp. However, it is also important to examine the effects of mild heat on the bacterium itself. The time/temperature combinations used to process shredded cabbage

provides minimal lethality for *Listeria* spp. and the sensitivity to heat varies substantially depending on strain. In order to gain insight into strain variations in heat tolerance of *L. monocytogenes*, a transposon library was constructed in a single strain, Lm568, to prospect for mutants with deactivated genes resulting in a thermotolerant phenotype. The insertions were mapped to genes involved in a wide array of functions, including nucleic acid, protein and sugar metabolism, transport, motility and structure. Changing the thermal resistance via deactivation of such a diversity of genes indicates that cell death due to heat is not likely the result of failure of a single target within the cell, but is the cumulative effect of damage to multiple sites. In addition, it is evident that the factors responsible for variations in heat resistance among strains are complex and what makes one strain more resistant to heat than another strain may not hold true for a third strain. Perhaps the most remarkable observation regarding these thermal resistance studies is the relative plasticity of *L. monocytogenes*. A single strain (Lm568) can be altered through transposon mutagenesis to possess thermal resistances more than 100 fold greater than its wild type. This extreme increase in thermal resistance may bring into question current margins of safety (i.e. 4D processes) regarding thermal inactivation of *L. monocytogenes* in real world food processing situations.

One of the most thermal resistant mutants (32F11) was subjected to an in-depth analysis to elucidate the reason for the enhanced heat tolerance. It was discovered that this mutant was defective in the production of a phosphotrehalase enzyme and as a result accumulated intracellular trehalose-6-phosphate. Ultimately this resulted in increased levels of cytoplasmic trehalose, a known compatible solute, via the dephosphorylation of

trehalose-6-phosphate. It is not known whether natural trehalose-accumulating strains of *L. monocytogenes* exist; however, other pathogens have been shown to accumulate the disaccharide. The results provided in this study may serve as a model system to help understand the occurrence of enhanced stress resistance due to the presence of trehalose in other Gram-positive pathogens.

As a continuation of the research presented in this thesis, several areas could be pursued. Intriguing is the effect of heat on shredded cabbage. Further investigations are warranted as serious risk implications could result from extending the sensory life of this product which clearly provides a favourable environment for the growth of *L. monocytogenes*. A thorough biochemical analysis on the pre- and post-heated cabbage may provide answers to the mechanisms involved. Secondly, the rapid and strong binding of *Listeria* spp. to cut and intact cabbage tissues raises questions regarding the extent of this interaction. Only a few reports are available regarding the attachment of *Listeria* spp. to fresh or minimally-treated produce surfaces, thus more commodities need to be tested. More importantly, the mechanisms involved in the binding to these surfaces need to be better understood. This information is required for the development of remedial processes to remove this pathogen from vegetable surfaces. Thirdly, the positions of the Tn917 insertions for most of the thermotolerant *L. monocytogenes* 568 mutants were identified. However, full elucidation of the thermal tolerant phenotypes needs to be conducted through the construction of deletion mutants. The effect of these mutations on thermal resistance and other important stress factors should be considered. Ultimately, characterized mutants should also be examined in terms of pathogenicity. Finally,

although the transposon library was screened specifically for heat tolerant mutants, other factors pertaining to the minimal thermal processing of cabbage need to be examined. In particular, cold tolerance following a prior heat shock. Again, it would be beneficial to examine the pathogenicity of such mutants following exposure to the complete processing regimen. Insight into these areas will better our understanding of how *Listeria monocytogenes* is affected by minimally processes designed for the improved quality of cut produce.

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